2017 | Faculty of Medicine and Life Sciences

UHASSELT
KNOWLEDGE IN ACTION

KU LEUVEN

Doctoral dissertation submitted to obtain the degrees of
- Doctor of Biomedical Sciences | tUL
- Doctor of Biomedical Sciences | KU Leuven

Silke Smolders

DOCTORAL DISSERTATION

Embryonic brain development is hampered by environmental and genetic insults

BIOMED
BIOMEDICAL
RESEARCH INSTITUTE
UHASSELT

Promoters: Prof. Dr Bert Brône | UHasselt
Prof. Dr Carlos Dotti | KU Leuven
Co-promoter: Dr Annette Gärtner | KU Leuven

D/2017/2451/5025

"If a cluttered desk is a sign of a cluttered mind, of what, then, is an empty desk a sign?"

- Albert Einstein -

Members of the jury

Prof. Dr. Marcel Ameloot, Universiteit Hasselt, Diepenbeek, Belgium, Chairman

Prof. Dr. Bert Brône, Universiteit Hasselt, Diepenbeek, Belgium, Promotor

Prof. Dr. Carlos Dotti, KULeuven, Leuven, Belgium, Copromotor

Dr. Annette Gärtner, KULeuven, Leuven, Belgium, Copromotor

Prof. Dr. Jean-Michel Rigo, Universiteit Hasselt, Diepenbeek, Belgium

Prof. Dr. Niels Hellings, Universiteit Hasselt, Diepenbeek, Belgium

Dr. Evgenia Salta, KULeuven, Leuven, Belgium

Prof. Dr. Eve Seuntjes, KULeuven, Leuven, Belgium

Prof. Dr. Urs Meyer, University of Zurich, Zurich, Switzerland

Table of contents

Table of contents I
List of abbreviations VI
Chapter 1 1
General Introduction 1
1.1 The central nervous system 3
1.2. Development of the cortex 5
1.2.1 Corticogenesis 5
1.2.2 Microglia 10
1.3 Neuropsychiatric disorders 17
1.3.1 Schizophrenia 17
1.3.2 Autism Spectrum Disorders 19
1.3.3 A role for microglia in schizophrenia and autism spectrum disorder 21
1.3.4 Maternal infection as a shared risk factor for schizophrenia and autism spectrum disorder 22
1.4 Animal models for maternal infection 23
1.4.1 Consequences of MIA in the offspring 24
1.4.2 Mechanisms contributing to the effects of MIA 29
1.4.3 MIA in non-rodent models 31
1.4.4 MIA and schizophrenia and ASD comorbidities 33
1.4.5 The role of MIA in the 'double hit' hypothesis 34
1.4.6 Prevention of MIA-induced behavioral deficits 35
1.5 Disrupted in schizophrenia 1 36
1.6 Objectives of this thesis 41
Chapter 2 43
Effects of maternal immune activation on activation status of embryonic microglia 43
2.1 Abstract 45
2.2 Background 45
2.3 Materials and methods 46
2.3.1 Animals 46

2.3.2 Maternal immune activation 47
2.3.3 Fluorescent immunostaining of embryonic brains 47
2.3.4 BV-2 cell culture 48
2.3.5 Isolation of microglia and flow cytometry experiments 49
2.3.6 Analysis and statistics 50
2.4 Results 51
2.5 Discussion 64
Chapter 3 69
Inducible genetic deletion of PU.1 does not result in microglia depletion 69
3.1 Abstract 71
3.2 Background 71
3.3 Materials and methods 73
3.3.1 Animals 73
3.3.2 Genotyping 73
3.3.3 Tamoxifen 74
3.3.4 Immunohistochemistry 74
3.4 Results 75
3.5 Discussion 78
Chapter 4 83
Behavioral outcome of prenatal immune challenge in wild type and CX3CR1 deficient mice 83
4.1 Abstract 85
4.2 Background 85
4.3 Materials & Methods 86
4.3.1 Animals 86
4.3.2 Serum IL6 determination 88
4.3.3 Genotyping 88
4.3.4 Statistics 89
4.3.5 Behavioral experiments 89
4.4 Results 93
4.5 Discussion 101
Chapter 5 105

Influence of maternal immune activation on cortical development 105
5.1 Abstract 107
5.2 Background 107
5.3 Materials and methods 109
5.3.1 Animals 109
5.3.2 *In utero* electroporation 109
5.3.3 Confocal microscopy and analysis 111
5.3.4 Serum IL6 determination 112
5.3.5 Genotyping 112
5.3.6 Statistics 112
5.4 Results 113
5.5 Discussion 117
Chapter 6 119
The function of DISC1 in microglia 119
6.1 Abstract 121
6.2 Background 121
6.3 Materials and methods 123
6.3.1 Animals 123
6.3.2 Primary microglia isolation 123
6.3.3 Cell lines 123
6.3.4 RT-PCR 124
6.3.5 Western blot 125
6.3.6 Immunocytochemistry 125
6.3.7 Constructs 126
6.3.8 Transfection 127
6.3.9 Flow cytometry to determine transfection efficiency 127
6.3.10 Phagocytosis assay 128
6.3.11 ELISA 128
6.3.12 Microscopy 128
6.3.13 Statistics 128
6.4 Results 129
6.5 Discussion 135

Chapter 7 139
General discussion 139
7.1 Maternal immune activation as an environmental risk factor for neuropsychiatric disease 141
7.1.1 Factors influencing the outcome of MIA on the offspring 142
7.1.2 The gender bias in autism and schizophrenia reflected in the MIA model 150
7.1.3 Cytokines as main responsibles for conveying the effects of MIA on the offspring 153
7.1.4 MIA and other neurodegenerative disorders 155
7.2 Neuronal development deficits in MIA and other ASD and schizophrenia models 156
7.3 Microglia as mediators of neuropsychiatric disease 158
7.3.1 The role of microglia in MIA 159
7.3.2 The role of genetic risk factors in microglia 162
English summary 165
Nederlandse samenvatting 169
References 173
Curriculum Vitae 215
Acknowledgements 219

List of abbreviations

(e)GFP	(enhanced) Green fluorescent protein
AD	Alzheimer’s disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	Autism spectrum disorder
BDNF	Brain-derived neurotrophic factor
BLBP	Brain lipid binding protein
CNS	Central nervous system
CNTNAP2	Contactin Associated Protein-Like 2
CSF1R	Colony stimulating factor 1 receptor
Ctip2	Chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2
Cux1	Cut Like Homeobox 1
CX3CR1	CX3C chemokine receptor 1
DA	Dopamine
DAP12	DNAX activation protein of 12kDa
DAPI	4,6-diamidino-2-phenylindole
DAT	Dopamine transporter
DISC1	Disrupted in schizophrenia 1
DMEM	Dulbecco's Modified Eagle Medium
E	Embryonic day

EMP	Erythro-myeloid progenitor
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FMRP	Fragile X mental retardation protein
Fw	Forward
GABA	γ-aminobutyric acid
GAD65/67	Glutamate decarboxylase 65/67
GFAP	Glial fibrillary acidic protein
Iba1	Ionized calcium binding adaptor molecule 1
ID	Intellectual disability
IFNγ	Interferon γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
I.P.	Intraperitoneal
IPC	Intermediate progenitor cell
IRF8	Interferon regulatory factor 8
IUE	*In utero* electroporation
I.V.	Intravenous
KCC2	K-Cl co-transporter 2
LGE	Lateral ganglionic eminence
LI	Latent inhibition
LiCl	Lithium chloride

LPS	Lipopolysaccharide
MecP2	Methyl CpG binding protein 2
MGE	Medial ganglionic eminence
MIA	Maternal immune activation
MMP	Matrix metalloproteinase
NaCl	Sodium chloride
NKCC1	Na-K-Cl cotransporter 1
NMDA	N-methyl-D-aspartate
P	Postnatal day
PBS	Phosphate buffered saline
PD	Parkinson's disease
PFA	Paraformaldehyde
Poly IC	Polyinosinic:polycytidylic acid
PP	Prepulse
PPI	Prepulse ihibition
Rev	Reverse
RGC	Radial glia cell
RUNX1	Runt-related transcription factor 1
S.C.	Subcutaneous
SP	Startle pulse
Tbr2	T-box brain protein 2
TH	Tyrosine hydroxylase

VIII

TLR	Toll like receptor
TNFα	Tumor necrosis factor α
TREM2	Triggering receptor expressed on myeloid cells 2
TSC	Tuberous sclerosis
TSPO	Translocator protein
TTX	Tetrodotoxine
VGAT	Vesicular GABA transporter
YS	Yolk sac

Chapter 1

General Introduction

1.1 The central nervous system

The brain together with the spinal cord makes up the central nervous system (CNS). The CNS is the integration center of the body where information to and from all other organ systems is processed. The brain is made up of different cell types that all work together to assure proper functioning. They can be divided in two main groups: neuronal cells and glial cells. Neuronal cells make up about 20% of the total amount of cells in the brain and are responsible for transducing signals from one cell to another via release of neurotransmitters into the synapse which can either activate or inhibit the other cell by binding on its receptor. The two main types of neurons are pyramidal neurons and interneurons with pyramidal neurons typically activating other cells and making long distance connections while interneurons make up smaller networks that generally inhibit the activity of other cells. Glial cells constitute 80% of the brain cells and can be divided in three main subgroups: astrocytes, oligodendrocytes and microglia. Although astrocytes were long believed to be the support cells of the brain, providing a scaffold for neurons, research from the last decades showed that these cells serve many more purposes. Astrocytes take part in the formation of the blood brain barrier to restrict access from blood molecules to the brain and they also participate in the synapse, where they play an important role in the metabolism of neurotransmitters. The main function of oligodendrocytes is to produce myelin sheets that are wrapped around neuronal processes to ensure a fast transmission of neuronal signals. Microglia are the resident macrophages of the brain that are specialized in the defense against foreign material and damage by secreting a plethora of immune molecules and growth factors. Besides their well-known role in the adult brain, they also serve many purposes in the developing brain[1].

Made up by a mixture of these different cell types, the brain is divided in several anatomically distinct structures that are all involved in different neurological functions. The cerebral cortex forms the outer layer of the brain and is divided in several areas. The sensory areas are those receiving information from the outside world through seeing, hearing and feeling. The motor areas are important for the fine control of voluntary movements and the association areas, including the prefrontal cortex, are involved in the integration of information

from other brain areas to allow planning and decision making and modulating social behavior. The cortex is characterized by a special organization of the pyramidal neurons in six different layers. The hippocampus and the amygdala belong to the limbic system and play important roles in the consolidation of information from short-term memory to long-term memory, decision-making and emotional reactions. The striatum coordinates multiple aspects of cognition, including motor and action planning, decision-making, motivation, reinforcement, and reward perception and the cerebellum is important for fine motor control, balance and coordination of the body but also plays a role in cognition[1].

Neurons from different brain regions communicate with each other using neurotransmitter systems. Synapses can be divided in 3 main subclasses: excitatory, inhibitory and modulatory. The main excitatory neurotransmitter in the brain is glutamate which exerts its function via binding to several receptors: NMDA, AMPA, kainate and mGluR receptors. GABA accounts for most of the inhibitory neurotransmission and functions through binding of the GABAa and GABAb receptors. However, during development, GABA receptor activation leads to depolarization instead of hyperpolarization of the cell owing to a different relative expression of the Na-K-Cl transporter (NKCC1) and the K-Cl cotransporter (KCC2) compared to mature neurons. The modulatory neurotransmitter dopamine is mainly expressed by neurons from the hypothalamus, the substantia nigra pars compacta and the ventral tegmental area in the brainstem and its signaling is mainly involved in regulating reward, attention, motivation and movement. Impairments in the dopaminergic system are associated with disorders such as Parkinson's disease, schizophrenia and substance abuse. The main source of serotonin are the neurons from the raphe nuclei of the brain stem which regulate mood, attention, sleep and appetite and dysregulation of the serotonergic system is involved in depression[2].

1.2. Development of the cortex

1.2.1 Corticogenesis

The central nervous system develops entirely from the ectodermal germ layer, starting with the formation of the neural tube in the earliest stages of embryonic development. In this neural tube, three primary vesicles are formed that will later form the hindbrain, the midbrain and the forebrain, the latter of which later grows into the two cerebral hemispheres that contain most of the brain structures described above. While cortical and striatal interneurons originate from the ventral part of the brain including the medial and the caudal ganglionic eminence (MGE and CGE respectively) and the preoptic area (POa) and migrate tangentially into the cortical plate, pyramidal neurons are born in the neurogenic zone dorsally from the lateral ventricle and reach the cortical plate by radial migration (Figure 1.1).

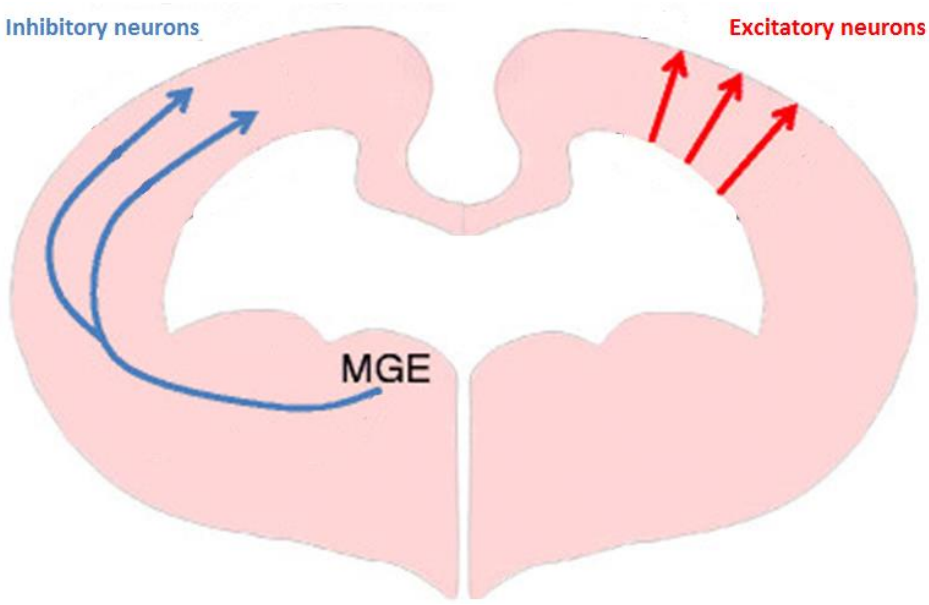


Figure 1.1: Coronal brain slice of an embryonic mouse brain showing tangential migration by inhibitory neurons (left, blue) and radial migration by excitatory neurons (right, red). MGE: medial ganglionic eminence (caudal ganglionic eminence and preoptic area are not shown here). Adapted from Moffat *et al.* 2015[3].

Early in cortical development, neuroepithelial cells, the stem cells of the nervous system, become radial glia cells as they start to express typical astrocytic markers, including the intermediate filament protein glial fibrillary acidic protein (GFAP) and brain lipid binding protein (BLBP). These radial glia are the neuronal precursors that have a fairly typical characteristic: throughout development, they span the entire developing cortex by extending a single long fiber while keeping their cell body close to the ventricular lining. Radial glia cells divide symmetrically, giving rise to another radial glia cell and thereby exponentially increasing the pool of neuronal precursors. Radial glia also divide asymmetrically, producing a radial glia cell and an intermediate neuronal progenitor cell or a neuron[4-7] (Figure 1.2A). Intermediate progenitors are Tbr2 positive and migrate up to the subventricular zone where they undergo another round of division[8-10]. Neurons, either derived directly from asymmetric division of radial glia or from division of intermediate progenitors, then acquire a multipolar phenotype, extending and retracting several protrusions in all directions[6, 11]. Although it is not yet clear what the exact function of the multipolar phase is, it has been suggested that multipolar cells explore the environment to determine which process will become the trailing and the leading process. Alternatively, cells could in this way adjust their timing to enter the cortical plate[12]. Many genes have been found to play a role in the multipolar to bipolar transition, including transcription factors and kinases, but most downstream effectors are involved in cytoskeletal remodeling[13].

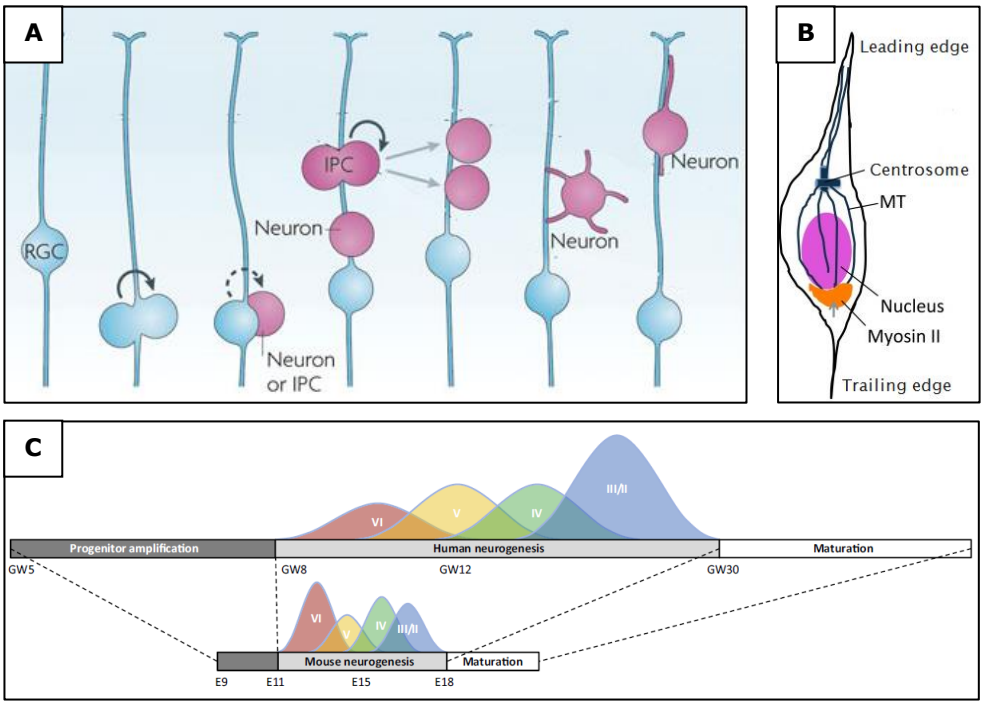


Figure 1.2: Generation of neurons during corticogenesis (A) RGCs in the ventricular zone divide either symmetrically or asymmetrically, the latter producing either an IPC or a neuron. IPCs migrate to the subventricular zone and divide symmetrically into two neurons. After a transient multipolar stage, neurons acquire a bipolar phenotype and migrate up to the cortical plate. RGC: radial glia cell, IPC: intermediate progenitor cell. Adapted from Hur and Zhou 2010[14]. (B) Machinery involved in radial glia guided migration of neurons. MT: microtubule. Adapted from Ohtaka-Maryuma and Okado 2015[13] (C) Timeline of humane and mouse neurogenesis. GW: gestational week, E: embryonic day. Adapted from van den Ameele *et al.* 2014[15].

After transition into bipolar cells, neurons associate with the radial glia fibers and use them as a scaffold for radial migration into the cortical plate using locomotion. Among adhesion molecules involved in adherence of migrating cells to radial glia are N-Cadherin and the gap junction components Connexin 26 and 43. Knock down of either of these proteins results in defective migration into the cortical plate[16-18]. During radial glia guided migration, translocation of the nucleus is achieved by coupled movement of the centrosome and the nucleus coordinated by the cytoskeleton. The centrosome is positioned at the base of the leading process and microtubuli extend into the tip of the leading process on one

side and surround the nucleus on the other side, providing a pulling force on the nucleus[19] (Figure 1.2B). The motor proteins of the dynein complex mediate the movement of the centrosome into the tip of the leading process and the pulling of the nucleus towards the centrosome[20-22]. At the trailing edge, a pushing force is generated on the nucleus by activated myosin II[23]. When migrating neurons reach their final destination in the cortical plate, locomotion is terminated, mediated by factors including the transcription factors Lmx1a and Foxc1 and G-protein coupled receptor 56. Absence of these factors results in overmigration of neurons into the meningeal space and a disorganized laminar organization[24-26]. For detachment from the radial glia fiber, the anti-adhesive activity of the matricellular protein SPARC-like1 appears to play an important role[27]. Finally, detached neurons terminally translocate to occupy their final location in the cortex, a process mediated by reelin signaling[28].

The earliest born neurons make up the first layer that is formed, the preplate. Layer VI neurons are the next to be produced in the ventricular zone, around embryonic day 11 (E11) in mice and gestational week 8 in humans[15] (Figure 1.2C). These neurons migrate up to the preplate and split it up in a lower subplate and an upper marginal zone. Meanwhile, neurons destined to become layer V neurons are generated in the ventricular zone. These neurons migrate past the previously formed layer VI and settle on top of it and successively form the six layered cortex in an inside-out-fashion[29]. The marginal zone (layer I) contains the Cajal-Retzius cells, a cell type that secretes the glycoprotein reelin. As recently reviewed by Zhao and Frotscher 2010, reelin appears to be involved in several steps of neuronal migration, including (1) invasion of layer VI neurons into the preplate, (2) attracting migrating neurons toward the cortical plate and enabling them to migrate past the previously formed layers, (3) provide a stop signal and induce detachment of migrating neurons from radial glia fibers and (4) stabilize the cytoskeleton of terminally translocating neurons[30]. Consequently, *reeler* mice that naturally lack reelin, display a complex phenotype with, among others, an inverted cortical layering. At the end of neurogenesis, radial glia generate astrocytes and oligodendrocytes.

During embryonic development of the cortex, neurons send out their axon to make connections with other cells. Pyramidal cells in layer II and III mainly

make connections with cortical neurons within the same hemisphere. Layer IV neurons communicate with cortical neurons in the other hemisphere and layer V and VI neurons make connections with subcortical structures. While the pyramidal neurons settle, interneurons that have migrated tangentially from the medial ganglionic eminence, take up their place in the cortical plate, interspersed between the pyramidal cells[31].

Already in the earliest stages of cortical development, even before the onset of synaptogenesis, several neurotransmitter systems play an important role in the regulation of neurodevelopment. Developing neurons express several GABA receptors and depending on which receptor is activated, it has a different effect on neuronal development. Activation of the GABAa receptor on neuronal progenitors in the subventricular zone stimulates cell cycle exit and initiation of migration. Stimulation of GABAb and GABAc receptors on neuronal progenitors in the intermediate zone maintains migration while GABAa receptor activation in the cortical plate terminates migration and stimulates integration of the new neuron in a network[32]. The main ligand of GABA receptors is GABA, but taurine has also been found to stimulate these receptors. Taurine is a semi-essential amino acid in the embryo: the embryo depends on transport from the mother across the placenta. Depletion of taurine from the embryonic brain results in increased neuronal migration of excitatory neurons born in the dorsal telencephalon[33].

Proper development of the cerebral cortex requires this strict pattern of events that is regulated by a plethora of different factors. Evidently, absence or deficiency of any of these regulators can disturb normal cortical development and alter neuronal connectivity later in life.

1.2.2 Microglia

1.2.2.1 Microglia ontogeny and development

As evident from the description above, all cell types in the cortex derive from the radial glia, apart from microglia. Microglia are of mesodermal origin and are born outside the brain, in the blood islands from the yolk sac, before the onset of neurogenesis, around E7,5 in mice[34] (Figure 1.3). Development of these erythro-myeloid precursors (EMPs) into microglia is controlled by several molecules, including transcription factors PU.1 and IRF8. EMPs, which express the transcription factor c-kit but not the receptor-linked protein tyrosine phosphatase CD45 or the fractalkine receptor CX3CR1, are converted to immature microglia by PU.1 and are characterized by lowered expression of c-kit and onset of expression of CD45 and CX3CR1. Subsequently, IRF8 is required for the transition into the more mature state which is characterized by absence of c-kit expression and high levels of CD45 and CX3CR1. Microglial progenitors then migrate to the developing brain using the circulatory system where they enter the brain parenchyma via the meninges and the lateral ventricles using matrix metalloproteinases (MMPs). MMPs are enzymes that are involved in the remodeling of extracellular matrix and MMP8 and 9 were found to be highly expressed in migrating microglia. Moreover, inhibition of MMP function by Batimastat significantly diminished microglia spreading in the developing brain[35]. After entering the brain, microglia proliferate and mature, a process which is dependent on the CSF1 receptor (CSF1R)[34, 36]. During migration, microglia display an amoeboid morphology but once they reach their final destination they start to ramify, a process that was shown to be dependent on transcription factor RUNX1[37, 38]. Using these ramifications, microglia constantly scan their environment for possible threats. Upon detection of such a threat, which is in general either cell damage or infection, microglia transform into an activated state in which they acquire a more amoeboid shape, phagocytose cellular debris and secrete a plethora of cytokines.

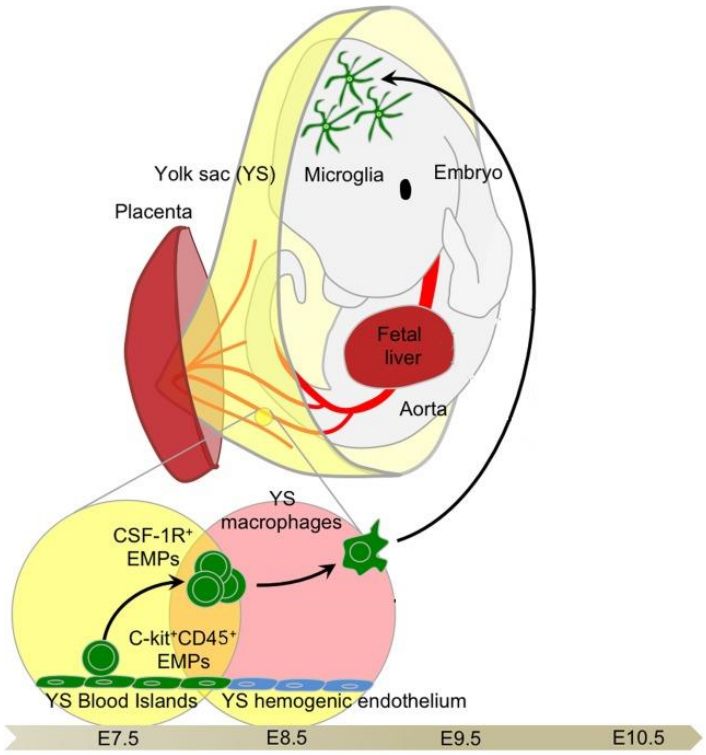


Figure 1.3: Ontogeny and development of microglia. EMPs from the YS blood islands at E7.5 develop into primitive microglia that migrate to the embryonic brain via the blood circulation around E9.5 in the mouse. YS: yolk sac, EMP: erythro-myeloid progenitor, E: embryonic stage. Adapted from Hoeffel and Ginhoux 2015[39].

1.2.2.2 Microglia tasks in the developing and adult brain

While microglia were long believed to serve only immune functions in the adult brain, research from the last decade has shown that they also contribute to the proper development of the embryonic brain. Several methods have been developed to deplete microglia from the brain in order to investigate their physiological role in neuronal development. Microglia can be depleted *in vivo* either by a genetic approach or by administration of compounds. Parkhurst *et al.* 2013 generated a mouse model in which the diphtheria toxin receptor was expressed only in CX3CR1-expressing cells. Upon administration of diphtheria toxin, cells of the monocytic lineage are specifically targeted and as a consequence, in the central nervous system mostly (but not only) microglia are

affected and thus depleted[40]. Other genetic microglia depletion models, such as CSF1R or PU.1 knock-out mice[34, 35, 41], efficiently eliminate cells of the monocytic lineage already during development, but are not compatible with life after birth. As a non-genetic approach to deplete microglia, liposomal chlodronate is often used. This cytotoxic drug is encapsulated in lipid droplets which are selectively taken up by phagocytic cells and subsequently induce apoptosis. Nelson and Lenz 2017 and Van Ryzin *et al.* 2016 successfully depleted microglia in early born rats by repeated bilateral intracerebroventricular injection of chlodronate[42, 43]. Elmore *et al.* 2014 achieved microglia depletion in adolescent mice by systemic treatment with the CSF1R-signaling inhibitor PLX3397[36]. Finally, Squarzoni *et al.* 2014 achieved microglia depletion during embryonic development after maternal administration of CSF1R antibodies[44].

As many mouse lines used to investigate microglia also target other members of the mononuclear phagocyte system such as monocytes and macrophages, many groups have recently searched for a molecular marker that allows to distinguish between microglia and other immune cells in the brain. As such, Buttgereit *et al.* 2016 recently showed the expression of the transcriptional repressor Sall1 to be largely restricted to microglia in the adult brain, although some cells in the liver, kidney and heart were also found to express this marker[45]. Similarly, Bennett *et al.* 2016 identified Transmembrane protein 119 (Tmem119) as a microglia-specific marker in the human and mouse CNS[46]. However, Tmem119 immunoreactivity appeared to be developmentally regulated, with no reactivity until P3, 25% positive microglia by P7 and the amount of positive microglia comparable to adulthood around P10-P14. Thereby, the use of this marker in embryonic stages is limited.

Microglia have been shown to have effects on neuronal cells in almost all stages of development: proliferation, migration, differentiation and integration (as reviewed in [47-49]). Beside their involvement in neuronal development, it is also suggested that they play a role in development of the brain vasculature[50] (Figure 1.4).

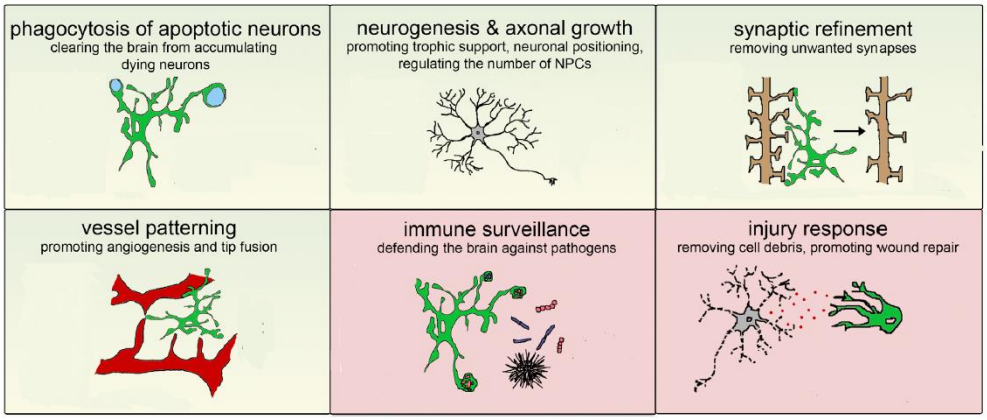


Figure 1.4: The multiple roles of microglia in the developing and adult brain. Adapted from Casano and Peri 2015[51].

Role in vasculogenesis

Initial evidence for the involvement of microglia in brain vasculogenesis came from Checchin *et al.* 2006 who showed that a depletion of microglia resulted in a decrease in vascular density in the retina which could be restored by exogenous administration of microglia[52]. Similarly, Kubota *et al.* 2009 and Fantin *et al.* 2010 reported a decreased branching in the vascular plexus in the absence of microglia, in spite of a normal number of endothelial tip cells and filopodia, suggesting a role for microglia in branching anastomosis but not tip cell extension[53, 54]. Further elucidating the communication between microglia and developing blood vessels, Rymo *et al.* 2011 used microglia co-cultured in collagen matrix with mouse aortic rings to demonstrate that only medium from cultured microglia is sufficient to induce vascular branching, although less potent[55]. This suggests that microglia may release soluble factors to stimulate sprouting/branching which is enhanced by physical microglia-blood vessel contact. Finally, Stefater *et al.* 2011 demonstrated the involvement of the Wnt-Flt1 pathway specifically in microglia in regulation of vascular branching[56].

Role in neuronal precursor pool maintenance

During neurogenesis, an excess of neuronal progenitors is produced in the proliferative zone of the brain. Cunningham *et al.* 2013 and Swinnen *et al.* 2013 recently showed that embryonic microglia colonize the proliferative zones of the developing cortex already at early ages, and even more so by the end of neurogenesis[38, 57]. Even more, Cunningham *et al.* 2013 showed that these embryonic microglia phagocytose neuronal precursor cells while these precursor cells do not show signs of cell death or apoptosis. In addition, manipulation of microglial activation status altered the amount of precursor cells in the ventricular zone[57]. Microglial stimulation with the bacterial cell wall component lipopolysaccharide (LPS) decreased the amount of neuronal precursors while treatment with the antibiotic tetracycline increased the neuronal precursor pool, likely through respectively increased and decreased phagocytic activity of microglia[57].

Not only are microglia involved in the removal of neuronal precursors, through secretion of several neurotrophic factors they can also promote cell survival, differentiation and maturation. Aarum *et al.* 2003 showed that soluble factors released from microglia direct the migration of cultured neuronal precursors and have the capacity to influence precursor differentiation into a neuronal phenotype[58]. In another study addressing the relevance of microglia for neuronal precursor development, cells were cultured from PU.1 knock-out mice which are depleted of microglia. Compared to neuronal cultures containing microglia, these precursors displayed decreased proliferation and astrogenesis. Reintroduction of microglia to the depleted cultures rescued the otherwise observed deficits in precursor development[59]. Additionally, Shigemoto-Mogami *et al.* 2014 described both *in vitro* and *in vivo* the promoting effect of microglial secreted proteins on neurogenesis and oligodendrogenesis as treatment with the antibiotic minocycline suppressed these processes[60].

Role in axonal outgrowth and fasciculation

While older studies already showed that microglia physically associate with developing axon tracts[61-63], more recent studies described the detrimental effects of microglia depletion on the development of these tracts. Squarzoni *et*

al. 2014 employed two different models of microglia depletion, a PU.1 knock-out mouse line and injection with a CSF1R antibody, to show that dopaminergic axons of the thalamo-cortical tract extending into the striatum exhibited increased outgrowth in the absence of microglia at E14.5. At birth, this altered extension of dopaminergic axons was associated with an imbalance of dopaminergic innervation of the striatum, which could contribute to the development of neurological disorders[44]. Additionally, in the study of Pont-Lezica *et al.* 2014 it was shown that mice depleted of microglia (PU.1 knock-out) or deficient of the adaptor molecule DAP12 protein, which is important for phagocytosis, have impairments in axon fasciculation in the corpus callosum[64].

Role in synaptogenesis

In the initial stages of neuronal network formation, neurons make an excess of connections with other cells. Elimination of the weaker and inappropriate synapses and the strengthening of the appropriate ones occurs in a process called synaptic pruning, which has been shown to be modulated by microglia. One of the first hints towards the involvement of microglia in synaptic remodeling came from a study by Stevens *et al.* in 2007[65]. They showed that the complement cascade, which is part of the innate immune system, at least partially, mediates synaptic elimination. Mice deficient in complement protein C1q or its downstream effector molecule C3 displayed increased amounts of synapses. Since microglia are the only cell type in the brain that express the receptor for C3, Stevens *et al.* proposed that they could likely be the executors of synapse elimination. Three years later, Tremblay *et al.* 2010 used the visual system to monitor microglia interactions with synapses with either high or low levels of neuronal activity[66]. Specifically, mice were either exposed to light or deprived from it, the latter causing a decrease in neuronal activity. Microglial behavior was subtly altered by sensory deprivation: they displayed an altered morphology and apposed and phagocytosed synaptic elements more often, thereby supporting a role for microglia in synaptic modulation. Paolicelli *et al.* 2011 were the first to actually demonstrate engulfment of pre- and postsynaptic material (SNAP25 and PSD95 respectively) by microglia in the healthy brain (using immunohistochemistry and 3D reconstruction)[67]. Mice lacking the fractalkine receptor CX3CR1 displayed an increased dendritic spine density in the

hippocampus accompanied by synaptic characteristics reminiscent of an immature connectivity. Complementary to Paolicelli *et al.* 2011, the group of Beth Stevens reported on an activity dependent engulfment of synaptic material by microglia, albeit mediated by the complement system[68]. Mice received an intraocular injection of anterograde tracers enabling the detection of fluorescently labeled synaptic material within microglial processes and cell soma. Blockage of cellular activity by intraocular injection of tetrodotoxine (TTX) resulted in an increased amount of synaptic material inside microglia. Finally, microglial depletion of the complement receptor 3 led to a decrease in engulfment of synaptic material and a sustained increase in synaptic density[68].

In 2013, Ji *et al.* used hippocampal brain slices in which they inhibited (using the tripeptide macrophage/microglial inhibitory factor Thr-Lys-Pro) or depleted microglia (with chlodronate)[69]. There was increased synaptic activity in the absence of microglia (but not with microglia inhibition) along with an increase in glutamatergic dendritic spines, both of which could be reverted by replenishment of microglia. Only recently, Kim *et al.* 2016 showed that microglial autophagy is also important during synaptic pruning[70]. Using a microglia specific knock-out of atg7 (a crucial protein of the autophagy pathway), they found an increase in dendritic spines owing to an impaired microglial degradation of synaptic material.

Microglia are not only involved in synaptic pruning, they can also stimulate the formation of new and maintenance of established synapses. When microglia plated on a porous cell culture insert were added to hippocampal neuronal cultures (to avoid direct contact), the amount of dendritic spines increased[71]. This effect on synapse formation was found to be dependent on IL10 as either an interleukin (IL) 10 neutralizing antibody or elimination of its receptor on hippocampal neurons reduced the amount of synapses. Interestingly, activation of microglia by LPS induced secretion of IL1β which in turn inhibited synapse formation[71]. In addition, Parkhust *et al.* 2013 demonstrated the importance of microglial brain derived neurotrophic factor (BDNF) in learning induced synaptic plasticity[40]. Finally, while depletion of microglia in the adult retina did not change the amount of synapses, synapses showed a degenerative morphology[72].

Taken together, the proper development of the brain is a tightly orchestrated process where already subtle alterations can lead to an altered network formation. In addition, microglia are clearly implicated in the normal development of neurons and their connections and suggest that deficits in their proper functioning could contribute to altered brain function. Indeed, the neurodevelopmental origin of neuropsychiatric disorders and the role of microglia in their pathogenesis is a field of intense investigation, as will be reviewed in the next paragraph.

1.3 Neuropsychiatric disorders

The term 'neuropsychiatric disorders' is an umbrella term often used to describe a group of disorders that affect normal functioning of the brain. Unlike brain disorders such as traumatic brain injury, neuropsychiatric disorders are characterized by a general lack of clear causality and diagnosis is thus often based solely on symptom presentation. Although the cause for most neuropsychiatric disorders is often not known, several of them are believed to have a neurodevelopmental origin. Two hallmark neurodevelopmental disorders are schizophrenia and autism spectrum disorder (ASD). Although both disorders share a subset of symptoms, they differ greatly in the age of disease onset. While ASDs are usually diagnosed during early childhood, schizophrenia emerges around adolescence.

1.3.1 Schizophrenia

Schizophrenia is diagnosed in about 1% of the population, usually around late adolescence, based on clinical presentation as no diagnostic tests are available so far. The consequences of schizophrenia represent a big burden on the affected individual, their family and the society as schizophrenia patients usually require life-long treatment and unemployment is high.

Schizophrenia is characterized by a combination positive, negative and cognitive symptoms although the clinical presentation can vary greatly between patients. The term 'positive symptoms' refers to symptoms that are present in affected

individuals and include mainly hallucinations (auditory or visual) and delusions. Negative symptoms are characteristics normally present in unaffected individuals but deficient in patients and include deficits in affection, motivation and sociability. Cognitive symptoms mainly reflect in difficulties with working memory. While the positive symptoms usually 'come and go', negative and cognitive symptoms are more chronic and are often the main cause of functional impairments[73]. Schizophrenia occurs more often in men than in women, and men usually have an earlier age of onset, wider range of symptoms and a lesser response to treatment[73, 74].

Schizophrenia has a high heritability, with concordance rates of up to 50% in identical twins, implicating an important contribution of genetic factors to the development of the disease[75]. However, a concordance which is not 100% means that environmental factors are also posing substantial risk to develop schizophrenia.

As schizophrenia is a highly variable disorder with overlap with several other neuropsychiatric disorder, no single anatomical or functional abnormality has been found in all patients. Nevertheless, many patients have been reported to display enlarged lateral ventricles alongside reduced gray matter volume, especially in the hippocampus, the thalamus and the cortex. In addition, white matter tracts such as the corpus callosum are often reduced in schizophrenia patients (as reviewed by [76]). Finally, the amount of synapses has often been found to be reduced[77-79].

As reviewed by Bolkan *et al.* 2016, several neurotransmitter systems have been implicated in schizophrenia pathophysiology[77]. Dopamine hyperactivity is believed to be associated with positive symptoms as Dopamine D2 receptor (D2R) blockers alleviate these symptoms while hypoactivity of the dopamine system in the cortex would result in the negative and cognitive symptoms[80]. In post mortem brains of schizophrenia patients, they found increased levels of Dopamine D2 receptors[81, 82]. Concerning the glutamate system, several studies have found morphological alterations in synapses and dendrites of glutamatergic neurons and increased tissue levels of glutamate[83, 84]. Also GABA system dysfunction can be related to cognitive and negative symptoms of schizophrenia. Post mortem brain analyses revealed decreased expression of the GABA

synthesizing enzyme GAD67 and the GABA transporter GAT1 and altered expression of GABA receptor subunits

Current treatment usually consists of antipsychotic medication alongside psychological treatment. Haloperidol, risperidone and clozapine are the most widely used antipsychotics. Most, if not all, antipsychotics work via blocking the Dopamine D2 receptor while several also affect serotonin neurotransmission. Although most antipsychotics are effective in treating positive symptoms, they are accompanied with several side effects such as weight gain, movement disorders and sedation. In addition, negative and cognitive symptoms are usually not alleviated with these medications[73].

Schizophrenia is believed to be caused by either genetic and/or environmental risk factors. Over a 100 common genetic alterations have been identified by genome wide association studies to increase the risk for developing schizophrenia, although with a fairly small effect. On the other hand, 11 large and rare variants have been found to confer a much higher risk for schizophrenia[85-88]. Environmental risk factors for schizophrenia include cannabis exposure during adolescence and prenatal adversities such as infection, malnutrition and hypoxia[89]. Interestingly, many genetic risk factors are implicated in normal brain development and many environmental risk factors also occur during development, strengthening the idea of a neurodevelopmental origin of schizophrenia.

Of note, schizophrenia is associated with several comorbidities. As reviewed by Buckley *et al.* 2009, many schizophrenia patients also suffer from anxiety disorders, depression or substance abuse[90]. These comorbidities can substantially influence the clinical presentation of schizophrenia symptoms: depression can aggravate negative symptoms while panic attacks and substance abuse can worsen positive symptoms.

1.3.2 Autism Spectrum Disorders

Prevalence rates of ASD in the general population have been estimated around 1 in 100. ASD is diagnosed more often in males than in females with an incidence ratio of 4 to 1. Diagnosis of ASD is made solely on behavioral assessment, with

patients presenting symptoms in three core domains (1) restricted interests and repetitive movements (2) deficits in social interaction (3) impaired communication (either verbal or non-verbal). The precise clinical presentation can vary tremendously as well as the onset, severity and extent of disability. ASD is also associated with a few syndromes such as Fragile X, Prader-Willi or Angelman's syndrome, Tuberous Sclerosis and others[91].

Much like for schizophrenia, concordance in identical twins is about 60% for ASD suggesting a high genetic predisposition. Moreover, 10-20% of ASD cases can be explained by a genetic cause although no specific gene accounts for the majority of ASD cases[92]. Nevertheless, several environmental factors have been found to increase the risk of developing ASD.

As no cure or specific medication exists, ASD is usually managed by behavioral intervention, providing speech, occupational and behavioral therapy, individualized education, family support, etc. For 'challenging' behaviors that do not respond to behavioral therapy, including aggression and irritability, antipsychotics such as risperidone or aripiprazole may be used[91, 93-95].

As for schizophrenia, no two patients present with the same symptoms and abnormalities found in ASD are divergent. The most reproducible abnormalities include brain overgrowth in early childhood, loss of cerebellar purkinje cells and increased amount of synapses, although with an immature phenotype[79, 96].

Similar to schizophrenia, ASD is believed to be caused by an interaction between several low penetrance common genetic alterations and/or environmental risk factors. Some rare genetic mutations, such as in the Fragile X Mental retardation protein (FMRP) or tuberous sclerosis protein (TSC), cause Fragile X syndrome and Tuberous Sclerosis respectively, and these syndromes are highly associated with autistic traits. Notably, syndromic autism is often characterized by dysmorphic features and an equal male:female ratio (as reviewed by [97]). Most environmental risk factors are shared with schizophrenia and mainly occur during pregnancy, including prenatal infection and maternal (mal)nutrition and alcohol use[98].

A quarter of ASD patients suffers from comorbid epilepsy, which in turn is correlated with more severe symptoms and a lower IQ[99]. Other co-morbid conditions reported in ASD patients include cognitive impairment and gastrointestinal disorders (such as constipation, diarrhea and food allergy)[100].

Of note, although there are substantial differences between schizophrenia and ASD, also many similarities can be noted, suggesting a possible common deficit. Indeed, interneurons are often suggested to be implicated in both disorders and in other disorders characterized by a disturbed excitation/inhibition balance such as epilepsy[101]. Using optogentic manipulation of interneurons, Cho *et al.* showed the involvement of prefrontal interneurons in cognitive flexibility; while inhibition of interneurons in wild type mice induced cognitive inflexibility, interneuron stimulation at γ-frequencies was able to restore these deficits in a genetic model for schizophrenia[102]. In addition, transplantation of MGE-derived interneurons in newborn wild type mice resulted in decreased anxiety-like behavior compared to non-transplanted controls[103]. Finally, clinical trials with the diuretic bumetanide, an NKCC1 antagonist that reduces intracellular chloride concentrations and thereby reinforces GABAergic inhibition, was able to ameliorate hallucinations and social interactions in schizophrenia patients [104, 105] and improved symptoms in patients suffering from ASD or Asperger Syndrome [106, 107].. Hence, targeting GABAergic neurotransmission seems to be an attractive therapeutic target for neuropsychiatric disorders.

1.3.3 A role for microglia in schizophrenia and autism spectrum disorder

As described above, microglia exert several important functions in the normal brain, and microglia dysfunction might contribute to neuropsychiatric disorders such as schizophrenia and ASD. Several post mortem and live imaging studies provided evidence for an activated state of microglia in schizophrenia and ASD patients[108]. In the earliest post mortem brain studies, microglia activation was assessed by either an increase in the number/density of microglia or by their morphology. In post mortem brain of schizophrenia patients, increased microglia density was found in several brain regions, including frontal cortex and dorsolateral prefrontal cortex[109, 110]. Busse *et al.* 2012 also found an increased

microglial density but only in paranoid compared to residual schizophrenia patients[111]. In ASD patients, an increased density of microglial cells has also been found in dorsolateral prefrontal and visual cortex[112-114]. Using markers that are upregulated on the surface of activated microglia, such as [^{3}H]-PK11195, [^{3}H]-PBR28 and CD11b, Kreisl *et al.* 2013 and Rao *et al.* 2013 reported on increased reactivity in post mortem brain of schizophrenia patients[115, 116]. *In vivo* positron emission tomography (PET) imaging studies using radioligands for the translocator protein (TSPO), have also suggested increased microglia activation in schizophrenia and ASD patients[117-120].

Whether microglia activation is a cause or a consequence of the disease is currently unknown. However, supporting a role for dysregulation of the immune system in the pathogenesis of schizophrenia and ASD is their shared environmental risk factor: infection during pregnancy.

1.3.4 Maternal infection as a shared risk factor for schizophrenia and autism spectrum disorder

As discussed above, genetic risk can't explain 100% of the susceptibility for schizophrenia and ASD, suggesting an important role for environmental risk factors as well. Among these environmental insults associated with neurodevelopmental disorders, the activation of the maternal immune system during pregnancy has received much attention. With the recent ZIKA virus outbreak, awareness about the fact that viral, bacterial and parasitic infections during pregnancy increase the risk for development of neurological disorders in the offspring has increased. However, even before the ZIKA virus epidemic, several epidemiological studies have been published indicating associations between maternal infections and deviating behavior in children from infected mothers[121].

Four different studies, the Child Health and Development Study, the Collaborative Perinatal Project, the Rubella Birth Defects Evaluation Project and the Copenhagen Perinatal Cohort, reported on the association between maternal infection and an increased risk for schizophrenia. Based on maternal antibodies during pregnancy, it was shown that infection with influenza or Toxoplasma Gondii during early to mid-pregnancy increased the risk of developing

schizophrenia by three and twofold respectively[122, 123]. Increased levels of IL8 or Tumor necrosis factor α (TNFα) in maternal blood were found in mothers of schizophrenia patients[124, 125]. It was found that 20% of subjects whose mothers were infected with rubella during pregnancy were later diagnosed with schizophrenia[126]. Additionally, it was demonstrated that maternal genital-reproductive infection increased the risk for schizophrenia fivefold, while exposure to respiratory infection conferred a twofold risk for schizophrenia[127, 128]. Finally, an association between bacterial infection and schizophrenia was also reported[129].

Studies on the risk for ASD after maternal infection mainly consist of the Danish Medical Birth Register, the Early Markers for Autism Study and the Danish Historic Birth Cohort. When maternal viral or bacterial infection required hospitalization, this was associated with a significantly increased risk for ASD while hospitalization upon viral infections increased the risk up to threefold[130]. It was found that maternal levels of IL4, IL5 and Interferon γ (IFNγ) during pregnancy were significantly increased in mothers of ASD patients and also increased levels of TNFα and TNFα in the amniotic fluid were associated with ASD in the offspring[131, 132].

1.4 Animal models for maternal infection

Since the first indications that maternal infection increases the risk for neurodevelopmental disorders, several groups attempted to model this risk factor in rodents, often referred to as the maternal immune activation (MIA) model. Fatemi *et al.* developed the first model for infection during pregnancy by intranasaly infecting mice with influenza virus. This resulted in altered behavior in the offspring resembling schizophrenia and ASD symptoms[133, 134]. Afterwards, other models using bacterial infection and injection with viral/bacterial mimicking agents have been developed[135]. As a bacterial mimicking agent, LPS, a component of the cell wall from gram negative bacteria, is most often used. On the other hand, polyriboinosinic: polyribocytidilic acid (Poly IC) is a synthetic analogue of double-stranded RNA that is most often used to mimic a viral infection. LPS is mainly recognized by Toll-like receptor 4 on the

cell surface of various immune cells while Poly IC stimulates the Toll-like 3 receptors on intracellular compartments (such as endosomes) of these cells. Binding of these mimicking agents to their receptors results in the induction of a systemic cytokine storm to help clear the infection[136, 137].

Regardless of the infectious agent used, studies univocally reproduce symptoms reminiscent of ASD and schizophrenia in the offspring of infected mothers. Depending on the timing and dose of infection however, symptoms can differ in nature and severity. Infection early during pregnancy can interfere with embryo implantation, while during late pregnancy, infection can result in embryo loss or preterm delivery[138]. A relatively low dose administered at mid-pregnancy most frequently leads to behavioral alterations in the offspring without overt obstetric complications[138]. Since different infections (viral, bacterial, parasitic) lead to similar behavioral defects, it has been hypothesized that the maternal immune reaction, rather than the infectious agent itself, is responsible for the deleterious effects on the developing embryo[139]. Indeed, Smith *et al.* 2007 showed that injection of only the pro-inflammatory cytokine IL6 was sufficient to induce behavioral deficits in the offspring while genetic deletion of the gene encoding IL6 prevented the onset of behavioral deficits induced by Poly IC[140].

Of note, agents other than influenza virus, Poly IC or LPS are also used to model *in utero* risk factors for neuropsychiatric disorders, including stress, CMV infection, Group B Streptococcus, maternal allergic asthma, maternal autoimmune disease and alcohol and valproic acid exposure[141-146]. Most recently, ZIKA virus has received much attention because of its detrimental effects on the developing brain. Although these environmental insults have also been associated with alterations in behavior in the offspring, the results discussed below will focus on MIA induced by influenza virus, Poly IC or LPS.

1.4.1 Consequences of MIA in the offspring

Maternal immune activation in rodents revealed several alterations in the offspring, both in the short and the long term, and on several levels including behavioral and neuropathological changes.

1.4.1.1 Behavioral deficits

As the initial link in humans was made between MIA and schizophrenia, the earliest studies in rodents focused on behavioral changes reminiscent of schizophrenia symptoms. While psychosis, a symptom required for the diagnosis of schizophrenia, cannot be analyzed in mice or rats, other symptoms can be modeled in rodents. In a later phase, MIA was also linked to the development of ASD, for which also several behavioral tests in rodents exist. While a plethora of behavioral tests have been developed to examine different aspects of behavior modeling schizophrenia and ASD, I will only review the most used and most robust ones.

The prepulse inhibition (PPI) to acoustic startle test is a measure of sensorimotor gating that is also used in humans. In the PPI test, a weak non startle stimulus (prepulse) precedes the presentation of a stronger startle stimulus, the latter of which is inhibited by the prepulse. In schizophrenia, PPI is usually disrupted (meaning that the prepulse will not inhibit the startle stimulus) which is thought to reflect an inability to filter out non-relevant stimuli[147]. Upon MIA in rodents, this behavioral test is most reproducibly affected (as reviewed by [135]). Interestingly, in 2008, Meyer *et al.* reported that the timing of the infectious stimulus determines whether a deficit in PPI emerges later in life: PPI is affected after MIA during mid-gestation but not after MIA in late gestation[148]. Moreover, the age at which the offspring is tested is crucial to detect PPI deficits: in pubescent mice, PPI deficits usually do not occur while they do at adolescent ages, which corresponds to the onset of positive symptoms in schizophrenia[149, 150].

Schizophrenia patients typically have an increased sensitivity to dopamine stimulating drugs, including amphetamine. As a read-out for increased amphetamine sensitivity in rodents, locomotor activity before and after administration of the drug is measured. Although some studies report a decreased locomotor activity upon amphetamine administration, the vast majority shows an increase[151-155]. Unlike changes in PPI, increased locomotor activity in response to amphetamine is independent of the gestational age at which MIA is induced[148].

To assess working memory deficits in rodents, the delayed non-matching to sample test is used. In this test, a sample stimulus is presented to the animal. After a short time interval, the same sample stimulus is presented together with a novel stimulus and the animal is required to select the novel one in order to receive a reward. Working memory deficits are often described in offspring that was prenatally exposed to an inflammatory agent[156-158].

The social preference/social novelty test is widely used to detect deficits in social interaction in rodents, which can be related to both ASD and schizophrenia symptomatology. Rodents are social animals that tend to explore and interact with their conspecifics. Moreover, when presented with a familiar and an unfamiliar animal, they usually spend more time exploring the unfamiliar one. Most studies on MIA find a decrease in time spent with a conspecific, either or not accompanied by a decrease in preference for social novelty[138, 157, 159-161].

Finally, an increase in repetitive behavior, which is typically used to test for ASD-like behavior, is usually measured by the marble burying test. Here, the testing animal is placed in a cage containing glass marbles for a certain amount of time. At the end of the test, the amount of marbles buried is determined, whereby an increase in buried marbles is believed to correspond to increased repetitive behavior. Several studies focused on MIA report an increased marble burying in offspring prenatally exposed to inflammation[159, 162, 163].

Deficits in at least one of these behavioral measures have been found in all kinds of MIA models, using Poly IC, LPS or influenza virus in either rats or mice at different gestational time points of MIA exposure. On the other hand, no single MIA model captures deficits in all of these behavioral measures. This might not be too surprising as MIA in humans has been associated with both ASD and schizophrenia which are already two distinct disorders, and even within one disorder, there is high variability in symptom presentation between patients.

1.4.1.2 Neuropathological alterations

A large amount of studies providing hints towards anatomical, cellular and molecular deficits underlying the behavioral changes in MIA offspring have been published in the last decade. Abnormalities have been described on several

levels, including structural changes of the brain and neurons, altered synaptic function and protein expression, differences in neurotransmitters levels and disturbed neurodevelopmental processes.

Gross morphological brain changes described in several MIA models include decreased cortical thickness[164, 165] and increased lateral ventricular volume[166]. Total brain size has been found to be either increased[164, 165, 167] or decreased[168]. Several white matter abnormalities have been found including increased cell death and decreased myelin basic protein (MBP) immunostaining in white matter regions, decreased numbers of immature oligodendrocytes and oligodendrocyte precursors[169-173], impaired axon fasciculation in corpus callosum[64] and alterations in gene and protein expression in proteins involved in myelination[174, 175].

Several other abnormalities on the cellular level have been found, including a decreased dendritic length, arborization and synaptic density of hippocampal and prefrontal neurons[176, 177], decreased density of perineuronal nets[178] and decreased neurogenesis in the hippocampus[179-182]. In addition, at the level of the synapse, alterations in the levels of pre- (synaptophysin and bassoon) and postsynaptic (PSD95 and syngap) proteins were reported[183, 184], as well as reduced spine dynamics[162].

A hand full of studies reported alterations in functional network connectivity, including a reduction in theta oscillations[185], enhanced neuronal excitability[186] and a reduction in the coherence between the prefrontal cortex and the hippocampus[187]. In addition, Oh-nishi *et al.* 2010 found a higher short term synaptic plasticity but decreased long term synaptic plasticity[188].

Several studies described alterations in the dopaminergic system, hyperactivity of which is believed to contribute to the positive symptoms of schizophrenia, while hypoactivity of the dopamine system in the prefrontal cortex is thought to contribute to negative and cognitive symptoms[189]. Evidence is found both for increased and decreased dopamine neurotransmission: decreased tyrosine hydroxylase (TH) immunoreactive neurons and dopamine levels were found[190-199] as well as increased dopamine levels and TH immunoreactivity[197, 200-203]. Decreased D1R, D2R and dopamine transporter (DAT) expression[148, 204],

increased dopamine (DA) dopamine turnover and decreased D2R binding[205] have also been found. Finally, spontaneously active dopamine neurons were found to be reduced[201].

The GABAergic system, which is believed to cause cognitive symptoms in schizophrenia and ASD, also seems to be affected in MIA offspring: almost all components of the system have been found to be either increased or decreased depending on the brain region investigated: GAD65, GAD67, VGAT, NKCC1, KCC2, GABA and GABAa receptor subunits[157, 191, 206-208]. GABAergic transmission specifically from parvalbumin positive interneurons was found to be reduced[206] as well at the expression of genes that regulate interneuron migration[209].

Other neurotransmitter systems that have been investigated in the context of MIA are serotonin, glutamate and choline. While serotonin could contribute to impairments in emotional processing and affection, glutamate dysfunction could play a role in positive and cognitive symptoms[210]. The amount of choline acetyl transferase and the number of cholinergic neurons were increased[211]. On the other hand, serotonin levels are either increased or decreased[168, 199, 203, 212, 213] while expression of the serotonin transporter protein and 5HT2a receptors is increased[214, 215]. A reduction in NR1 receptor subunit immunoreactivity and GABA levels were described [148, 191] as well as an increased ratio of AMPA/NMDA receptor current[216, 217].

1.4.1.3 Inflammation of the fetal brain

Several studies have pointed towards fetal neuroinflammation as a potential mediator of MIA on the developing brain, as they found increased cytokine levels in the brains of prenatally exposed fetuses. The source of these cytokines however is not always clear, as it can originate from either the mother, the placenta, or the fetus itself. The pro-inflammatory cytokine IL6 has been shown to be able to cross the placenta after induction of MIA in mid-gestation[218-220], suggesting a contribution of maternal cytokines to the fetal neuroinflammation. On the other hand, the placenta itself is also capable of producing cytokines in response to MIA. Hsiao *et al.* 2011 showed a 16-fold increase in both IL6 mRNA and protein in the placenta, 3 hours after induction of MIA[221]. Alternatively,

cytokines could originate from the fetal brain, as evidenced by increased mRNA levels of several cytokines in fetal brain lysates[170, 181, 222, 223]. Aside from these acute effects on cytokine levels in the fetal brain, Garay *et al.* 2013 measured the levels of 23 cytokines in the brain of MIA offspring at different postnatal ages and found chronic region- and age-specific changes in brain cytokine levels[224]. As the resident immune cells of the brain, microglia have been suggested to be the source of inflammatory cytokines in the brains of MIA offspring. However, findings on microglia activation in MIA offspring are inconsistent, with several groups reporting an activated microglial phenotype while others find no difference compared to controls (a.o.[161, 182, 225-227]).

1.4.2 Mechanisms contributing to the effects of MIA

1.4.2.1 A role for epigenetics in MIA

How such a relatively short environmental insult causes such detrimental effects later in life is still a matter of debate. Even long after the inflammatory stimulus, several cytokines and chemokines have been found to be upregulated in MIA offspring. One plausible way to explain this phenomenon is through epigenetics, which refers to the process where gene expression is affected by changes in the chromosome that are not changes in the nucleotide sequence. The best known epigenetic mechanism is methylation, where a methyl residue is placed on cytosine nucleotides in the promotor region of a gene to repress gene transcription. Also histone acetylation, where an acetyl group is transferred to a histone resulting in a more relaxed structure of the chromatin that is associated with greater levels of gene transcription, represents an important mechanism for epigenetic regulation. So far, only a hand full of studies investigated the effect of MIA on epigenetic changes. In juvenile and adolescent MIA offspring, a global decrease in histone acetylation and methylation was found respectively[228, 229]. Looking at promotors from specific genes, MIA offspring displayed decreased methylation of the MeCP2 gene, which itself is an important epigenetic regulator[228], while hypermethylation occurred on GAD1 and GAD2 (the genes encogin GAD65 and GAD67) promotor regions[230] and histone 3 and 4 acetylation increased on serotonin transporter gene[231]. Most recently, Richetto *et al.* 2016 investigated genome wide DNA methylation in the prefrontal cortex

of adult offspring exposed to mid or late pregnancy MIA[232]. After MIA in mid-pregnancy, methylation changes occurred mainly in genes involved in the Wnt-signaling pathway while MIA in late pregnancy mainly induced altered methylation in genes belonging to the GABAergic system. 167 genes were altered in both conditions, including genes associated with neurodevelopmental disorders, such as the cell adhesion molecules Neuregulin 1 and Neurexin 2, the transcription factor NeuroD6 and the receptor protein-tyrosine kinase Ephrin B3[232].

1.4.2.2 The placenta as an important mediator of MIA effects

The placenta, which ensures exchange of nutrients, waste and gases between fetal and maternal blood, promotes immunological tolerance during pregnancy to prevent repulsion of the fetus. However, placental immune cells retain the capacity to detect and react to maternal infection and inflammation[138]. Disruption of the maternal milieu can influence vital aspects of placental structure and function, including integrity of the protective transplacental barrier, nutrient and oxygen exchange and placental endocrine action[233]. Few studies in rodents have examined the effects of MIA on the placenta.

In 2010, Girard *et al.* showed that MIA induced by LPS resulted in decreased placental perfusion and increased cellular loss and macrophage infiltration. In addition, IL1β, IL6 and TNFα were increased. Strikingly, maternal administration of a IL1 receptor antagonist was able to rescue these placental abnormalities[234]. In 2011, Hsiao and Patterson showed an increase in IL6 mRNA and maternally derived IL6 protein in the placenta as well as an increase in acute phase genes[221]. Increases in placental macrophages, granulocytes and natural killer cells indicated placental immune activation and the growth hormone-insulin-like growth factor axis was disrupted[221]. Bronson and Bale 2014 provided evidence for placental alterations in the pro-apoptotic factor Fas ligand which, within the placenta, mediates immune tolerance of the embryo, vascular remodeling and trophoblast turnover. Additionally, transcription of several inflammatory genes was upregulated in male placentas but not in female placentas, possibly contributing to the sex bias in ASD and schizophrenia[235]. The maternal-fetal LIF signaling pathway seems to be affected by MIA as well, leading to a reduction in fetal LIF which impairs neural stem/progenitor cell

proliferation[236]. Additionally, MIA causes an increase in placental synthesis of serotonin and delivery to the fetus, resulting in disruption of fetal serotonergic axon outgrowth[237]. Most recently, Wu *et al.* 2016 highlighted the key role of placental IL6 signaling in mediating effects of MIA onto the offspring. Specifically, they showed that deletion of the IL6 receptor (IL6Ra) specifically in placental trophoblasts prevented the inflammatory cascade in placenta and fetal brain. Moreover, loss of cerebellar purkinje cells and onset of behavioral deficits in the offspring were no longer observed [160].

1.4.3 MIA in non-rodent models

While rodent models are very useful for examining the effects of MIA on the offspring, they suffer from a few experimental draw-backs. Firstly, anatomically speaking, brain development at birth in rodents is comparable to that of beginning of third trimester in humans. As reviewed by Semple *et al.* 2013, oligodendrocyte maturation and establishment of the blood brain barrier occurs in gestational week 23-32 in humans but after birth (postnatal day 1-3) in rodents[238]. Furthermore, the peak in gliogenesis and the brain growth spurt occurs around P7-10 in rodents but in the final weeks of gestation in humans. Secondly, placental composition is different between rodents and humans, with humans having only one layer of trophoblast separating maternal from fetal blood circulation while in rodents, there are 3 layers of trophoblast[239]. As such, transfer of nutrients across the placenta might be different between humans and rodents. Finally, neuropsychiatric disorders are characterized by deficits in complex behaviors that are often mediated by the prefrontal cortex, a cortical structure that is not as well developed in rodents. This prompted several research groups to confirm results found in rodents in species that have characteristics more closely resembling humans. Among the best studied non-rodent species is the nonhuman primate, including the rhesus monkey, which shows many similarities with human physiology and anatomy. Importantly, the rhesus monkey lives in a complex, hierarchical social system and communicates in a human-like fashion using facial expressions and social gestures, making them more suitable to study neuropsychiatric disorders[240].

The first study in monkeys was published in 2010. Short *et al.* induced MIA in pregnant rhesus macaque monkeys using the H3N2 influenza virus in the third trimester of pregnancy[241]. In this study, they found decreased gray matter volumes and increased lateral ventricular size in young MIA offspring. However, when MIA was induced with LPS, Wilette *et al.* 2011 found an increase in total brain and white matter volume with no changes in ventricular size[242]. While stress-induced cortisol levels were increased in offspring from LPS treated mothers, this was not the case in offspring from influenza infected mothers. Offspring from LPS treated mothers showed deficits in PPI but not in social or exploratory behavior.

Another study on monkeys from 2014 used Poly IC to induce MIA either at the end of the 1st or 2nd trimester of pregnancy[240]. While offspring from both 1st and 2nd trimester infected mothers displayed repetitive behaviors, only offspring from 1st trimester MIA mothers showed altered social interaction. This deficit in social interaction was recently investigated in more depth using non-invasive eye-tracking [243]. 1st trimester MIA offspring fixed less on the eyes when monkey faces with fearful face expression were displayed, suggesting abnormal attention to salient social information. In addition, they displayed inappropriate social behavior (low visual attention and high tendency to physically approach). MIA during the first trimester was also associated with brain pathological features in offspring of 3.5 years of age[244]. While basal dendrite and cell soma were not affected, apical dendrites were smaller in diameter and had a greater amount of oblique dendrites. The same research group recently showed that plasma cytokine levels as well as cellular responses of isolated peripheral immune cells from MIA offspring were increased both at 1 year of age and 4 years of age[245].

Detrimental effects of maternal immune activation could recently also be partly replicated in swine. Antonson *et al.* 2017 induced porcine reproductive and respiratory syndrome virus infection in late gestation and found that social interaction was reduced in adult offspring[246].

1.4.4 MIA and schizophrenia and ASD comorbidities

As ASD and schizophrenia are both characterized by a high rate of co-morbidity with other disorders, several studies have aimed to investigate the link between MIA and these comorbidities. The most prominent neurological disorder associated with ASD is epilepsy. In 2013, Pineda *et al.* showed that offspring from mothers treated with Poly IC or IL6+IL1β had higher baseline hippocampal excitability and developed earlier and more convulsions during hippocampal kindling[247]. Interestingly, induction of MIA either by IL6 or IL1β alone did not result in increased susceptibility to seizures. Similarly, Washington *et al.* 2015 and Yin *et al.* 2015 showed increased susceptibility to seizures in MIA offspring when seizures were induced by administration of kainic acid[248, 249].

Gastrointestinal abnormalities, including inflammatory bowel disease, are often reported in ASD patients. Hsiao *et al.* 2013 recently showed the complex interaction between commensal gut bacteria and behavior in relation to ASD symptomatology[250]. MIA offspring showed several abnormalities of the gut: permeability was increased, intestinal cytokine profiles were altered and the composition of the gut microbiome was changed. Strikingly, treatment with microbiota partially prevented these gut alterations along with stereotypical and anxiety-like behavior and deficits in sensorimotor gating. As suggested by Rao *et al.* 2016, dysfunction of the enteric nervous system, which plays an important role in the integrity of the intestinal barrier, might underlie these gastrointestinal abnormalities[251].

Schizophrenia patients have a high tendency to develop depression. Khan *et al.* 2014 and Depino *et al.* 2015 both showed depression-like behavior in MIA offspring, either as a reduction in sucrose preference (anhedonia) and/or increased immobility in the forced swim test (behavioral despair)[212, 214]. Both studies showed accompanying alterations in the hippocampus, including a decrease in adult hippocampal neurogenesis and a decrease in serotonin and noradrenalin levels. Aiming to elucidate the mechanism behind the increase in depressive-like symptoms after MIA, Reisinger *et al.* 2016 showed an increase in acetylation of histone 3 and 4 at the promoter of the serotonin transporter in the hippocampus, resulting in an increased expression[231].

1.4.5 The role of MIA in the 'double hit' hypothesis

As infection during pregnancy obviously is not fully correlated with the development of neuropsychiatric disease later in life, it is suggested that MIA interacts with other genetic and environmental risk factors and that this precise interaction determines the outcome. This phenomenon is called the double-hit hypothesis and few studies so far have investigated the interaction between MIA and a second risk factor, mostly a genetic risk factor. The most studied genetic risk factor in this regard is DISC1 (Disrupted in Schizophrenia 1).

In mice constitutively expressing a truncated version of the DISC1 protein, MIA-induced deficits in working memory, social interaction and dopaminergic sensitivity (as assessed by MK801-induced hyperactivity). While the antipsychotics clozapine and haloperidol improved MK801-induced hyperactivity, only clozapine improved working memory deficits and social interaction was not improved by either of the antipsychotics[252, 253]. Similarly, inducible expression of truncated DISC1 throughout life in combination with prenatal infection induced anxiety and depression-like behavior and deficits in social interaction. Curiously, when truncated DISC1 was expressed only during a restricted amount of time (prenatal and early postnatal or only late postnatal), behavioral deficits did not occur[254]. Finally, in mice carrying a point mutation in the Disc1 gene, MIA induced a worsening of deficits in social interaction, working memory and prepulse inhibition[255].

Likewise, MIA offspring with a heterozygous deletion of the Neuronal acetylcholine receptor subunit α7gene displayed increased anxiety-like behavior, Nurr1 (a nuclear receptor) deficiency aggravated hyperactivity and deficits in PPI and latent inhibition and mutations in Tsc2 worsened deficits in social interaction[256-258].

One of the environmental risk factors recently investigated as a second hit is postnatal stress. While Yee *et al.* 2011 did not find evidence for an interaction between MIA and postnatal stress on anxiety-like behavior, Giovanoli *et al.* 2013 showed synergistic effect of MIA and peripubertal stress on deficits in PPI and amphetamine sensitivity. Applying stress during adolescence instead of peripuberty abolished these synergistic effects on behavior[259, 260].

1.4.6 Prevention of MIA-induced behavioral deficits

Having established that offspring exposed to inflammation *in utero* display symptoms reminiscent of neuropsychiatric disorders and having identified molecular pathways that could be involved in the etiology, few groups have employed this knowledge on possible pathological mechanisms to study the prevention of symptom onset. Most recent research showed that intervention with several kinds of substances is able to either prevent or revert some of the behavioral deficits and/or neuropathological abnormalities caused by MIA.

Minocycline is a tetracycline antibiotic with anti-inflammatory properties that is used in schizophrenia patients as add-on therapy as it has been shown to improve cognitive and negative symptoms. In 2014, Zhu *et al.* showed that treatment with minocycline during adolescence was able to prevent deficits in PPI, locomotor activity and social interaction in adulthood after MIA during early gestation[161]. Similarly, Mattei *et al.* 2014 showed in rats that PPI deficits induced by MIA in mid-gestation could be ameliorated by minocycline treatment during adulthood[261]. Even more, Giovanoli *et al.* 2016 were able to prevent the additive effects of a second hit (postnatal stress) by simultaneous minocycline treatment[262]. Curiously, while in the study of Zhu *et al.* 2014 and Giovanoli *et al.* 2016 microglia activation could be prevented by minocycline, this was not the case in the study of Mattei *et al.* 2014.

Another treatment that is used for its anti-inflammatory properties is the dietary supplementation of omega 3 polyunsaturated fatty acids. Enriched diet in MIA offspring either from adolescence on or already during pregnancy could prevent anxiety, deficits in PPI, social interaction and repetitive behavior but not increased sensitivity to amphetamine[263, 264]. Finally, Tronnes *et al.* 2016 administered the anti-inflammatory hormone progesterone to the pregnant mother before induction of MIA and showed that this treatment was able to prevent the effects of MIA on cortical neurogenesis in the embryo (altered amount of Tbr2+ intermediate neuronal progenitors) but not microglia activation[265].

Improvements in behavior could also be achieved by treatments impacting on specific signaling pathways. While MIA induced a decrease in cortical,

hippocampal and striatal D-serine, an endogenous co-agonist of the NMDA receptor, in adult offspring, early supplementation with D-serine prevented the onset of working memory deficits[266]. Nicotine, an Nicotinic acetylcholine receptor agonist, could not rescue working memory deficits but improved PPI and latent inhibition deficits[158]. Choline treatment throughout pregnancy until weaning improved anxiety-like and repetitive behavior but not deficits in PPI[258]. Antipurinergic therapy was able to prevent deficits in social behavior and sensorimotor coordination as well as synaptosomal structural abnormalities and cerebellar purkinje cell loss[267]. Supplementation with a Tropomyosin receptor kinase B (TrkB) agonist ameliorated PPI and working memory deficits and rescued BDNF-TrkB signaling and parvalbumin immunoreactivity in prefrontal cortex and hippocampus[150].

In conclusion, activation of the maternal immune system during pregnancy, either alone or in combination with other genetic or environmental risk factors, induces neuropathological and behavioral changes in the offspring, reminiscent of schizophrenia and/or ASD. While the exact mechanism is not clear, several factors appear to be involved in mediating the effects of MIA on the offspring, including the maternal and fetal immune system, the placenta and epigenetic modification of the fetal genome. Nevertheless, recent findings provide evidence for the benefits of early intervention in the behavioral outcome of MIA offspring.

1.5 Disrupted in schizophrenia 1

As described above, microglia play an important role in the normal development and functioning of the brain and microglial dysregulation is likely to contribute to the development of neuropsychiatric disorders. While the role of several genetic risk factors for schizophrenia and ASD in neurons has widely been investigated, data on their function in microglia are scarce. One such hallmark risk gene for neuropsychiatric disease is disrupted in schizophrenia 1 (DISC1). The Disc1 gene was initially reported to be involved in a Scottish family where there was a segregation of mental illnesses, including schizophrenia, bipolar disorder and depression. The Disc1 gene is located on chromosome 1q42 and consists of 13 exons which give rise to more than 40 different transcripts of Disc1. Although it

is not yet completely clear how many of these transcripts are actually translated into functional proteins, western blot experiments showed that multiple DISC1 protein species exist, the most commonly described having a molecular weight of around 100 or 75 kDa[268].

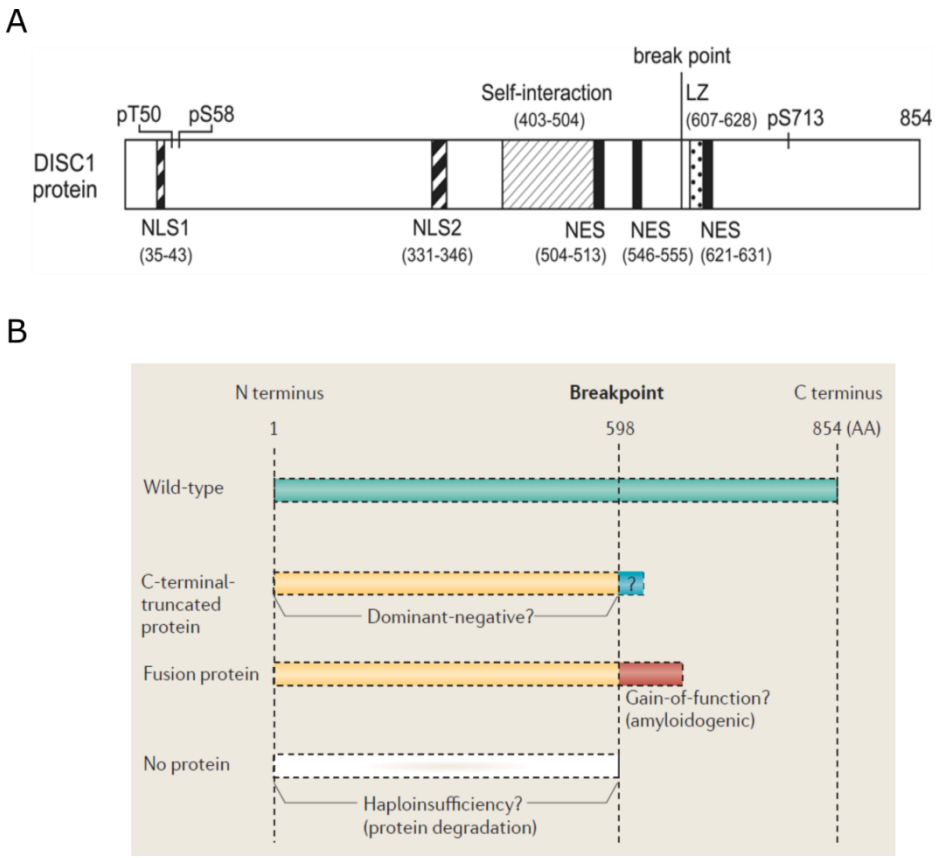


Figure 1.5: The DISC1 protein and consequences of its truncation. (A)Functional domains of the DISC1 protein. The self-association domain, phosphorylation sites (pT50, pS58 and pS713), leucine zipper (LZ) motif, nuclear localizing signals (NLSs), and nuclear export signals (NESs) are indicated with the amino acids between brackets. The break point created by the t(1,11) chromosomal translocation is shown. Figure adapted from Hikida *et al.* 2012[269]. (B) Possible mechanisms of dysfunction of truncated DISC1. The t(1,11) chromosomal translocation found in the Scottish pedigree creates a stop codon at amino acid 598. This could lead to the formation of either a truncated protein with possible dominant negative function, a fusion protein with possible gain of function or no protein resulting in haploinsufficiency. Figure adapted from Brandon and Sawa 2011[270].

The full length DISC1 protein consists of 854 amino acids and contains nuclear localizing and export signals which are important for intracellular localization of the protein (Figure 1.5A). Via the self-interaction domain in the middle of the protein, DISC1 can form homodimers or oligomers which is required for its proper functioning. Finally, DISC1 has at least three phosphorylation sites (T50, S58 and S713) which control the switch from one interaction partner to another[269].

In the Scottish family, a balanced chromosomal translocation between chromosome 1 and 11 results in a truncated DISC1 protein lacking 256 C-terminal amino acids. The exact consequence of this truncation is not entirely clear, but a few hypotheses exist[270] (Figure 1.5B). Firstly, the truncated protein could be prone to degradation and thereby causes haploinsufficiency. Alternatively, the truncation might result in a stable protein that retains part of its functionality or exerts dominant-negative effects by binding to and thereby neutralizing the function of full length DISC1. Finally, a fusion protein could be formed with the remaining sequence on chromosome 11 (the Bowmay gene), resulting in a new protein with unknown functions. Of note, besides the disruption of the Disc1 gene by chromosomal translocation, also more subtle single nucleotide polymorphisms have been associated with psychiatric disorders, the most common being R264Q, L607F and S704C.

The DISC1 protein is a versatile protein due to its many interactions with other proteins in different subcellular locations. A comprehensive list of DISC1 interacting proteins was provided by Camargo *et al.* 2007, identifying 158 interactions with 127 proteins and therefore called the DISC1 158 network. Some of the DISC1 interactors also interact with each other, suggesting they take part in common pathways. DISC1 connects several pathways involved in neurodevelopment and synapse formation, including Akt/mTOR, GSK-3, D2R, PDE4/cAMP and GSK-3/ β-catenin (Figure 1.6).

DISC1 is located at the nucleus where it interacts with transcription factors to globally modulate gene expression. DISC1 is also associated with centrosomes, which make up the main cytoskeleton-arranging centers of the cell and thus regulate mitosis, differentiation and migration. Another location where DISC1 can be found is the mitochondria which provide the main source of energy for

cells. Finally, DISC1 localizes to motor proteins that provide trafficking of proteins and vesicles to the synapse and recycling back to the cell soma[271].

Given its plentiful interaction partners, it is of no surprise that DISC1 mediates several functions during brain development and maintenance: interference with normal DISC1 expression resulted in altered neuronal proliferation and neuronal migration, impaired neuronal morphology and dendritic growth and altered synaptic plasticity (as reviewed by [271]).

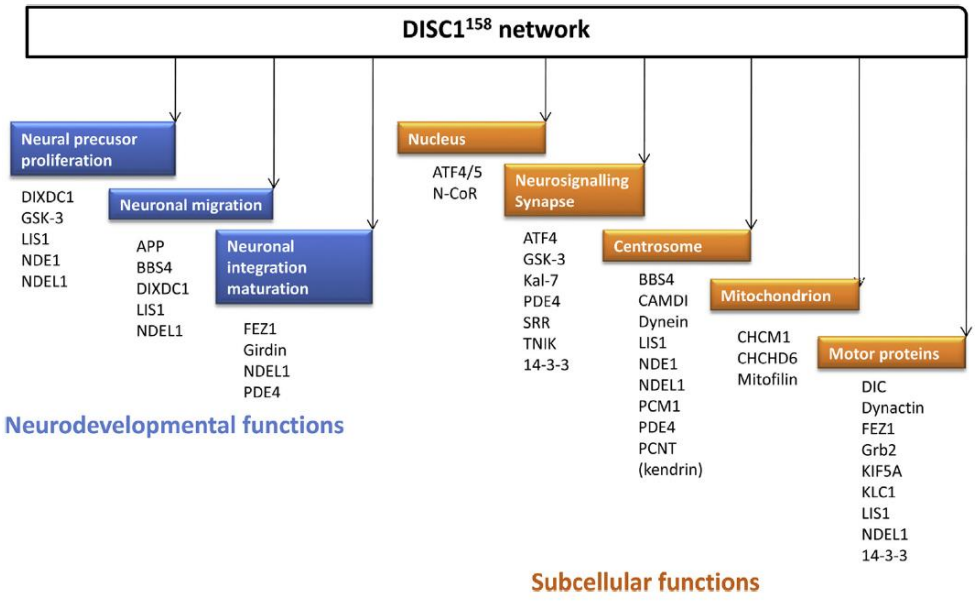


Figure 1.6: The DISC1 158 network. DISC1 interaction proteins are classified by their neurodevelopmental and/or subcellular functions. Figure from Lipina and Roder 2014[271].

While most studies had focused on the function of DISC1 in neurons, Wood *et al.* 2009 provided the first evidence towards expression of DISC1 in oligodendrocytes in zebrafish[272]. In 2010, Seshadri *et al.* reported on the expression of DISC1 in astrocytes, oligodendrocytes and microglia both in human cortical sections and rat primary cortical cultures[273]. Although results on the precise function of DISC1 in glial cells is scarce, Katsel *et al.* 2011 showed a functional role of DISC1 in oligodendrocytes: expression of human mutant DISC1 in mouse forebrain induced increased proliferation of oligodendrocyte precursors and premature differentiation[274]. In addition, Hattori *et al.* 2014

used RNA interference and overexpression of full length or truncated DISC1 to establish the effect on oligodendrocyte differentiation *in vitro*[275]. Overexpression with full length DISC1 reduced oligodendrocyte differentiation while either knockdown of DISC1 or overexpression of truncated DISC1 promoted oligodendrocyte maturation.

The group of Pletnikov developed a mouse model with selective expression of mutant human DISC1 in astrocytes to investigate the functions of DISC1 in this cell type. Ma *et al.* 2013 implicate a role for DISC1 in astrocytic D-serine production and consequently NMDA neurotransmission[276]. Specifically, overexpression of mutant DISC1 in astrocytes disrupted its binding to serine racemase, the enzyme that generates D-serine, leading to degradation of the latter and thus decreased production of D-serine. Using primary astrocyte cultures and hippocampal tissue from these mice, Abazyan *et al.* 2014 showed an altered expression of vesicular glutamate transporters and NMDA receptor subunits upon overexpression of mutant DISC1, possibly compensating the decreased D-serine production[277]. Finally, co-culturing of wild type neurons with astrocytes expressing mutant DISC1 resulted in decreased dendritic arborization and density of excitatory synapses, both of which could be rescued by supplementation with D-serine[278]. Strikingly, data on the function of DISC1 in microglia are currently lacking.

1.6 Objectives of this thesis

In this thesis, we hypothesized that risk factors for neuropsychiatric disorders such as prenatal infection and mutations in the Disc1 gene affect normal development of the brain through alteration of microglia function and thereby contribute to the increased disease risk (Figure 1.7). To test this hypothesis, the objective of this thesis was to:

(1) assess schizophrenia and ASD-like behavioral changes in male offspring prenatally exposed to a single Poly IC injection at E11.5 (**Chapter 4**).

(2) investigate the effect of maternal immune activation by single or double injection of the viral mimic Poly IC on the activation of embryonic microglia and the positioning of pyramidal neurons. Microglia activation was assessed in the developing cortex and hippocampus by determining microglia density and expression of activation markers IL1β, Mac-2 and iNOS. For analyzing cortical positioning of pyramidal neurons, progenitors were fluorescently labeled *in utero* by electroporation at different embryonic ages and distribution of labeled neurons in the cortex determined (**chapter 2 and 5**).

(3) develop a genetic mouse model for microglia depletion by tamoxifen inducible recombination of the PU.1 gene, which is an important transcription factor for microglia development and maintenance (**Chapter 3**).

(4) investigate the expression of DISC1 in a microglia cell line and in primary microglia and modify endogenous DISC1 protein levels to investigate its role in microglial phagocytosis. Phagocytosis is a process that is highly dependent on cytoskeletal rearrangement and dynamic remodeling of the cytoskeleton has been shown to be altered in neurons lacking DISC1. As microglial phagocytosis is important during brain development (in for example progenitor pool size and synaptic refinement), impairment of this process by DISC1 mutations might affect normal brain development and thereby contribute to neuropsychiatric disease pathogenesis (**Chapter 6**).

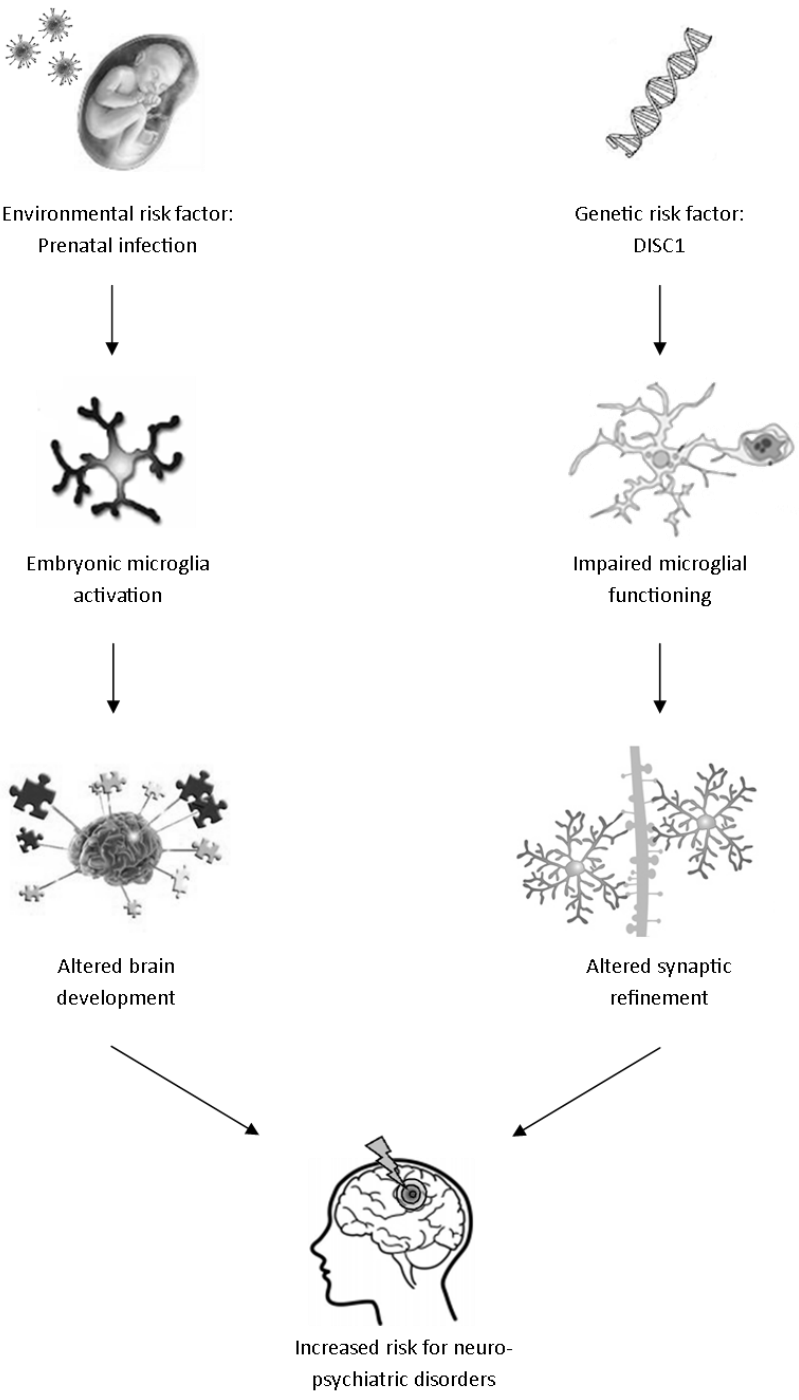


Figure 1.7: Schematic representation of the hypothesis of this thesis.

Chapter 2

Effects of MIA on the activation status of embryonic microglia

Based on:

Smolders S, Smolders SMT, Swinnen N, Gärtner A, Rigo J-M, Legendre P and Brône B (2015) Maternal immune activation evoked by polyinosinic:polycytidylic acid does not evoke microglial cell activation in the embryo. Front. Cell. Neurosci. 9:301.

2.1 Abstract

Several studies have indicated that inflammation during pregnancy increases the risk for the development of neuropsychiatric disorders in the offspring. Morphological brain abnormalities combined with deviations in the inflammatory status of the brain can be observed in patients of both ASD and schizophrenia. It was shown that acute infection can induce changes in maternal cytokine levels which in turn are suggested to affect fetal brain development and increase the risk on the development of neuropsychiatric disorders in the offspring. Animal models of MIA reproduce the etiology of neurodevelopmental disorders such as schizophrenia and ASD. In this study, the Poly IC model was used to mimic viral immune activation in pregnant mice in order to assess the activation status of fetal microglia in these developmental disorders. Because microglia are the resident immune cells of the brain, they were expected to be activated due to the inflammatory stimulus. Microglial cell density and activation level in the fetal cortex and hippocampus were determined. Despite the presence of a systemic inflammation in the pregnant mice, there was no significant difference in fetal microglial cell density or immunohistochemically determined activation level between the control and inflammation group. These data indicate that activation of the fetal microglial cells is not likely to be responsible for the inflammation induced deficits in the offspring in this model.

2.2 Background

MIA has been shown to increase levels of cytokines in the fetal brain, originating from maternal, placental and/or embryonic tissue. The pro-inflammatory cytokine IL6 is known to induce activation of adult microglial cells, leading to the production of pro-inflammatory factors, such as nitric oxide, reactive oxygen species, proteolytic enzymes and TNFα by microglial cell cultures[279], microglial proliferation (*in vitro*)[280] and infiltration (*in vivo*)[281] or the upregulation of microglial CX3CR1, making them more sensitive to fractalkine signaling[282]. An imbalance in cytokine levels caused by MIA might thus be able to activate embryonic microglia, even at early developmental stages, and alter their normal functions. This can trigger a cascade of events that could lead to developmental

defects observed in the offspring of LPS or Poly IC treated pregnant mice. Indeed, MIA evoked by LPS injection evoked microglia activation and enhanced phagocytosis of neural precursors by microglia at prenatal stages in rats[57]. However, whether Poly IC-induced MIA results in the activation of embryonic microglia during fetal development is currently unknown.

To determine to what extent MIA evoked by Poly IC can alter cortex invasion by microglia and/or change embryonic microglial cell activation state, we evoked MIA using a single (at E11.5) or a double injection (at E11.5 and E15.5) of Poly IC. This developmental time window is an important time point for cortex invasion by immature microglia as their cell density dramatically increases during this period[38]. In the cortex and hippocampus of embryonic MIA offspring, we determined microglial cell density and determined the expression of inflammatory markers inducible Nitric oxide synthases (iNOS), the lectin Mac2 and IL1β.

2.3 Materials and methods

2.3.1 Animals

All experiments were conducted in accordance with the European Community guiding principles on the care and use of animals and with the approval of the Ethical Committee on Animal Research of Hasselt University. Mice were maintained in the animal facility of the Hasselt University in accordance with the guidelines of the Belgian Law and the European Council Directive. To visualize microglia in the embryonic cortex, the transgenic CX3CR1-eGFP knock-in mice [283] were used in which the first 390 base pairs of the gene encoding CX3CR1 were replaced by the gene encoding eGFP. The $CX3CR1^{+/eGFP}$ embryos used in this study were obtained by crossing wild type C57BL/6 females with homozygous $CX3CR1^{eGFP/eGFP}$ male mice (obtained from the European Mouse Mutant Archive – EMMA with the approval of Stephen Jung[283]). The day of conception was designated as E0.5.

2.3.2 Maternal immune activation

At day E11.5 (single injection) or at E11.5 and E15.5 (double injection) mice received i.p. a dose of Poly IC (20mg/kg) (Sigma-Aldrich, Bornem, Belgium) or vehicle (saline). Five hours after injection the maternal blood was collected, the serum was aliquoted and stored at -80°C until the IL6 assay was performed[140, 284]. The maternal IL6 concentrations were determined using the Mouse IL6 ELISA Kit from Thermo Scientific (Rockford, Illinois, USA), following the manufacturer's instructions. The analysis was conducted using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

2.3.3 Fluorescent immunostaining of embryonic brains

Pregnant mice were sacrificed and embryonic tissue processed as described before[38]. The heads of E11.5 and E12.5 embryos were fixed in 4% paraformaldehyde (PFA) for 3 hours at 4°C and 5 hours for E17.5 embryos. After fixation, the embryonic heads were cryoprotected overnight in phosphate-buffered saline (PBS) + 30% sucrose, frozen in optimal cutting temperature compound (Tissue-Tek) and stored at -80°C until sectioned. Ten micrometer-thick coronal tissue sections were cut on a Leica CM1900 uv cryostat, mounted on Superfrost Plus glasses and stored at -20°C until staining.

To check whether embryonic microglia can be directly activated by Poly IC, IL6 or LPS, 300-µm thick coronal brain slices (E15.5) were cultured for 24 hours with either saline, Poly IC (50 µg/ml), IL6 (10 ng/ml) or LPS (1 µg/ml). To this end, pregnant mothers were euthanized at E15.5. Embryonic brains were isolated in ice-cold PBS-glucose (pH 7.4; 25mM), embedded in 3% low melting agarose (Fisher Scientific) and sliced coronally at a thickness of 300 µm using a Microm HM650V Vibrating Blade Microtome. Slices were mounted on MilliCell organotypic inserts (Millipore) and maintained in semi-hydrous conditions at 37°C and 5% CO2 for 24 hours. The media consisted of Neurobasal medium supplemented with 2mM L-glutamine, B27 supplement, N2 supplement and 0.5% penicillin-streptomycin (all from Invitrogen) with either saline, Poly IC (50 µg/ml), IL6 (10 ng/ml) or LPS (1 µg/ml) added. Afterwards slices were fixed for 1 hour in 4% PFA and cryoprotected overnight in PBS + 30% sucrose, frozen in optimal cutting temperature compound (Tissue-Tek) and stored at -80°C until

sectioned. Ten micrometer-thick coronal tissue sections were cut on a Leica CM1900 uv cryostat, mounted on Superfrost Plus glasses and stored at -20°C until staining.

In order to determine the activation state of the microglia, we used antibodies against IL1β, iNOS and Mac-2/Galectin-3 [57, 285]. All primary antibodies and working solutions are listed in Table 2.1.

Table 2.1: Overview of the antibodies used for immunostainings and flow cytometry experiments.

Antibody	Company	Reference	Dilution
Immunohistochemistry			
Anti-IL1β (rabbit polycl.)	Abcam	ab9722	1:100
Anti-iNOS (rabbit polycl.)	Abcam	ab15323	1:250
Anti-Mac-2 (rat monocl.)	American Type Culture Collection	TIB-166	1:250
Flow cytometry			
Anti-IL1β PE (rat monocl.)	LifeSpan BioSciences	LS-C184791	1:300
Anti-iNOS PE-Cy7 (rat monocl.)	eBioscience	25-5920	1:300
Anti-Mac-2 PE (rat monocl.)	eBioscience	12-5301	1:300

2.3.4 BV-2 cell culture

The immortalized mouse microglial cell line BV-2 (kindly provided by Dr. F. Stassen, Maastricht, The Netherlands) was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), 2mM glutamine and 1

% penicillin streptomycin (all from Life Technologies). For flow cytometry, cells were detached by incubation with PBS-EDTA 20 mM for 10 minutes at room temperature.

2.3.5 Isolation of microglia and flow cytometry experiments

Brains were isolated from CX3CR1$^{+/eGFP}$ E17.5 embryos from mothers subjected to a single saline or Poly IC injection at E11.5, or a double Poly IC injection at E11.5 and E15.5. All steps were performed at 4°C or on ice, unless stated otherwise, to avoid microglia activation. Meninges were removed, the cortical area identical to the immunohistochemical analysis was dissected out and incubated during 30min at 30°C in DMEM/F-12(1:1) + GlutaMAX (Life Technologies) containing 48U/ml Papain from papaya latex (Sigma). Papain containing supernatants was discarded and the tissue was mechanically disrupted in medium through fast pipetting using a 1 ml pipet. Afterwards, the homogenate was centrifuged at 400g during 5 min, resuspended in 40% isotonic Percoll (GE Healthcare) and centrifuged at 700g during 10 min without break. The pellet was resuspended in PBS and both the cell suspension and detached BV2 cells were filtered through a 35µm cell strainer. Cell suspension and BV2 cells were fixed and permeablized in Cytofix/Cytoperm buffer (BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit, BD Biosciences) during 20 min on ice, washed and incubated on ice for 30 min in Perm/Wash buffer with a mix of fluorochrome-conjugated rat anti-mouse antibodies: iNOS-PE-Cy7 (clone CXNFT, eBioscience), Mac-2-PE (clone eBioM3/38, eBioscience) and IL1β-PE (clone 11n92, LifeSpan BioSciences) (Table 2.1). The following isotype controls were used: Rat IgG2aκ PE-Cy7, Rat IgG2aκ PE and Rat IgG2b PE (all from eBioscience). After washes, cells were resuspended in FACS buffer (PBS, 2% FCS, sodium azide), acquired in a FACS Aria II and analyzed with FACS Diva 6.1.3 software (BD Biosciences). Isotype-marker overlay graphs were created in FlowJo 10.0.8 Software. Inside the singlet population, the eGFP positive microglia (1000-12000 cells per experiment) were gated (Figure 6A), and within this population, the percentage of Mac-2, iNOS and IL1β positive microglia was analyzed. Isotype controls were used to gate the positive cell population (Figure 6B). Per group, embryos were derived from one to three different mothers

(saline, single Poly IC, double Poly IC). BV-2 cells were used as positive controls for the different antibodies.

2.3.6 Analysis and statistics

Quantitative analysis of microglial cells was performed on images of coronal embryonic brain sections. The regions of interest are depicted in figure 2.1. We focused our analysis on the cerebral cortex area located dorsally to the LGE and MGE, containing the frontal and pariental cortex on E11.5 and E12.5, and the somatosensory and motor cortex at E17.5. Sections were included from the first appearance of the LGE until the start of the fusion of the LGE and MGE. For the quantifications of the hippocampal area at E17.5, only the dorsal hippocampus was included in the analysis.

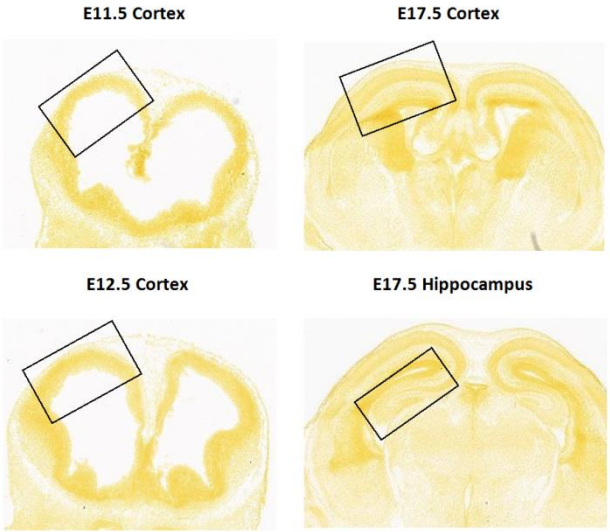


Figure 2.1: Regions of interest for analysis of microglial density. The region of interest for the cortex at E11.5, E12.5 and E17.5 are shown, as well as the region of interest for the hippocampus at E17.5. Adapted from the Allen Developing Mouse Brain Atlas[286].

Images were taken with a Nikon Eclipse 80i microscope and a Nikon digital sight camera DS-2MBWc (10x Nikon plan objective (numerical aperture (NA) of 0.25) and a 20x Plan Fluor objective (NA of 0.5)). Images (1600 x 1200) were analyzed with ImageJ 1.45e software (NIH, USA; http://rsb.info.nih.gov/ij/). Only eGFP-positive cell bodies were taken into account for the measurements.

Density analysis was performed by counting the number of eGFP positive cell bodies per mm² [38]. For analysis of activation state we calculated the percentage of the eGFP positive cells that were also showing immunoreactivity for the activation marker. All values are expressed as mean ± S.E.M. The number of sections used is indicated as n, the number of embryos or blood samples as N; # sections/# embryos is thus designated in the text as n/N. Statistical significance was assessed by nonparametric Mann Whitney test or Kruskal-Wallis test, P-values smaller than 0.05 were considered significant.

2.4 Results

An increase in IL6 levels in the maternal blood is a crucial factor in the development of MIA-induced deficits and changes observed in the offspring[140]. To control that the Poly IC injection procedure we used evoked an increase in IL6 levels in the maternal blood, we measured IL6 in the maternal serum samples 5 hours after injection of either saline or Poly IC. We found a significant increase ($P < 0.0001$; Mann Whitney test) in the level of IL6 in the sera of female mice primed with Poly IC (1876.0 ± 389.2 pg/ml, N = 22) when compared to those injected with saline (14.8 ± 3.3 pg/ml, N = 26), thus indicating that the mice in the Poly IC group effectively suffered from a systemic immune response.

Recent studies indicated that adult Poly IC MIA offspring show an increase in microglial density in different brain regions [225, 287]. To determine if Poly IC-evoked MIA alters the embryonic microglial cell colonization process in the fetal brain, we compared cell density after single injection of Poly IC, double injection of Poly IC or saline treatment, in the cortex at E11.5, E12.5 and at E17.5 (single injection) or at E17.5 (double injections) and in the hippocampal area at E17.5 (single and double injections). At all ages tested, we did not find any significant difference in microglia cell density (Mann Whitney test; $P > 0.05$, for detailed P-values see Table 2.2) in the cortex or in the hippocampus after a single or after double injections (Figure 2.2 and Table 2.2), thus suggesting that Poly IC-evoked MIA does not alter early invasion of the cortex and the hippocampus by microglial cells in the embryo.

Table 2.2: Microglial cell density in the cortex and hippocampal area of embryos derived from the control group and the group that was subjected to maternal inflammation at E11.5 or at E11.5 and E15.5.

	Single injection at E11.5			
Brain structure	*Cortex*			*Hippocampus*
Embryonic age	*E11.5*	*E12.5*	*E17.5*	*E17.5*
Saline	48.6 ± 8.8	34.9 ± 2.8	59.6 ± 5.8	122.5 ± 4.9
Poly IC	32.2 ± 5.7	37.8 ± 2.9	56.5 ± 4.3	111.6 ± 5.7
***P* value**	*0.191*	*0.375*	*0.573*	*0.435*
	Double injection at E11.5 and E15.5			
Brain structure	*Cortex*			*Hippocampus*
Embryonic age	*E17.5*			*E17.5*
Saline	59.8 ± 3.1			95.3 ± 4.8
Poly IC	57.8 ± 2.2			91.0 ± 5.7
***P* value**	*0.931*			*0.699*

Values are mean ± SEM of the number of microglial cells per mm², Mann Whitney test was used for statistical analysis. When injected at E11.5 the numbers of embryonic brains in the saline and Poly IC group were respectively: E11.5 = 4/5; E12.5 = 12/7; E17.5 cortex = 6/8; E17.5 hippocampus = 5/8. When injected at E11.5 and E15.5 numbers of embryonic brains in the saline and Poly IC group were respectively: E17.5 cortex = 5/6; E17.5 hippocampus = 6/6.

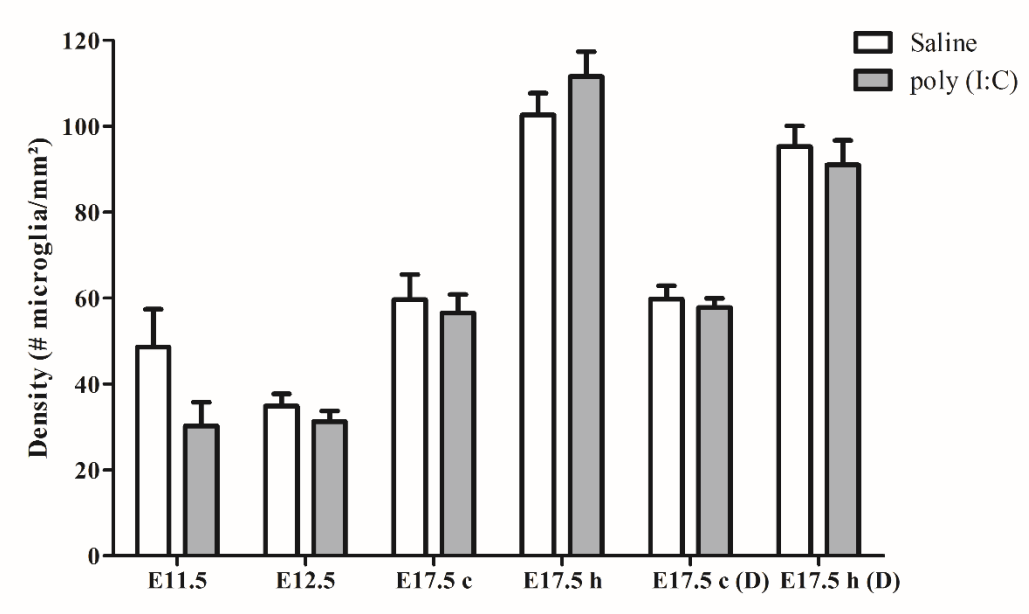


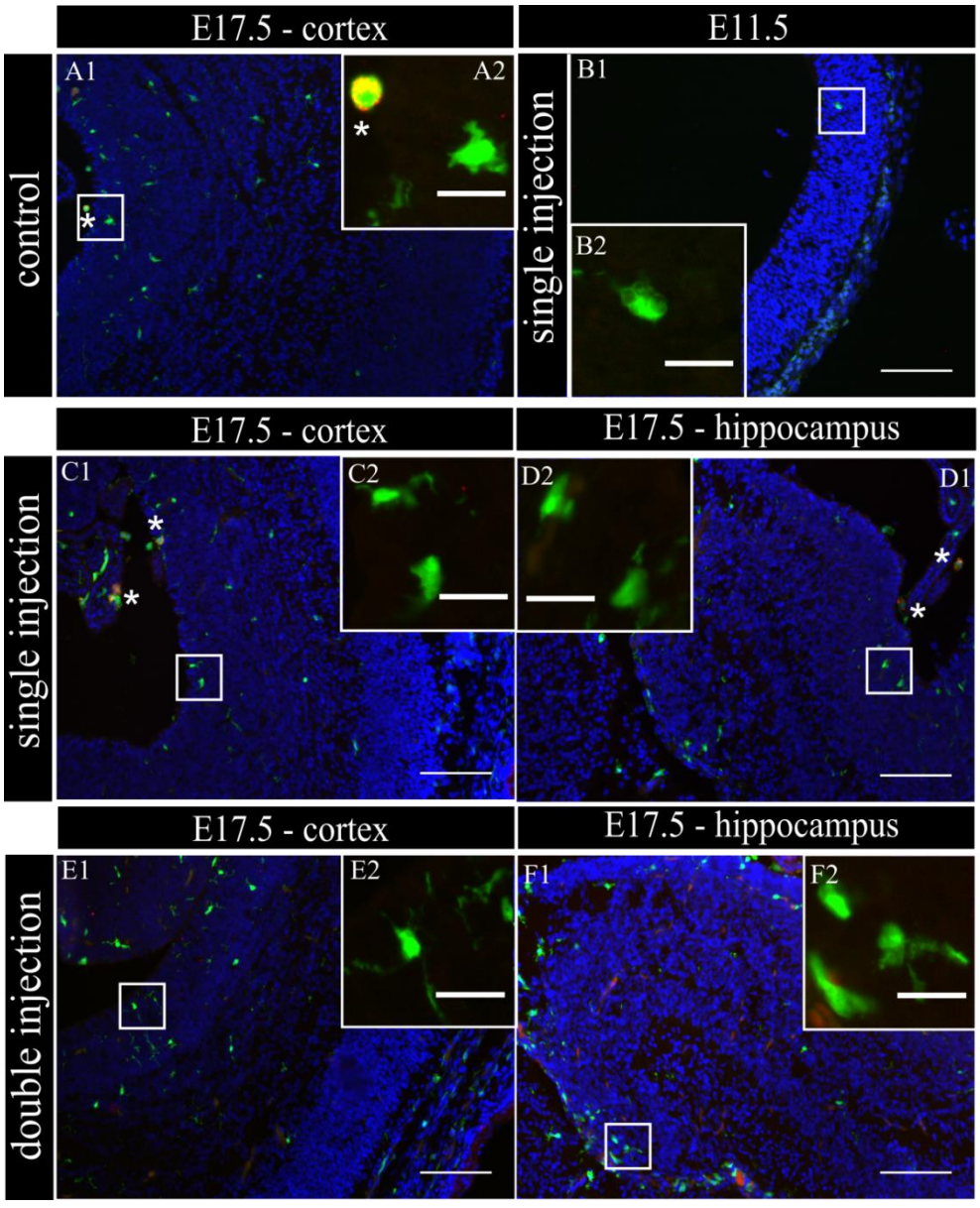
Figure 2.2: Embryonic microglial cell density is not increased after single and double injection of Poly IC. Microglial cell density in the cortex and hippocampal area was not affected after Poly IC-induced MIA. Values are mean ± SEM of the number of microglial cells per mm², Mann Whitney test was used for statistical analysis. When injected at E11.5 the numbers of embryonic brains in the saline and Poly IC group were respectively: E11.5 = 4/5; E12.5 = 12/7; E17.5 cortex = 6/8; E17.5 hippocampus = 5/8. When injected at E11.5 and E15.5 numbers of embryonic brains in the saline and Poly IC group were respectively: E17.5 cortex = 5/6; E17.5 hippocampus = 6/6. c, cortex; h, hippocampal area; (D), double injection.

To determine if MIA induced a change in microglial activation level after a single Poly IC injection (E11.5), we performed an immunostaining for three different activation markers: Mac-2/Galectin-3, iNOS and IL1β at E11.5 and E17.5. Mac-2/Galectin-3 is a marker of microglial phagocytic activation state[288, 289] while iNOS and IL1β are markers of a cytotoxic activation state[57]. At E11.5 none of the microglia located in the cortex was immmunopositive for Mac-2 staining both after saline injection (n/N = 14/3) and after Poly IC challenge (n/N = 18/3) (Figure 2.3 B1 and B2). At E17.5, 2.5 ± 0.5% (n/N = 38/4) of the microglia in the cortex (Figure 2.3 A1 and A2) and 3.2 ± 0.7% (n/N = 27/4) of the microglia in the hippocampal area expressed Mac-2 after saline injection. We did not find any significant difference (Kruskal-Wallis test; P = 0.448) after Poly IC

challenge. After Poly IC challenge, 1.9 ± 0.7% (n/N = 23/4) of the microglia in the cortex and 2.5 ± 1.0% (n/N = 15/4) of microglia in hippocampal area expressed Mac-2 (Figure 2.3 C1, C2, D1 and D2). We next investigated the expression of IL1β and iNOS[57] to determine if embryonic microglia can adopt a cytotoxic activation state after a single injection of Poly IC. Induction of MIA by a single injection of Poly IC did not result in a significant increase in the percentage of microglia expressing IL1β either at E11.5 and E17.5 (Kruskal-Wallis test; P = 0.136). In control conditions, 0 ± 0% (n/N = 6/3) and 2.2 ± 1.0% (n/N = 15/4) of microglia located in the cortex expressed IL1β at E11.5 and E17.5 (Figure 2.4 A1 and A2) respectively while 3.1 ± 1.3% (n/N = 17/4) expressed IL1β in the hippocampal area (E17.5). After Poly IC challenge, 3.3 ± 3.3% (n/N = 10/3) and 3.5 ± 1.0% (n/N = 19/4) of microglia located in the cortex expressed IL1β at E11.5 (Figure 2.4 B1 and B2) and at E17.5 (Figure 2.4 C1 and C2) respectively, while 7.2 ± 2.6 % (n/N = 17/4) expressed IL1β in the hippocampal area (E17.5) (Figure 2.4 D1 and D2). We found similar results when analyzing iNOS expression at E11.5 and E17.5 in the cortex and in the hippocampal area (E17.5). Cortical iNOS expression in control conditions (E11.5: 8.3 ± 5.7%, n/N = 10/3; E17.5: 2.0 ± 1.1%, n/N = 15/4 (Figure 2.5 A1 and A2)) was not significantly different when compared to the Poly IC condition (E11.5: 0 ± 0%, n/N = 8/3 (Figure 2.5 B1 and B2); E17.5 :1.9 ± 1.1%, n/N = 12/4 (Figure 2.5 C1 and C2) (Kruskal-Wallis test; P = 0.471).

Figure 2.3 (next page): Embryonic microglial cell population is poorly immunoreactive to the Mac-2/Galectine-3 antibody after single and double injection of Poly IC. (A-F1) Coronal sections of embryonic brains, with cell nucleus staining in blue (DAPI) and microglial (CX3CR1-eGFP) cells in green. Immunohistochemical staining using a Mac-2 antibody (red) showed that at E17.5 almost no microglial cells in the cortex were immunoreactive for Mac-2 (A2) after injection with saline. At E11.5 (B2) and E17.5 (C2 and E2) in the cortex and E17.5 hippocampal area (D2 and F2) there was no increased percentage of microglial cells expressing the activation marker after Poly IC challenge compared to control. White square indicates the location of the cells in the tissue showed in the inset; * indicates a Mac-2 positive eGFP cell. Examples of one control brain area and Poly IC group only as they were not significantly different. For regions of interest, the reader is referred to the materials and methods section. Scale bar = 100 µm and for insets = 20 µm.

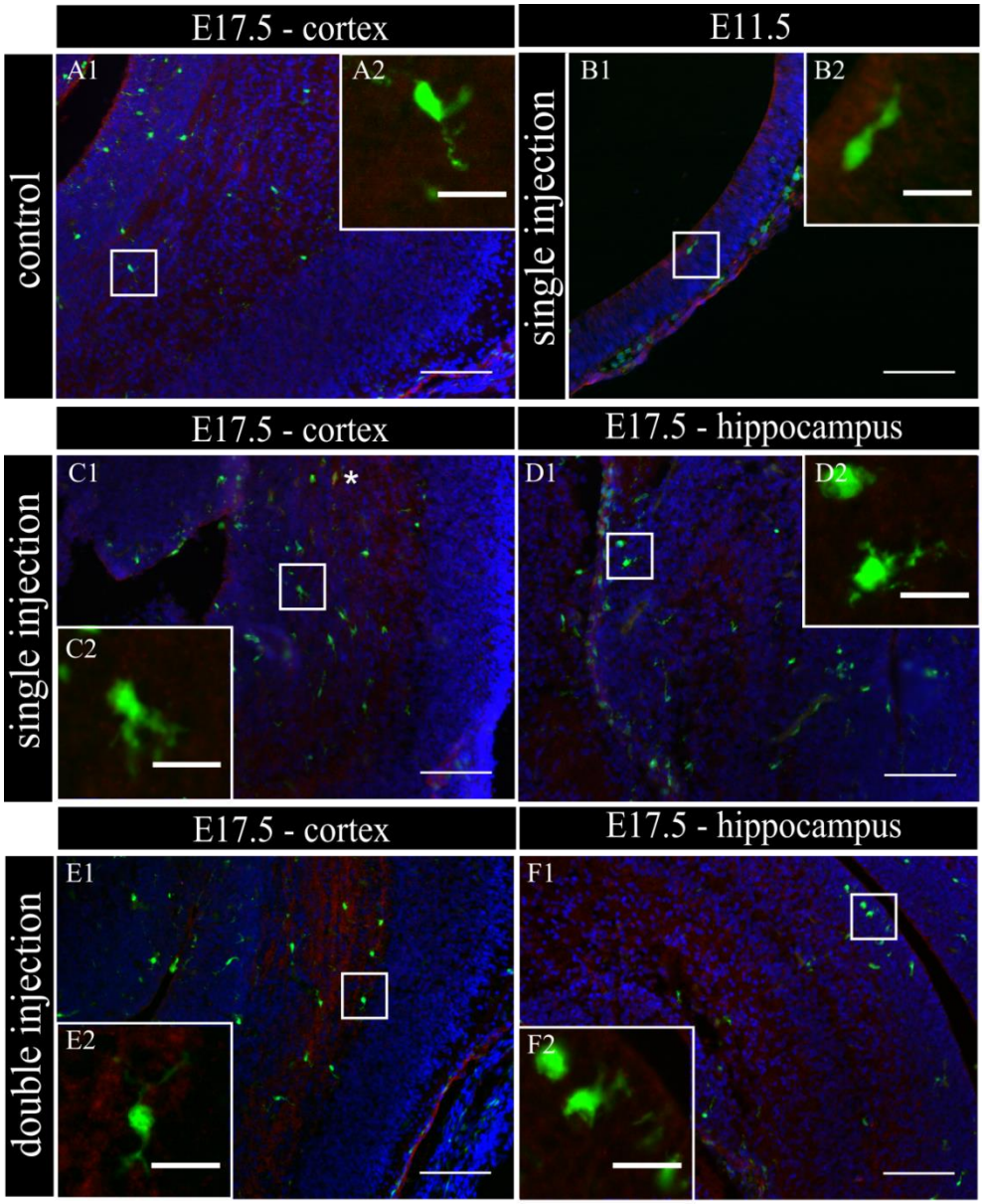
In the hippocampal area, 1.5 ± 1.0% of microglia (n/N = 14/4) expressed iNOS in control conditions while 0 ± 0%, of microglia (n/N = 10/4) expressed iNOS after Poly IC challenge (Figure 2.5 D1 and D2, being not significantly different (Kruskal-Wallis test; P = 0.471)).

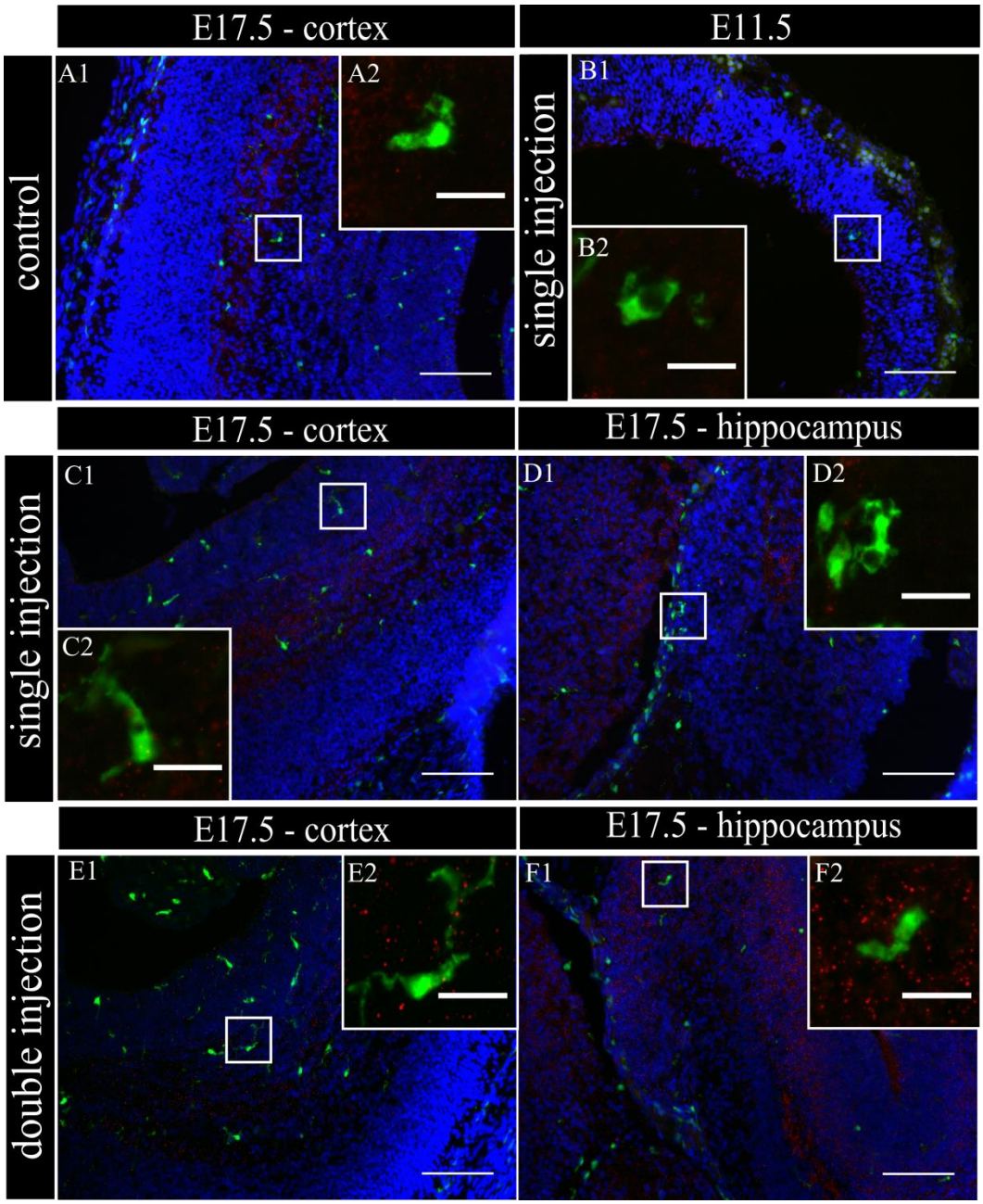


This lack of change in embryonic microglia activation state after a single Poly IC injection could possibly lead only to a "primed" microglial state. Indeed, two injections of LPS were necessary in rat to elicit MIA induced microglia dysfunction during phagocytosis of cortical neural precursor cells[57], suggesting that the microglial phenotype could become only fully altered after the second inflammatory challenge. To determine if this is also the case for Poly IC, we reanalyzed microglial density and activation level after a repeated injection of Poly IC. Consequently, the mothers suffered from a double immune stimulation (on E11.5 as well as on E15.5). Despite the presence of a maternal immune response after both injections, there was no significant increase in microglial cell density (Mann Whitney test; $P > 0.05$, for detailed P-values see Table 2.2) (Figure 2.2 and Table 2.2). Microglial activation states were analyzed at E17.5 as described above. We did not find any significant difference (Kruskal-Wallis test; Mac-2, $P = 0.139$; IL1β, $P = 0.945$; iNOS, $P = 0.093$) in the percentage of microglia expressing Mac-2, IL1β or iNOS between control conditions and after double injections of Poly IC. After double injections of Poly IC, the percentage of microglia immunoreactive for Mac-2 was 0 ± 0% (n/N = 29/6) in the cortex (Figure 2.3 E1 and E2) and 2.0 ± 0.7% (n/N = 22/6) in the hippocampal area (Figure 2.3 F1 and F2). In the cortex (Figure 2.4 E1 and E2) and hippocampal area (Figure 2.4 F1 and F2) 1.4 ± 0.7% (n/N = 34/6) and 1.4 ± 1.0% (n/N = 25/6) of the microglial cells showed immunoreactivity for IL1β, while 1.8 ± 0.7%

Figure 2.4 (next page): Embryonic microglia show no increased expression of IL1β after single and double injection of Poly IC. (A-F1) Coronal sections of embryonic brains, with cell nucleus staining in blue (DAPI) and microglial (CX3CR1-eGFP) cells in green. Immunohistochemical staining using an IL1β antibody (red) showed that at E17.5 almost no microglial cells in the cortex were immunoreactive for IL1β (A2) after injection with saline. At E11.5 (B2) and E17.5 (C2 and E2) in the cortex and E17.5 hippocampal area (D2 and F2) there was no increased percentage of microglial cells expressing the activation marker after Poly IC challenge compared to control. White square indicates the location of the cells in the tissue showed in the inset; * indicates an IL1β positive eGFP cell. Examples of one control brain area and poly (I:C) group only as they were not significantly different. For regions of interest, the reader is referred to the materials and methods section. Scale bar = 100 μm and for insets = 20 μm.

(n/N = 34/6) and 0 ± 0% (n/N = 23/6) of the microglia were positive for iNOS in the cortex (Figure 2.5 E1 and E2) and hippocampal area (Figure 2.5 F1 and F2), respectively. These results indicate that even double injections of Poly IC did not evoke microglia activation in the embryo.





In addition to the immunohistochemical stainings, the presence of the activation markers on microglial cells at E17.5 was investigated by flow cytometry. The positive controls and gating strategy are shown in Figure 2.6 and Figure 2.7 A and B.

Figure 2.5 (previous page): Embryonic microglia cell population is poorly immunoreactive to the iNOS antibody after single and double injection of Poly IC. (A-F1) Coronal sections of embryonic brains, with cell nucleus staining in blue (DAPI) and microglial (CX3CR1-eGFP) cells in green. Immunohistochemical staining using an iNOS antibody (red) showed that at E17.5 almost no microglial cells in the cortex were immunoreactive for iNOS (A2) after injection with saline. At E11.5 (B2) and E17.5 (C2 and E2) in the cortex and E17.5 hippocampal area (D2 and F2) there was no increased percentage of microglial cells expressing the activation marker after Poly IC challenge compared to control. White square indicates the location of the cells in the tissue showed in the inset. Examples of one control brain area and poly (I:C) group only as they were not significantly different. For regions of interest, the reader is referred to the materials and methods section. Scale bar = 100 µm and for insets = 20 µm.

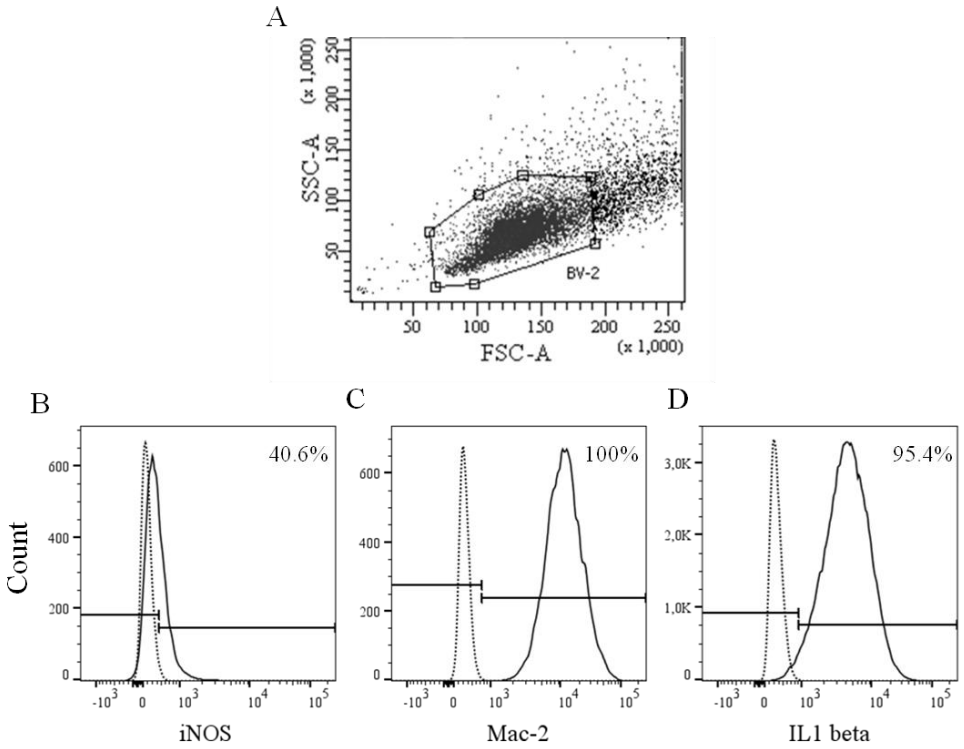
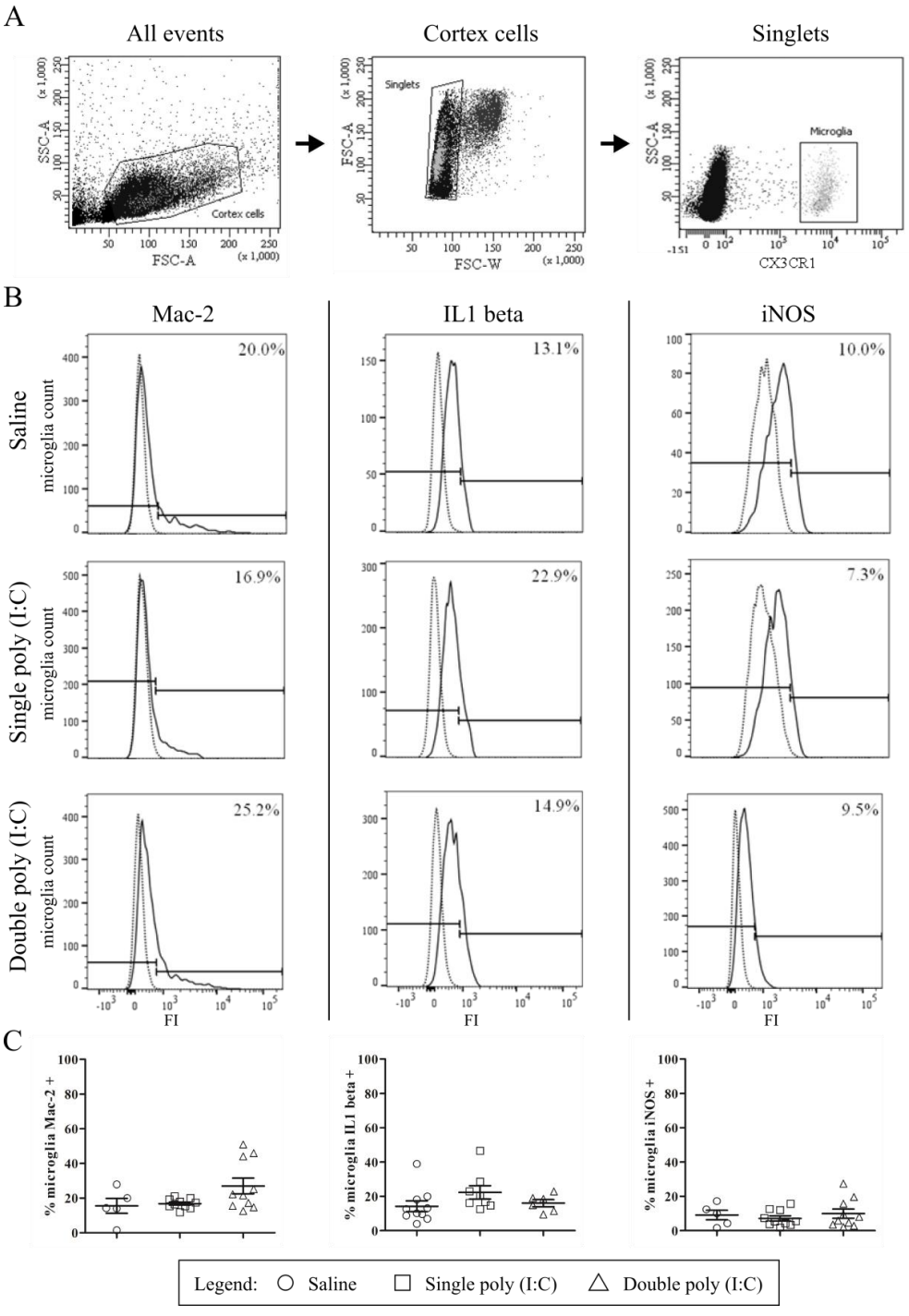


Figure 2.6: BV-2 cells are positive for iNOS, Mac-2 and IL1β(A-D). BV-2 cells (A) were processed identical to the embryonic cortex cell suspension for flow cytometric staining. Using the same antibody concentration we find immune reactivity for iNOS (40.6%, B), Mac-2 (100%, C) and IL1β(95.4%, D) (full lines), which indicates that the antibody is capable to recognize the antigens. Gates for positive populations were drawn based on the isotype fluorescence intensity (dotted lines).

The results of the flow cytometric quantifications were similar to those obtained by immunohistochemistry. There was no significant difference in the proportion of microglial cells that were positive for Mac-2 after single Poly IC injection (16.8 ± 0.0 %; N = 10) or double Poly IC injection (27.0 ± 4.6 %; N = 10) when compared to the control group (15.5 ± 4.3; N = 5) (Figure 2.7 C, left panel; Kruskal-Wallis test, P = 0.161). The proportion of microglial cells that were positive for IL1β in the control group (14.2 ± 3.1 %; N = 10) was not significantly different (Figure 2.7 C, middle panel; Kruskal-Wallis test, P = 0.093) from the percentage of microglia that was positive for IL1β after a single (22.3 ± 3.9 %; N = 8) or double Poly IC injection (16.0 ± 2.1 %; N = 6). The percentage of microglial cells positive for iNOS in the control group was 9.1 ± 2.8 % (N = 5). There was no significant effect (Figure 2.7 C, right panel; Kruskal-Wallis test, P = 0.816) of a single Poly IC (7.1 ± 1.5 %; N = 10) or double Poly IC challenge (9.9 ± 2.7 %; N = 10) on the percentage of microglia expressing this marker.

Figure 2.7 (next page): Flow cytometry reveals that embryonic microglial cells show a poor expression of activation markers Mac-2, IL1β and iNOS. (A) Gating strategies for the microglial cells. In the whole embryonic cortex cell suspension, a gate was created on the non-debris population (left). Inside this population, single cells were selected (middle) and within this population, the microglial cells were gated based on CX3CR1-eGFP intensity (right). SSC, Side scatter; FSC, Forward scatter. **(B)** Gating strategies for positive Mac-2, iNOS and IL1β populations. Microglial cell count of representative samples is shown for Mac-2 (left), IL1β (middle) and iNOS (right; full lines) for embryos derived from saline, single poly (I:C) and double poly (I:C) injected mothers. Gates for positive populations were drawn based on the isotype fluorescence intensity (dotted lines). FI, fluorescence intensity. **(C)** Left panels: At E17.5 only a small percentage of microglial cells shows reactivity for Mac-2. There is no significant effect of Poly IC injection on this percentage. Number of embryos tested: Saline N = 5; single Poly IC N = 10 and double Poly IC N = 10. Middle panels: In control conditions, less than 15 % of the microglial cells is positive for IL1β. There is no significant effect of Poly IC injection on this proportion. Number of embryos tested: Saline N = 10; single Poly IC N = 8 and double Poly IC N = 6. Right panels: At E17.5 less than 10 % of the microglial cells is positive for iNOS. Poly IC challenge has no significant effect on this percentage. Number of embryos tested: Saline N = 5; single Poly IC N = 10 and double Poly IC N = 10.


A
All events
Cortex cells
Singlets
SSC-A
FSC-A
Cortex cells
FSC-A
FSC-W
Singlets
SSC-A
CX3CR1
Microglia
B
Mac-2
IL1 beta
iNOS
Saline
Single poly (I:C)
Double poly (I:C)
microglia count
20.0%
13.1%
10.0%
16.9%
22.9%
7.3%
25.2%
14.9%
9.5%
FI
C
% microglia Mac-2 +
% microglia IL1 beta +
% microglia iNOS +
Legend: Saline Single poly (I:C) Double poly (I:C)

The absence of activation marker expression by microglia after Poly IC challenge raised the question whether fetal microglia can be directly activated by a Poly IC challenge as suspected for LPS[57] and IL6[140]. To address this issue, we analyzed the activation state of microglia in acute embryonic brain slices (E15.5) after exposure to IL6, Poly IC or LPS. The percentage of microglial cells expressing Mac-2/Galectin-3, iNOS and IL1β were analyzed 24 hours after immune challenge of the slices (Figure 2.8 D). Figure 2.8 insets show examples of microglial cells that did (Figure 2.8 A-C2) or did not show immunoreactivity (Figure 2.8 A-C3) for the activation markers tested (Mac-2, IL1β and iNOS). In control conditions 31.0 ± 5.9%, (n/N = 23/4) of microglia were immunoreactive for Mac-2. This percentage was significantly higher (Kruskal-Wallis test; $P < 0.0001$) than that observed *in vivo* indicating that an *in vitro* environment promotes microglia phagocytic activation state. However there was no significant effect (Kruskal-Wallis test; $P = 0.274$) of IL6, Poly IC or LPS treatment on the percentage of microglia being immunoreactive for Mac-2 (Figure 2.8 D), being 34.0 ± 5.5% (n/N = 22/4) after IL6 exposure, 32.0 ± 6.7%, (n/N = 18/5) after Poly IC exposure and 47.0 ± 7.5% (n/N = 21/5) after LPS exposure (Figure 2.8 D). As observed for Mac-2, the percentage of IL1β immunoreactive microglia was significantly higher than in *in vivo* conditions (in control conditions 52.0 ± 6.8%, (n/N = 27/4) (Kruskal-Wallis test; $P < 0.0001$)) and for iNOS a trend to a higher percentage was observed under control conditions (in control conditions 18.0 ± 5.7%, (n/N = 23/4) (Kruskal-Wallis test; $P = 0.091$)). As shown in figure 2.8 D, treatment with IL6 or Poly IC did not significantly change the percentage of microglia immunoreactive for IL1β or iNOS antibodies. When looking at IL1β immunoreactivity, 36.0 ± 7.2% (n/N = 16/4) of the microglia was positive after IL6 exposure and 54.0 ± 7.5%, (n/N = 19/5) after Poly IC exposure (Figure 2.8 D). For iNOS they were 30.0 ± 6.5% (n/N = 19/4) after IL6 exposure and 25.0 ± 3.9%, (n/N = 25/5) after Poly IC exposure (Figure 2.8 D). However we found that LPS, contrary to IL6 or Poly IC, can directly activate microglia to a detrimental activation state. Indeed LPS exposure significantly increased the percentage of microglia immunoreactive for IL1β (Kruskal-Wallis test; $P = 0.025$) or iNOS (Kruskal-Wallis test; $P = 0.025$). In the presence of LPS 66.0 ± 5.5 (n/N= 22/5) and 42.0 ± 7.1% (n/N = 21/5) of microglia were immunoreactive for IL1β or iNOS respectively.

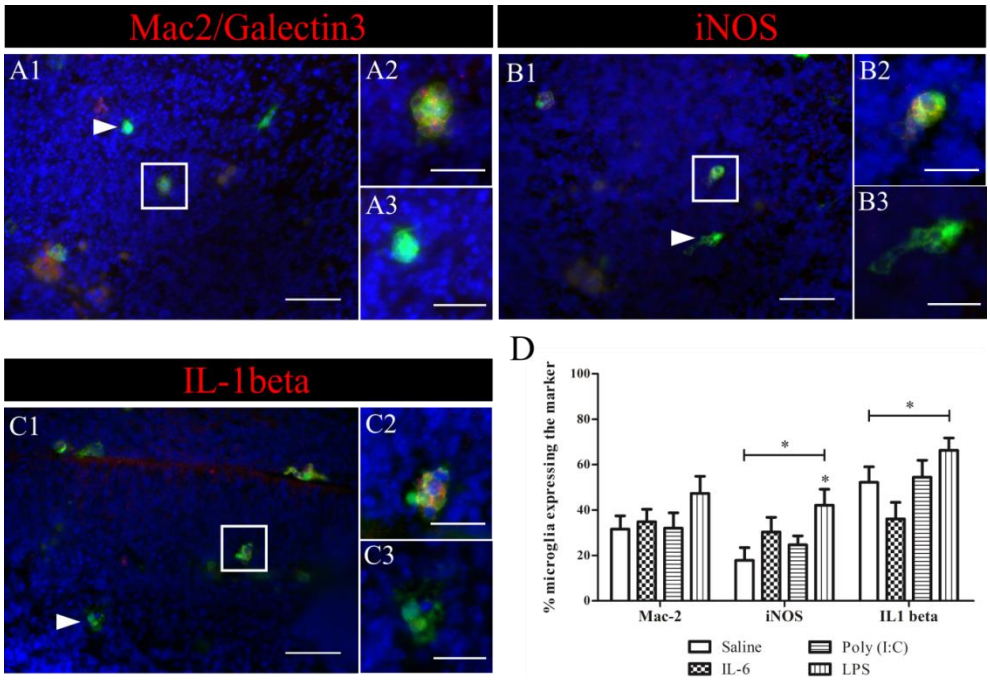


Figure 2.8: Microglial activation in acute brain slices. Example of activation marker stainings on acute slices treated with LPS. **(A)** Immunohistochemical staining for Mac-2/Galectin-3 (red), nuclei were visualized with DAPI (blue) (A1). Microglia (green) positive (A1 white square, A2) (red) and negative for Mac-2/Galectin-3 (white triangle, A3) were present in the slice. **(B)** Immunohistochemical staining for iNOS (red), nuclei were visualized with DAPI (blue) (B1). Microglia positive (white square B1, B2) and negative (white triangle B1, B3) for iNOS (red) were observed after LPS treatment. **(C)** Immunohistochemical staining for IL1β (red), nuclei were visualized with DAPI (blue) (C1). Microglial positive (white square C1, C2) and negative (white triangle C1, C3) for IL1β (red) were observed after LPS treatment. Examples of the different immunostainings were taken from slices treated for 24 hours with 1 µg/ml LPS. Scale bar = 50 µm and for inserts = 20 µm. White squares indicate the microglia positive for the marker and shown in higher magnification (A-C2), white triangles indicate microglia negative for the marker and shown in higher magnification (A-C3). **(D)** Quantification of the expression of three activation markers (Mac-2, iNOS and IL1β) by microglia in E15.5 brain slices cultured for 24 hours with IL6 (10 ng/ml), Poly IC (50 µg/ml) or LPS (1 µg/ml). Kruskal Wallis test was used for statistical analysis. Number of treated slices in control and IL6 group N = 4; LPS and Poly IC group N = 5. Number of cryosections for Mac-2/iNOS/IL1β in: saline group n = 23/23/27; IL6 group n = 22/19/16; Poly IC group n = 18/25/19; LPS group n = 21/21/22 (all derived from 3 different embryos). (* $p < 0.05$).

2.5 Discussion

MIA-induced behavioral and neurological alterations observed in the offspring at juvenile and adult stages in animals are believed to be correlated with the etiology of neuropsychiatric disorders in humans. Our study in mice demonstrates, for the first time, that MIA evoked by single or double Poly IC injections does not change microglia density or their activation state in the embryo *in vivo*. This suggests that the behavioral and neurological alterations in the offspring cannot be related to the alteration of the activation state of embryonic microglial cells. Our *in vitro* studies indicated that microglia cannot be directly activated by Poly IC or IL6 exposure, contrary to the activation observed upon LPS application.

Several observations suggest that the different infectious triggers induce differences in activation of embryonic microglia. The cytokine IL6 can cross the placental barrier *in vivo* when maternal inflammation was induced during mid-gestation[218-220], but it is not clear whether Poly IC can cross the placenta as well. LPS is shown to cross the placental barrier *in vivo* when maternal inflammation was induced during early gestation[220, 222], but this was not the case when LPS was injected at late gestation[218]. Although extrapolation of these results to a Poly IC challenge would suggest that embryonic microglia are directly or indirectly activated in response to Poly IC-induced MIA at mid-gestation, we could not find any evidence for microglia activation in this study. Previously, microglia dysfunction observed after Poly IC-induced MIA was only reported in offspring at postnatal and adult age[225, 226]. In that way, it is of interest to compare in parallel the effect of MIA induced by different infectious agents on the embryonic microglia. Studies using single or repeated LPS challenge showed that this leads to microglial activation: in the fetal sheep brain, microglial cell numbers increased as well as the number of activated/amoeboid cells[290-292]; in the rat embryo the percentage of microglia expressing iNOS and IL1β was increased[57] and postnatally a changed immunoreactivity by microglial cells was still observed[222]; and in mice Iba-1 reactivity was increased during late embryonic and early postnatal stages[167]. In conclusion, the time of injection and the nature of the infectious trigger determine whether an activation of the embryonic microglia does or does not

participate in developmental neurological defects observed in MIA offspring[224]. In addition, the microglial response might be species dependent. However, a thorough comparison of the effect of MIA in different species is difficult to make for several reasons. For example, some studies use the mRNA and/or protein expression level of different cytokines as read-out[224] while others use immunohistochemistry[57, 259] or cell number[226, 290] to investigate microglial cell activation after MIA. In addition, the effect of MIA is studied on several different postnatal and adult time points.

Microglial activation in postnatal to adult brains has been found to correlate to neurodevelopmental diseases. An active neuroinflammatory process, with microglial cell activation, was described in the brains of autistic [112, 114] and schizophrenic patients[109, 110, 293]. However, it remains unclear if microglia activation participates to neuronal disorders or reflects a normal microglia response to neural dysfunctions. Our results show that Poly IC-induced MIA does not lead to activation of embryonic microglia. Yet, they cannot exclude that the embryonic microglial cells become primed, which could result in a more vigorous response to a subsequent inflammatory stimulation in the adult. In some neurodegenerative disease models in rodents (for example Alzheimer's, Parkinson's and prion disease) the injection of LPS or Poly IC leads to a more severe pathology. The combined exposure of a prenatal immune challenge (Poly IC at E9) and peripubertal stress (from P30 to 40) resulted in the development of sensorimotor gating deficiencies and led to increased dopamine levels in the adult hippocampus[259]. At peripubertal age, the combination of both stressors resulted in altered neuroimmune responses, presented as increased microglial cell number and elevated levels of IL1β and TNFα in the hippocampus and prefrontal cortex[259]. These latter changes were transient, as they were no longer present in the adult. Finally, low doses of Poly IC worsened the deficits in PPI and latent inhibition in 16 week old mice with mutations in a schizophrenia susceptibility gene but had no effect in wild type animals, thus indicating that genetic and environmental factors can interact to worsen the schizophrenia-related behavior[255].

MIA induces not only a cytokine response in the maternal unit but also alters several cytokine levels in the placenta and in the fetus[211, 294]. Under normal

conditions, cytokines are present in the placental unit where they play an important role in controlling the tissue homeostasis and balance of the different T-cell types present in this structure. In addition, toll-like receptors (TLR), such as TLR-2 and 4, are expressed on human chorionic villi[295]. Maternal injection with IL6 is known lead to endocrine changes in the placenta[221] and injection of a high dose of LPS results in placental inflammation[234] and induction of pro-inflammatory cytokines in the amniotic fluid[296]. In addition, a direct injection of LPS into the uteroplacental circulation leads to a reaction in the embryonic brain, suggesting the placental unit can contribute to perinatal brain damage through the induction of an inflammatory reaction as a response to infection during pregnancy[290]. This complicates elucidating the site where the cytokines act upon to potentially alter brain development since they can act directly on neural progenitors and neurons[297, 298]. For example, IL6 and LIF can influence the differentiation of neural progenitor cells[299].

These data, in combination with the lack of microglial activation in our MIA study suggests that the acute maternal inflammation induced by Poly IC could affect other systems or cell types during embryonic stages. These MIA-induced early abnormalities might result in an altered CNS environment in the offspring that in turn affects the microglial cells at later developmental stages. This hypothesis is supported by the observed changes in neurotransmitter systems in the adult offspring and not in the pre-pubertal period after challenge with Poly IC[226]. GABAergic gene expression, like GABA receptor subunits and vesicular transporters, can be altered in the adult prefrontal cortex after MIA[157] In addition, serotonin and glutamate signaling was altered[300]. These changes were not present at pre-pubertal ages. It is also important to note that, although microglia do not invade the CNS of mouse embryo at E9[38, 285], Poly IC challenge at this gestational stage resulted in the suppression of spatial exploration in the adult[181]. This reinforces the idea that embryonic microglia dysfunction, if any, is unlikely to be the main mechanism inducing developmental disorders featuring pathological behavior. Accordingly, Poly IC challenge at E9 did not evoke any increase in cytokine mRNA level in the fetal brain[181]. Poly IC might induce developmental deficits via direct action on neuronal development. However our results cannot exclude that Poly IC evokes an embryonic microglia priming resulting in an exaggerated response of

microglia to homeostatic disturbances at postnatal stages and subsequently makes neuronal dysfunction worse.

Of note, we used the CX3CR1-eGFP mouse line to visualize microglia. This mouse line was created by targeted replacement of the first 390 base pairs of the gene encoding CX3CR1 with the gene encoding eGFP. Hence, mice heterozygous for eGFP lack one allele for CX3CR1 while in homozygous eGFP mice, CX3CR1 expression is abolished completely [283]. Although microglial alterations have been described in CX3CR1 knock-out mice, including increased microglial cell body and arborization area[301-303] and increased phagocytosis[304, 305], such changes are not found in mice with one functional CX3CR1 allele. Thus, it seems unlikely that the use of CX3CR1 $^{+/GFP}$ mice would have affected the outcome of this study.

In conclusion, our findings show that a single and double injection of Poly IC is not sufficient to induce changes in fetal microglia activation phenotype during mid or late embryonic development. In addition they suggest a different response of the embryonic brain to MIA depending on the challenge procedure used.

Chapter 3

Inducible genetic deletion of PU.1 does not result in microglia depletion

3.1 Abstract

To investigate the contribution of microglia to several neurodevelopmental processes, models for microglia depletion *in vitro* and *in vivo* have been developed using either a genetic approach or administration of compounds. However, among the genetic *in vivo* models, only few are inducible in time. The CX3CR1-CreER(T2) mouse line that was recently developed by Yona *et al.* 2013 offers the opportunity to induce genetic recombination upon systemic administration of tamoxifen. As the transcription factor PU.1 plays an important role in microglial development, we aimed to generate a new inducible genetic microglia depletion model by crossing the CX3CR1-CreER(T2) mouse line with mice carrying a floxed PU.1 allele. We administered tamoxifen to young adult mice or to pregnant mice to induce genetic recombination and evaluated the presence of microglia in the embryonic or young adult brain at several time points after tamoxifen injection. While we showed that recombination of the PU.1 allele had occurred, we found that microglia were not depleted in the cortex. These data suggest that genetic recombination of the PU.1 gene is not sufficient to eliminate microglia, at least when Cre recombinase is under the control of the CX3CR1 promotor.

3.2 Background

As described in chapter 2, MIA induced by single or double injection with Poly IC did not affect embryonic microglia cell density in the cortex or hippocampus, nor did it influence the expression of activation markers Mac2, IL1β and iNOS, suggesting that embryonic microglia are not activated by the maternal immune stimulation. However, these markers are typically indicative of a pro-inflammatory M1 activation state of microglia and it cannot be excluded that embryonic microglia exposed to maternal inflammation respond with an anti-inflammatory M2 phenotype and thus express other markers. Given their physiological role during neuronal development, any disturbance of normal embryonic microglia activity by MIA might interfere with their neurodevelopmental tasks. To understand whether microglia can act as the mediators of the effects of MIA onto the developing brain, it is crucial to

examine the development of interneurons and projection neurons in immune challenged mice without microglia, i.e. in a microglia depletion mouse model.

Several mouse models for genetic targeting of microglia have been developed, expressing genes of interest under promotors such as CD11b, F4/80, CSF1R etc. (as reviewed by [306]). While a few of these mouse lines express Cre recombinase, designed to mediate gene recombination in microglia specifically, the expression of Cre is constitutive and recombination can thus only be regulated in place (cell type) but not in time. In 2005, an inducible mouse model for depletion of microglia was designed by Heppner *et al.* 2005, the CD11b-HSVTK mouse line[307]. In this mouse line, the suicide gene thymidine kinase was expressed under the CD11b promotor and treatment with ganciclovir eliminated these CD11b-positive cells. However, eliminating all CD11b-positive cells was lethal and elimination of microglia was only possible when bone marrow from wild type animals was transplanted in irradiated CD11b-HSVTK mice or when ganciclovir was administered intracerebroventricularly[308]. In 2013, Yona *et al.* developed a genetically modified mouse model which expresses CreER(T2) under the endogenous CX3CR1 promotor. CreER(T2) is a Cre recombinase fused to a modified estrogen receptor through which it is retained in the cytosol[309]. Upon administration of tamoxifen to the animal, ligand binding to this estrogen receptor induces translocation of the Cre recombinase to the nucleus and allows genetic recombination[310]. This mouse line was generated similar to the CX3CR1-eGFP mouse described in chapter 2, with a targeted replacement of the first 390 base pairs of the gene encoding CX3CR1 with the gene expressing CreER(T2)[309]. Thus, like the CX3CR1-eGFP mice, mice heterozygous for CreER(T2) retain one functional allele for CX3CR1 while homozygous CreER(T2) mice lack CX3CR1 expression.

Using these CX3CR1-CreER(T2) mice, animals expressing the diphtheria toxin receptor specifically in CX3CR1-positive cells were created. Upon systemic administration of diphtheria toxin, microglia were efficiently ablated but repopulated the brain within 5 days, possibly by proliferation of those microglia that escaped ablation[40, 311]. To obtain a more stable ablation of microglia, we aimed to generate a new genetic and inducible microglia depletion mouse model based on the genetic recombination of the PU.1 allele, a transcription factor

primarily expressed in monocytes that is important for microglia development, viability and function[312]. We crossed the CX3CR1-CreER(T2) mouse with a floxed PU.1 mouse and administered tamoxifen to pregnant mice to obtain genetic recombination in the embryo as well as to young adult mice to obtain microglial depletion at a later stage.

3.3 Materials and methods

3.3.1 Animals

All experiments were conducted in accordance with the European Community guiding principles on the care and use of animals and with the approval of the Ethical Committee on Animal Research of Hasselt University. Mice were maintained in the animal facility of Hasselt University in accordance with the guidelines of the Belgian Law and the European Council Directive. Animals were group-housed (unless otherwise stated) in a temperature and humidity controlled room with ad libitum access to food and water and a 12h/12h light dark cycle (lights on at 7.30AM).

The conditional PU.1 knock-out mice were a kind gift from Stephen Nutt[313] and the CX3CR1-CreER(T2) mice were obtained from the European Mouse Mutant Archive (EMMA) institute with the approval of Steffen Jung[309]. Both mouse lines were maintained on a C57Bl/6 background. $PU.1^{fl/fl}CX3CR1^{CreER(T2)/CreER(T2)}$ mice were obtained by breeding of $PU.1^{fl/fl}$ mice with $CX3CR1^{CreER(T2)/CreER(T2)}$ mice. For timed pregnancy mating, a $PU.1^{fl/fl}CX3CR1^{+/+}$ female was coupled overnight with a $PU.1^{fl/fl}CX3CR1^{CreER(T2)/CreER(T2)}$ male to obtain $PU.1^{fl/fl}CX3CR1^{+/CreER(T2)}$ embryos. By using $CX3CR1^{+/+}$ mothers, we avoid possible side effects by genetic recombination of the PU.1 gene in the mother. The morning when a vaginal plug was detected was designated as E0.5.

3.3.2 Genotyping

The KAPA mouse genotyping kit (KAPA Biosystems KK7352) was used according to the manufacturer's instructions.

For amplification of the floxed PU.1 allele, the following primers were used:

PU.1 Fw: 5'-CACCCGCTTTGCCTCCCACCAG-3' + PU.1 Rev: 5'-TGTGGCCCCTGGGAGTGCTTTGAA-3'.

For amplification of the wild type CX3CR1 allele, the following primers were used:

CX3CR1 Fw: 5'-TTCACGTTCGGTCTGGTGGG-3' + CX3CR1 Rev: 5'-GGTTCCTAGTGGAGCTAGGG-3'.

For amplification of the CreERT2 allele, the following primers were used:

CreER(T2) Fw: 5'-ACCTGGATAGTGAAACAGGG-3' + CreER(T2) Rev: 5'-CCTGTCCAAGAGCAAGTTAG-3'.

For amplification of the recombined PU.1 allele, the following primers were used:

PU.1 del Fw: 5'-CCAGCAGCCATGTTTCAGAGGTGT-3' + PU.1 Rev: 5'-TGTGGCCCCTGGGAGTGCTTTGAA-3'.

3.3.3 Tamoxifen

Tamoxifen (Sigma T5648) was dissolved in corn oil at 20mg/ml and stored at -20°C. Before use, tamoxifen was sonicated until it was completely dissolved. To induce gene recombination, tamoxifen was injected subcutaneously (s.c.), the precise dosage was dependent on the experiment. For subcutaneous injection in young adult or pregnant mice, 2 injections (48 hours apart) of 400µl tamoxifen solution (20mg/ml) were administered. For intraperitoneal injection in pregnant mice, 2 injections of tamoxifen (48 hours apart) were administered at a dose of 4mg tamoxifen/35g body weight.

3.3.4 Immunohistochemistry

For adult animals, mice were administered an overdose of Dolethal and transcardially perfused with 4% paraformaldehyde, the brain isolated and post fixed overnight at 4°C by immersion in 4% PFA. For embryos, the heads were fixed by immersion in 4% paraformaldehyde overnight at 4°C. After fixation, the

embryonic and adult heads were cryoprotected overnight in PBS + 30% sucrose, frozen in optimal cutting temperature compound (Tissue-Tek) and stored at -80°C until sectioned. Ten micrometer-thick coronal tissue sections were cut on a Leica CM1900 uv cryostat, mounted on Superfrost Plus glasses and stored at -20°C until staining.

Embryonic sections were washed three times in PBS, blocked with serum and permeabilized with Triton X-100 (Sigma-Aldrich). Subsequently they were incubated overnight at 4°C with the primary antibody against Iba1 (1:500, Wako 019-19741). After washing, the sections were incubated with Alexa-labeled secondary antibodies (1:600, Invitrogen) and 4,6-diamidino-2-phenylindole (DAPI) for 1h at room temperature and mounted with Vectashield (Vector laboratories).

3.4 Results

Because PU.1 is described as an important factor for development and survival of microglia, we tested whether microglia depletion could be induced by removal of this gene in adult mice. Young adult PU.1$^{fl/fl}$CX3CR1$^{+/CreER(T2)}$ mice, both males and females, were injected twice with 400µl tamoxifen (20mg/ml) by a subcutaneous injection in the neck, 48 hours apart, according to the original protocol of Yona *et al.* 2013. Mice were sacrificed 3, 5, 7 or 9 days after the last tamoxifen injection and brain sections were stained against Iba1 to evaluate the presence of microglia. In all conditions, microglia were still detectable, suggesting that PU.1 depletion had no effect on microglia survival in adult stages (Figure 3.1).

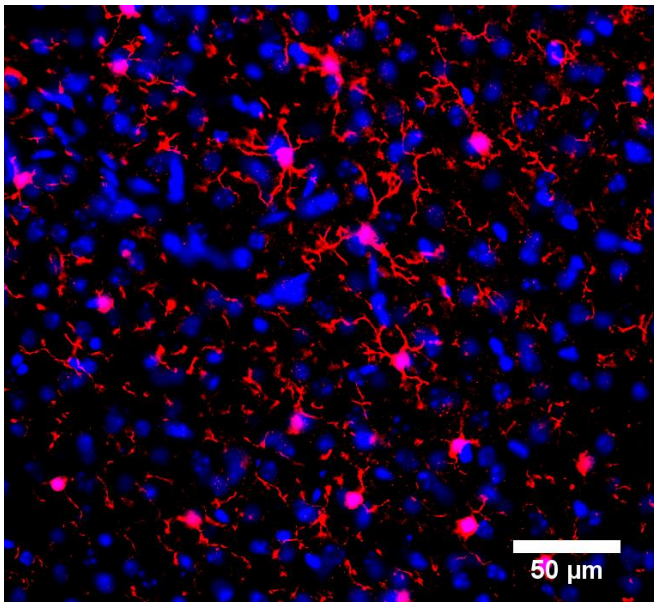


Figure 3.1: Microglia are not depleted in the adult cortex 3 days after tamoxifen treatment. Representative image of adult cortex, 3 days after the last tamoxifen injection. Blue = DAPI, Red = Iba1.

PU.1 is essential for microglia differentiation and as a consequence, the absence of PU.1 is expected to prevent monocytes to differentiate to microglia and to reach the brain. To investigate whether microglia could be depleted during embryogenesis, tamoxifen was administered to pregnant females to induce genetic recombination in the embryo. For mating, $PU.1^{fl/fl}CX3CR1^{+/+}$ females were used to avoid side effects of PU.1 deletion in the mother. As a high dose of tamoxifen (400µl tamoxifen at 20mg/ml) in pregnant females induced abortion, a lower dose was used for this experiment (4mg tamoxifen/35g body weight). Injections were administered at E8.5 and E10.5. These ages were chosen as they are 1 day before and 1 day after the start of the expression of CX3CR1, which occurs around E9.5. In this way, we aimed to interfere with PU.1 expression as early as possible. Embryos were sacrificed at E12.5, E15.5 and E18.5 and brain sections stained against Iba1 to evaluate the presence of microglia. Again, microglia were not depleted at any of the time points investigated (Figure 3.2). As we did not count the amount of Iba1 positive cells, we cannot state whether genetic PU.1 deletion caused a reduction in the amount of microglia compared to wild type animals.

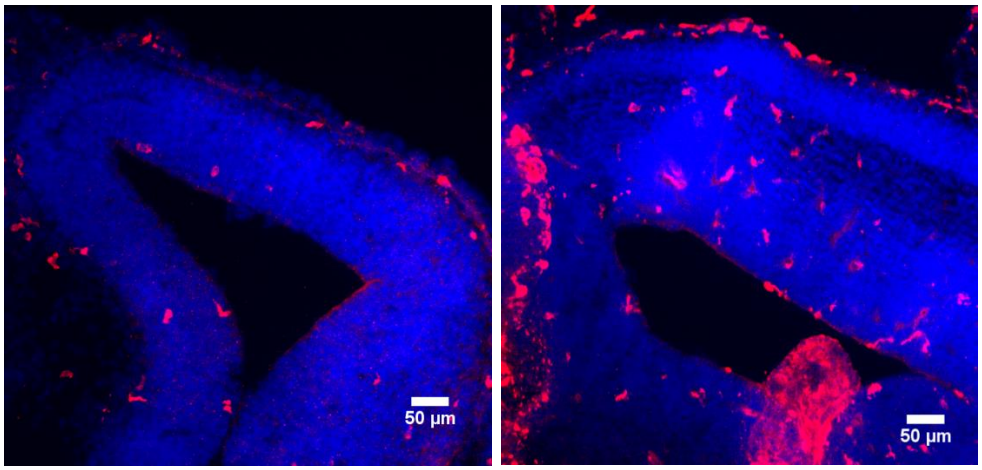


Figure 3.2: Microglia are still present in the embryonic cortex 2 (left, E12.5) and 5 (right E15.5) days after tamoxifen treatment of the mother. Blue = DAPI, Red = Iba1.

The absence of microglia depletion could be explained by a lack or incomplete PU.1 gene recombination after tamoxifen administration. To verify that gene recombination occurred in the embryonic brain, we performed a genotyping PCR on brain slices from each embryo using the primers shown in the figure below (Figure 3.3). If the PU.1 gene had recombined in microglia, we would expect a smaller band for the recombined allele and a larger band for the non-recombined allele in other cell types not expressing CreER(T2) (a.o. neurons and astrocytes).

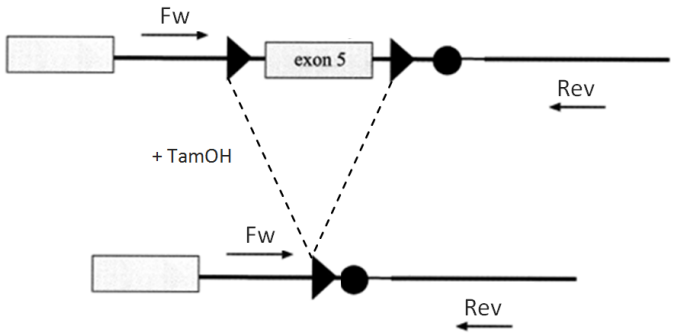


Figure 3.3: Schematic representation of the PU.1 allele before and after recombination upon tamoxifen injection. Locations of the genotyping primers are indicated. Fw: Forward primer, Rev: Reverse primer, TamOH: Tamoxifen

As shown in figure 3.4, genotyping PCR yielded 2 bands in all embryos from 1 litter and they thus all underwent genetic recombination, suggesting that the experimental setup, i.e. tamoxifen administration to the mother and distribution to the embryo, worked but embryonic microglia survived the PU.1 gene deletion.

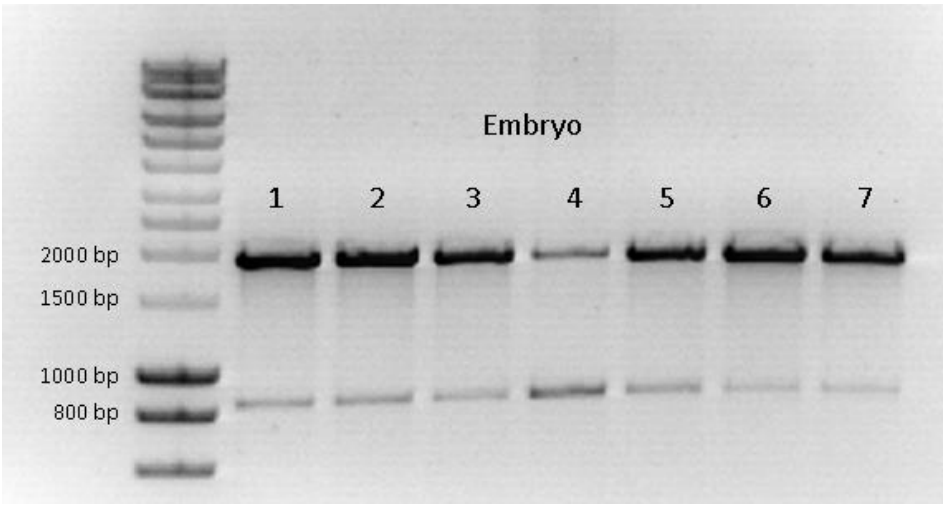


Figure 3.4: Genotyping PCR from all embryos in one litter, the lower band representing the recombined PU.1 allele and the upper band representing the wild type allele.

3.5 Discussion

Currently existing microglia depletion models can be divided in pharmacological or genetic deletion models, the latter being either constitutive or conditional. Constitutive genetic deletion models include mice lacking genes that are important for microglia development such as the CSF1R, PU.1 or Transforming growth factor β (TGFβ) knock-out mice. Although efficient in depleting microglia, the biggest drawback of these models is that the respective mutations are not compatible with life or that they induce defects in other organ systems as well[34, 35, 314]. Among pharmacologically induced microglia depletion models are those administering a CSF1R blocker or antibody (AFS98 or PLX3397) or chlodronate, both of which efficiently deplete microglia although for only a short amount of time. After PLX3397 administration, microglia repopulated within 5 days and treatment with AFS98 in early embryonic stages lead to complete microglial

repopulation after birth[36, 44]. For conditional genetic deletion, Parkhurst *et al.* 2013 generated a mouse model in which the diphtheria toxin receptor was expressed only in CX3CR1-expressing cells[40]. Upon administration of diphtheria toxin, cells of the monocytic lineage are specifically targeted and as a consequence, in the CNS only microglia will be affected and thus depleted. Unfortunately, also this model suffers from a fast microglial repopulation, within 5 days after injection of diphtheria toxin.

We aimed to create a new conditional microglia depletion mouse by tamoxifen-inducible, Cre-dependent recombination of the gene encoding PU.1, which has been shown to be of importance for the transition from EMPs to $CD45^{+}$c-kit^{Lo} EMPs[34]. PU.1 deficiency impairs the maturation of yolk sac-derived myeloid progenitors as evidenced by a failure of these cells to express terminal myeloid differentiation markers, including CSF1R, CD11b and CD64[315]. Constitutive knock-out of PU.1 results in severely immunocompromised offspring that die a few days after birth. These mice are deficient in circulating monocytes and tissue macrophages[316], but also parenchymal microglia in the brain are lacking[317, 318]. Importantly, PU.1 protein is also expressed in resting and activated microglia, suggesting it is not only important for the development of microglia but also for their maintenance and appropriate functioning. Accordingly, Smith *et al.* 2013 showed in cultured human microglia treated with siRNA against PU.1 that microglia cell viability and phagocytosis capacity was decreased[312].

Although we found that recombination of the PU.1 gene occurred in mice exposed to an injection of tamoxifen either *in utero* or in adulthood (data not shown), microglia were still present in the embryonic and adult brain respectively. We checked for the presence of microglia either shortly after the last tamoxifen injection (2-3 days) to avoid looking at repopulated cells and longer after tamoxifen injection (5-9 days) to 'give enough time' for the Cre recombination to occur and the remaining PU.1 protein to degrade. Although, to our knowledge, the exact half time of PU.1 protein is not known, Jego *et al.* 2014 and Kueh *et al.* 2013 suggest it is at least 10-12 hours[319, 320].

One possibility to explain why microglia depletion did not occur in the embryo could be the timing of PU.1 and CX3CR1 expression, with the onset of CX3CR1 expression occurring slightly later than that of PU.1 (Figure 3.5). As such,

expression of CreER(T2) and recombination of the PU.1 gene occur when PU.1 protein is already present. As Smith *et al.* 2013 showed that siRNA against PU.1 in cultured human microglia resulted in decreased PU.1 protein reactivity starting from 4-7 days after transfection, it could be possible that the PU.1 protein produced before genetic recombination remains for this period of time even though gene transcription no longer occurs. Alternatively, the PU.1 dependent transition from EMP to $CD45^{+}$c-kit^{Lo} EMP might have already occurred and the later maturation might no longer be dependent on PU.1.

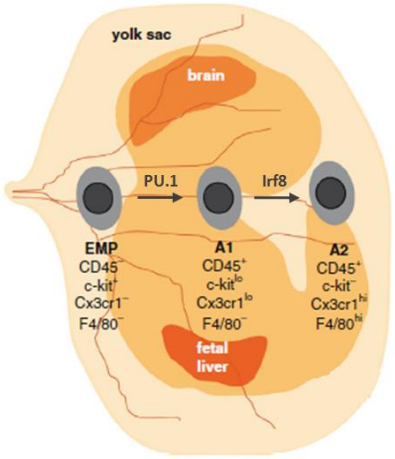


Figure 3.5: Maturation of yolk sac derived EMPs mediated by PU.1 and Irf8. EMP: erythro-myeloid progenitors. Adapted from Tay *et al.* 2016[321].

Although we did not assess abscence of PU.1 on the mRNA or protein level (qPCR, western blot, immunohistochemistry), we assured that genetic recombination occurred (and thus that the tamoxifen protocol worked) by performing a genotyping PCR that could distinguish between a recombined and a non-recombined PU.1 allele. For this PCR, we extracted DNA from whole embryonic brain that also contained other cell types, including neurons, which do not express CX3CR1 and thus CreER(T2), in which we would not expect recombination to occur. Hence, although we could conclude that recombination occurred, we could not distinguish between heterozygous or homozygous gene recombination within the microglia. This would require to look in isolated microglia. Nevertheless, if recombination would not occur efficient enough and 2

CreER(T2) alleles would be required, we would have to set up breeding pairs with homozygous CreER(T2) (and thus CX3CR1 deficient) females, thereby risking possible influences of (1) CX3CR1 deficiency and (2) PU.1 deletion in the mother on pregnancy parameters. In addition, bearing in mind that the final goal of this experiment would be to induce MIA in microglia depleted animals, maternal CX3CR1 deficiency might also affect the immune response.

As briefly touched upon in the introduction, several groups are in search of a molecular marker to more specifically target microglia in the brain. While several studies have reported on microglial markers that are more specific than CX3CR1, including Sall1 and Tmem119, the use of these genes in the genetic modification of mouse embryos remains to be determined. Buttgereit *et al.* 2016 described a high expression of Sall1 in adult mouse microglia, but whether this gene is also expressed in earlier developmental stages was not investigated[45]. In addition, Sall1 expression was also detected in liver, heart and kidney tissue, possibly conferring risk for side effects of PU.1 knock-out in these tissues during development. Alternatively, Bennett *et al.* 2016 reported Tmem119 to be a microglia-specific marker[46]. Although immunoreactivity for this protein was only detectable from P3 on, RNA sequencing analysis revealed that gene expression already occurred at E17.5 (but earlier embryonic stages were not analysed). Although further investigation of the expression profile of this protein is warranted, an inducible mouse model under the control of the Tmem119 promotor could be of great interest to manipulate embryonic microglia.

In conclusion, we developed this microglia depletion model based upon genetic recombination of the PU.1 gene in order to deplete microglia in different embryonic stages. While we failed to achieve full microglia depletion during development, this does not exclude the possibility that further optimization of the protocol could deplete or reduce microglia at later stages.

Chapter 4

Behavioral outcome of prenatal immune challenge in wild type and CX3CR1 deficient mice

4.1 Abstract

MIA in rodents has been shown to induce behavioral deficits in the offspring when induced at several gestational ages, albeit with slightly different aspects of symptomatology: while Poly IC in early-mid gestation induces deficits in sensory motor gating, late gestation MIA impairs working memory. In addition, genetic mutations that increase risk for neuropsychiatric disease can aggravate the behavioral deficits induced by MIA. Here we induced MIA by a single injection of Poly IC at E11.5 in wild type and CX3CR1$^{+/eGFP}$ mice to investigate the precise behavioral deficits induced by immune stimulation at this age and to study whether lack of one CX3CR1 allele would interact with the injection of Poly IC to induce a more severe phenotype. Although injection of Poly IC induced a systemic inflammatory response in the mother, as evidenced by increased levels of serum IL6, we found no effects of prenatal immune challenge on behavior in the offspring. CX3CR1 deficient mice however did show a reduced preference for social novelty in the social preference/novelty test.

4.2 Background

In the second chapter, we induced MIA in CX3CR1$^{+/eGFP}$ mice at E11.5 as Swinnen *et al.* 2013 showed that at this age, microglia density in the embryonic cortex peaks suggesting it is an important time point for microglial development[38]. However, the most common protocols in the literature are those administering Poly IC intravenously at 5mg/kg at either E9.5 or E17.5 or intraperitoneally at 20mg/kg at E12.5. These different embryonic ages represent different windows of vulnerability during pregnancy that have been related to slightly different aspects of behavioral outcome. In 2008, Meyer *et al.* demonstrated that MIA in early-mid gestation (E9.5) led to deficits in sensory motor gating while in late gestation (E17.5), MIA resulted in working memory deficits in the offspring[148]. The precise effects of MIA at E11.5 on behavioral deficits induced later in life have not yet been described.

In addition, the precise behavioral outcome of prenatal immune challenge is modified by the presence of genetic mutations. The best-studied example in this

regard is that of the Disc1 gene, which interacts with MIA to cause behavioral deficits that are more severe than with either of these risk factors alone[252-255]. Although the CX3CR1 gene is not typically associated with neuropsychiatric disease in humans, a recent meta-analysis by Bergon *et al.* 2015 showed a down-regulation of CX3CR1 in both blood and post mortem brain of schizophrenia patients with an inverse correlation between expression of CX3CR1 and symptom severity[322]. In mice, CX3CR1 deficiency results in decreased motor learning on the rotarod, impairments in contextual fear conditioning and Morris water maze performance[323] and impaired social interaction and increased repetitive behavior[324]. In contrast, Reshef *et al.* 2014 and Maggi *et al.* 2011 showed that CX3CR1 deficiency resulted in improved hippocampal-dependent learning and memory processes[303, 325]. Moreover, CX3CR1 deficient mice showed increased social withdrawal and depressive like behavior after acute LPS stimulation compared to controls[326]. So far, a possible interaction between MIA and heterozygous deletion of the CX3CR1 gene (as is the case in $CX3CR1^{+/eGFP}$ mice) has not been investigated.

To this end, we induced MIA at E11.5 by an intraperitoneal injection of Poly IC in wild type females mated with a $CX3CR1^{+/eGFP}$ male and explored the offspring's performance in several behavioral tests relevant to schizophrenia and ASD symptoms.

4.3 Materials & Methods

4.3.1 Animals

All experiments were conducted in accordance with the European Community guiding principles on the care and use of animals and with the approval of the Ethical Committee on Animal Research of Hasselt University. Mice were maintained in the animal facility of Hasselt University and Catholic University of Leuven in accordance with the guidelines of the Belgian Law and the European Council Directive. Animals were group-housed (unless otherwise stated) in a temperature and humidity controlled room with ad libitum access to food and water and a 12h/12h light dark cycle (lights on at 7.30AM).

For timed pregnancy mating, wild type C57Bl/6 females (to avoid possible effects of CX3CR1 heterozygosity on pregnancy parameters or immune reaction) were mated with CX3CR1$^{+/eGFP}$ males (on a C57Bl/6 background) overnight. The morning when a vaginal plug was detected was designated as E0.5. In the morning of E11.5, body temperature of pregnant mice was measured and weight was determined. Next, MIA was induced by an i.p. injection of Poly IC (Invivogen tlrl-picw, 20mg/kg, dissolved in NaCl, total volume of 100µl) between 9.00 AM and 9.30 AM. Five hours after the injection, body temperature was measured, pregnant females were sedated with 2% isoflurane and blood was collected by cutting a small piece of the tail. Body temperature and weight were again measured in the morning of E12.5 and E13.5. At E16.5, pregnant females were caged separately to give birth. 1 week after birth, pups were tattooed on the paw and a small piece of the tail was cut for genotyping. The resulting groups for male offspring are shown in table 4.1.

Table 4.1: Experimental groups used for behavioral experiments. Male offspring was either wild type or CX3CR1$^{+/eGFP}$ and received either NaCl or Poly IC during development.

	Offspring	Mothers
WT NaCl	17	8
WT Poly IC	16	8
CX3CR1 NaCl	12	7
CX3CR1 Poly IC	12	8

Three weeks after birth, pups were sexed and weaned, with 8 animals per cage (2 of each group). At 5 weeks of age, male offspring was transported to the animal facility of the Catholic University of Leuven. Tails of male offspring were colored with a permanent marker twice a week. Males were checked daily for signs of fighting. At 9 weeks of age, males were subjected to a battery of behavioral tests to assess ASD or schizophrenia-like behavior. Males were chosen given the fact that both schizophrenia and ASD are more prevalent in males. The timeline of all experiments and procedures is shown in figure 4.1.

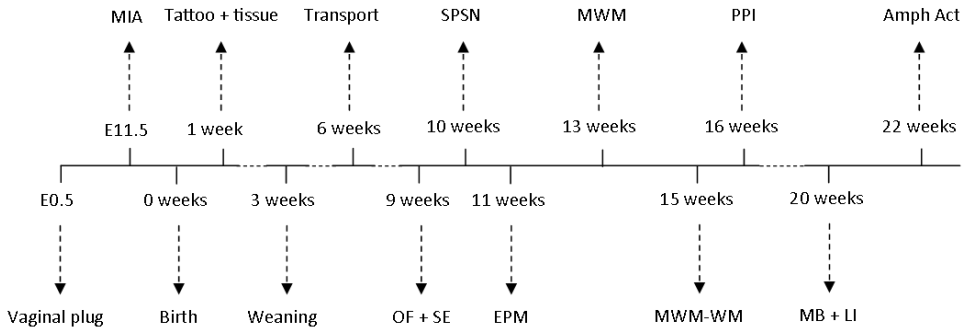


Figure 4.1: Timeline of experimental procedures and behavioral experiments. MIA: Maternal immune activation, OF: Open field, SE: Social exploration, SPSN: Social preference/social novelty, EPM: Elevated plus maze, MWM: Morris water maze, MWM-WM: Morris water maze working memory, PPI: Prepulse inhibition, MB: Marble burying, LI: Latent inhibition, Amph Act: Amphetamine-induced locomotor activity

4.3.2 Serum IL6 determination

100 µl maternal blood acquired via the tail was allowed to clot for 30 min at room temperature and centrifuged for 10 min, 8000 g at 4°C. Serum was aliquoted and stored at -80°C. IL6 concentration was determined using the mouse anti-IL6 ELISA kit (Affymetrix 88-7064-88) according to the manufacturer's instructions.

4.3.3 Genotyping

The KAPA mouse genotyping kit (KAPA Biosystems KK7352) was used according to the manufacturer's instructions.

For amplification of the wild type CX3CR1 allele, the following primers were used:

CX3CR1 Fw: 5'-TTCACGTTCGGTCTGGTGGG-3' + CX3CR1 Rev: 5'-GGTTCCTAGTGGAGCTAGGG-3'.

For amplification of the CX3CR1-eGFP allele, the following primers were used:

eGFP Fw: 5'-GATCACTCTCGGCATGGACG-3' + CX3CR1 Rev: 5'-GGTTCCTAGTGGAGCTAGGG-3'.

4.3.4 Statistics

Statistical analyses were performed with GraphPad Prism 5.0. For samples that passed the Kolmogarov-Smirnov test for normality test, a parametric test was performed while for those that were not normally distributed, a non-parametric test was performed. For serum IL6 levels and litter size, a student t-test was used. For all other data, a two way ANOVA with Bonferroni posttest was used. For maternal parameters, N represents the amount of mothers. For behavioral tests, the amount of subjects is provided in table 1. All values are presented as mean ± standard error mean.

4.3.5 Behavioral experiments

Only male offspring was tested. Before each behavioral experiment, mice were allowed to acclimatize to the room for 30 min.

4.3.5.1 Open field

Open field exploration was examined using a 50 cm x 50 cm arena. Each mouse was placed in the arena for 1 min of acclimation and 10 min testing. Movements were recorded using ANY-maze video tracking equipment and software (Stoelting Co., Illinois, USA). Total path length, path length in the center (defined as a circle with 30 cm diameter) and in the periphery, latency of first center approach and entries in the center were measured. Velocity was measured as control parameter.

4.3.5.2 Social exploration

The apparatus to examine social exploration was the same as for open field exploration. Only now 1 experienced stranger mouse was put in a wired cage placed in the center of the arena. Stranger mice were adult male C57Bl/6J mice that had served as stranger mice in other social exploration experiments. Mice were allowed to acclimatize for 1 min and to explore for 10 min. Total path length, path length in the center and in the periphery and latency to first approach were measured by recording movements using ANY-maze video tracking equipment and software (Stoelting Co., Illinois, USA). Velocity was measured as control parameter.

4.3.5.3 Social preference/Social novelty

The test arena consisted of a three chambered Plexiglas box in which chambers are connected by closable portals. The two side chambers contained cylindrical steel wired cages with white plastic top and floor in which a stranger mouse can be placed. In the acclimatization trial mice were allowed to explore the central chamber for 5 min. During the 10 min sociability trial, mice could enter the three chambers of which one side chamber contained an experienced stranger mouse and the other an empty wired cage. The side chamber containing the first stranger mouse was randomized. When a second stranger mouse was placed into the previous empty wired cage, the preference for social novelty was measured for 10 min. Between the different trials the testing animal had access only to the central chamber, allowing the researcher to place the stranger mouse into the wired cage in the side chambers. Time spent in a chamber and small periphery were used as parameters to measure social interaction.

4.3.5.4 Elevated plus maze

The apparatus consisted of a plus-shaped maze that was lifted 30 cm from the table surface. Two of the four arms (21 × 5 cm) were closed while the other two arms had no walls or roof. Animals were placed at the center of the maze and their exploratory behavior was recorded for 10 min by means of five infrared beams (four for arm entries and one for the open-arm) connected to a custom activity logger. The total number of arm visits was measured, as well as the percentage open arm visits (number of open arm visits/total number of arm visits), the ratio between the number of open and closed arm visits, and the time spent in the open arms.

4.3.5.5 Morris water maze

The water maze consisted of a large circular pool (150 cm diameter, 32.5 cm height) that was filled with 16 cm of water at 26 °C. A circular escape platform (15 cm diameter, height 15 cm) was hidden in a fixed position 1 cm below the water surface. Using non-toxic white paint the water was made opaque to prevent animals from seeing the platform. To locate the platform, animals thus had to rely on spatial information. Visual cues, such as posters, computer, and

furniture, were available to the animals. Also, the position of the experimenter during swimming trials remained constant.

Mice were trained during 10 acquisition days (Mondays through Fridays, weekends at rest) to locate the hidden platform. Four trials were performed per acquisition day from four fixed starting positions (the order of the starting positions was randomly altered each day). Mice that failed to reach the platform within 2 min were gently guided towards the platform where they were allowed to visually explore the surroundings for 15 s before being returned to the home cage. Ethovision video tracking equipment and software (Noldus, The Netherlands) were used to calculate escape latency, path length and swimming velocity. After five acquisition days there were two resting days. The following day (Monday morning before continuation of acquisition trials), a probe trial was conducted. During these probe trials, the platform was removed from the pool and the search pattern of the mouse was recorded for 100 s. Path length, swimming velocity, time spent in each quadrant, latency to the first entrance in the target quadrant and target position were calculated.

In the reversal learning consisted of five swimming trials of 2 min, each trial starting from a different position: the first trial was regarded as a cue trial, followed by four swimming trials, which were used to calculate average escape latency. The first and the last trial started from the same starting position.

4.3.5.6 Prepulse inhibition

PPI experiment was conducted in an acoustic startle box (Med Associates, St Albans, VT). Mice were placed in a restraining cubicle, which is mounted on a motion sensitive platform located inside the sound-attenuated startle box, and connected to a PC. Acoustic stimuli and background noise were delivered through speakers placed next to the restraining cubicle inside the sound attenuating box. Each test session started with 5 min acclimation time with background white noise at 65 dB maintained throughout the session. Thereafter, five startle pulses (startle pulse (SP) (115 dB)) were administered followed by 70 stimuli, randomly alternating between three different PPI trial types: (1) SP alone, (2) SP preceded by prepulse (PP) or (3) PP alone. Three different sound pressure levels were used for the PP (70-75-80dB) with 100 ms time interval

between PP and SP. Trial types were counterbalanced and inter-trial intervals were varied to reduce predictability (8–23 s). Pressure changes caused by movement of test subject on motion sensitive platform were recorded for every SP (for 200 ms after onset SP). For each recording window, the first peak value was noted and peak values averaged by trial type for each animal. Percentage PPI was calculated with the standard formula: [1 – (SP after PP)/(SP alone)] × 100. For habituation of startle response, peak values of first habituation block were averaged (stimulus block 1), peak values of 12 SP alone trials of PPI block were split into two six-trial blocks and averaged per block (stimulus block 2 and 3), and also values of last six SP alone trials were averaged (stimulus block 4).

4.3.5.7 Marble burying

Mice were placed individually in transparent plastic cages (15 × 26 × 42 cm) containing 5 cm of sawdust and 20 identical glass marbles (~1.5 cm diameter) evenly spaced 2 cm from the cage wall. The cages were placed on a platform 80 cm above the floor and under bright illumination. After 30 min, the mice were returned to their home cage, and the number of marbles buried 50% by saw dust was counted.

4.3.5.8 Latent inhibition

Latent inhibition consisted of four phases: training, pre-exposure (PE), conditioning and testing. The first phase consisted of two daily 30 min drinking sessions on 3 consecutive days (day 1-3), training water deprived mice to drink from modified Falcon tubes (15 ml). Twelve hours after the last training session, 3 PE days were scheduled (day 4-6). During PE trials, animals were presented with saccharin solution (0.5% saccharin in tap water), instead of unflavoured water. On day 7, mice were subjected to a conditioned taste aversion procedure: after 30 min drinking session with saccharin solution, mice were conditioned by injection of Lithium Chloride solution (25 mg/ml LiCl in saline, i.p. at 1% body weight, 10 min after saccharin exposure). In the afternoon session, only water was presented. Twenty four hours later, on the 8th day of the experiment, mice were given the choice between saccharin solution and water. The amount of water ingested was recorded by weighing the drinking tubes

before and after each drinking session. Aversion index is calculated using: [water intake/(water intake + saccharin solution intake)].

4.3.5.9 Amphetamine-induced locomotor activity

Animals were tested individually in small animal cages (20 cm × 30 cm same type as home cages) fitted with 3 horizontal infrared photo beams (150 mm above the floor, two on the long side, one on the short side) using a lab-built activity logger to record locomotion. A thin layer of saw dust was placed on the bottom of the cages. No water or food was available. At the start of the experiment, mice were injected i.p. with saline and activity measured for 1h. Photo beam breaks caused by test subject ambulating in test cage were registered for every 5 min-interval using an interfaced PC. After the first hour, the animal was briefly removed to be injected with d-amphetamine (in saline, i.p. injected at 3mg/kg) and placed back in the activity cage to be monitored for 2 h (post-injection) interval).

4.4 Results

After mating of wild type females with CX3CR1$^{+/eGFP}$ males, MIA was induced by a single injection of Poly IC at E11.5. To ensure that the maternal immune system was indeed activated after injection of Poly IC, IL6 was measured in maternal serum five hours after induction of MIA. In addition, weight and temperature were measured before and after MIA to assess indications of sickness behavior. As shown in figure 4.2A pregnant mice receiving NaCl gained weight 24 hours after the injection, while Poly IC treated animals lost weight. 24 hours later (48 hours after injection), maternal weight gain recovered to a level comparable to that of control animals. Similarly, Poly IC treated mice suffered from hypothermia 5 hours after injection which returned to normal 24 hours after injection (Figure 4.2B). Serum IL6 was also significantly increased after 5 hours (Figure 4.2C). Litter size at birth on the other hand was not decreased, suggesting that Poly IC stimulated the maternal immune system but not too harsh to cause embryonic lethality (Figure 4.2D).

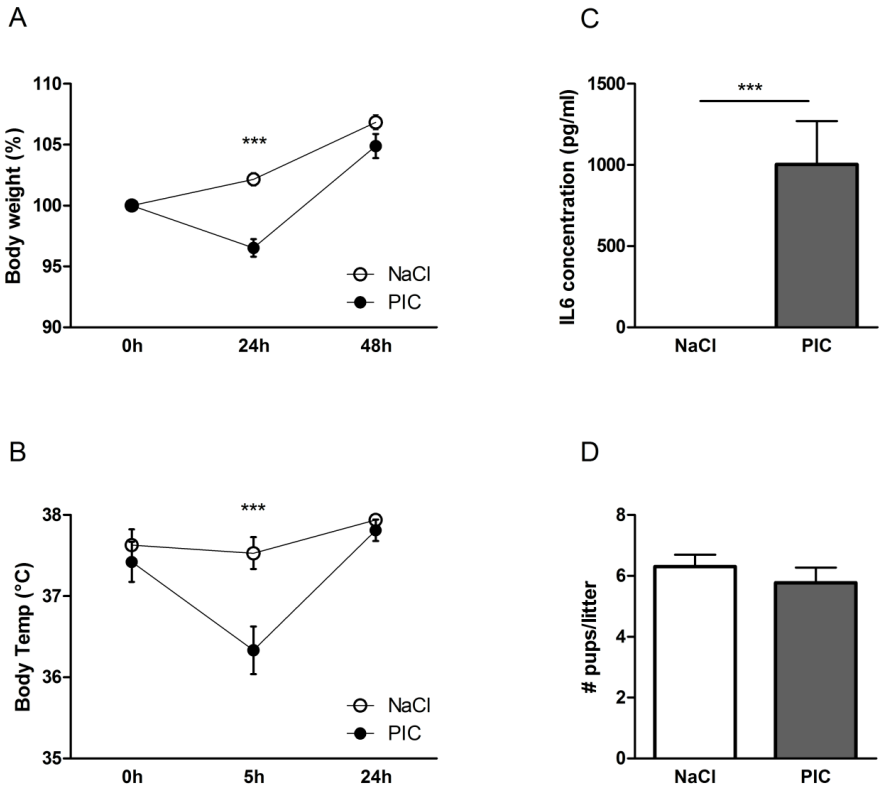


Figure 4.2: Maternal parameters of sickness behavior. (A) Increase (or decrease) in bodyweight 24 and 48 hours after injection of NaCl or PIC, expressed as percentage of bodyweight before injection. (Repeated measures Two way ANOVA with Bonferroni posttest) For NaCl N=10, for PIC N=9. (B) Increase (or decrease) in body temperature 5 and 24 hours after injection of NaCl or PIC. (Repeated measures Two way ANOVA with Bonferroni posttest) For NaCl N=10, for PIC N=9. (C) IL6 concentration (pg/ml) in maternal serum 5 hours after injection of NaCl or PIC. (Student T-Test) For NaCl N=10, for PIC N=9. (D) Mean amount of pups per litter after injection of NaCl or PIC. (Student T-Test) For NaCl N=10, for PIC N=9. *** = $p<0.0001$. PIC = Poly IC

To measure anxiety and explorative behavior in the MIA offspring, we used the open field and the elevated plus maze test. In the open field test, mice are placed in a bright lit arena where they are allowed to explore for a certain amount of time. Less time spent in the center of the arena is correlated with more anxious behavior. In the elevated plus maze, mice are placed in a plus-shaped apparatus with 2 open and 2 closed arms, at a certain distance from the ground. Increased time spent in the closed arms compared to the open arms is

again correlated with anxiety-like behavior. Offspring prenatally exposed to Poly IC did not show signs of increased anxiety and also CX3CR1$^{+/eGFP}$ mice behaved similar to wild type mice. In the open field test, the percentage of time spent in the center of the arena was comparable in all groups (Figure 4.3A). Spontaneous locomotor activity was not affected as there was no difference in total distance traveled (data not shown). In the elevated plus maze, the percentage of open arm crossings (data not shown) nor the percentage of time spent in the open arms was affected by either treatment nor genotype (Figure 4.3B).

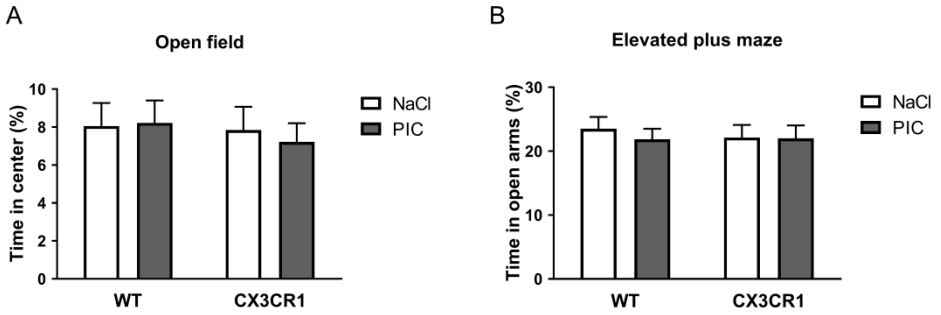


Figure 4.3: Anxiety-like behavior tested in the open field and elevated plus maze test. (A) In the open field test, the amount of time spent in the center, expressed as percentage of total time of the test, is shown. (B) In the elevated plus maze test, the amount of time spent in the open arms, expressed as percentage of total time of the test, is shown.

To assess sociability of MIA offspring, both a social exploration and a social preference/novelty test were performed. In the social exploration test, a stranger mouse was placed under a wired cage in the middle of the open field arena. The percentage of time spent in the center of the arena, and thus in proximity of the stranger mouse, was similar in all four groups, suggesting normal interest in social interactions in Poly IC exposed offspring (Figure 4.4A).

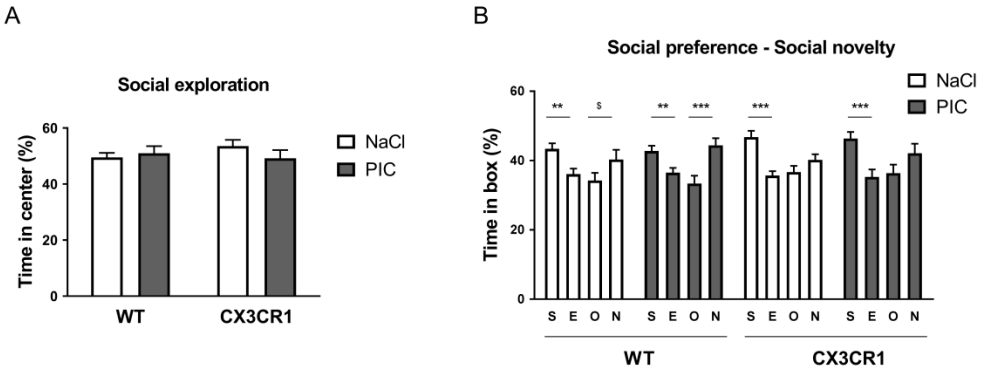


Figure 4.4: Social behavior tested in by the social exploration and social preference/social novelty test, (A) In the social exploration test, the amount of time spent in the center in proximity of the stranger mouse, expressed as percentage of total time of the test, is shown. (B) In the social preference/social novelty test, the amount of time spent in each chamber, expressed as percentage of total time of the test, is shown. In the first part of the test, time spent in the stranger compartment (S) is compared to the time spent in the empty wired cage compartment (E). in the second part of the test, time spent in the old stranger compartment (O) is compared to the time spent in the new stranger compartment (N). §=p<0.1, ** = p<0.01, *** = p<0.001.

The social preference/novelty test on the other hand was conducted in a 3-chamber apparatus where in the first part of the test an empty wired cage and a stranger mouse are presented. All groups show preference for the social chamber compared to the object chamber (Figure 4.4B). In the second part of the test, the empty wired cage is replaced by a novel stranger mouse to evaluate social affiliation, recognition and memory. More time spent with the 'old stranger' indicates either preference of interaction with a familiar compared to an unfamiliar mouse or deficits in social memory. The wild type animals prenatally exposed to Poly IC spent significantly more time in the chamber with the novel stranger compared to the old stranger. For wild type animals prenatally exposed to NaCl, there was a clear trend in the same direction. On the other hand, CX3CR1$^{+/eGFP}$ mice prenatally exposed to either NaCl or Poly IC did not spent significantly more time in the compartment with the novel stranger, suggesting a reduced preference for social novelty or a deficit in social memory.

Spatial learning and memory was assessed in the standard hidden platform Morris water maze test, where the testing mouse is expected to find an escape platform hidden in the water. Average swimming speed was similar in all groups, indicating no locomotor deficits (Figure 4.5A). Spatial learning was not affected by either treatment or genotype, as the average distance travelled before finding the platform was similar in all groups on all training days (Figure 4.5B). On the last day of the test, a probe trial was performed in which the target platform was removed from the water and the time searching for the platform in each quadrant was measured for 2 minutes. All groups spent significantly more time in quadrant 4, the quadrant in which the target platform was previously located (Figure 4.5C). These data indicate normal spatial learning and memory in Poly IC treated and CX3CR1 deficient mice. In the Morris water maze, we also assessed reversal learning/working memory to reveal whether they can extinguish their initial learning of the platform's position and acquire a direct path to the new position. To this end, the escape platform was placed in a different quadrant each day. Figure 4.5D shows the ratio between the time to reach the platform in trial 1 vs trial 5 for all training days. These are the first and the last trial of each day and were started from the same position. A ratio lower than 1 means that they found the platform faster in the last compared to the first trial and working memory is thus intact. No significant differences were found in working memory among the different groups.

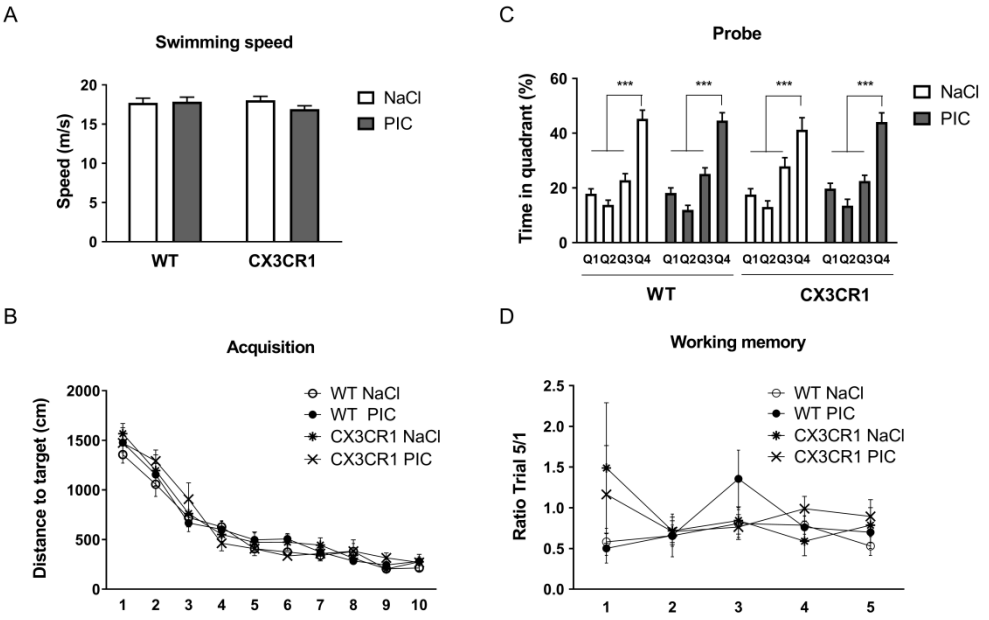


Figure 4.5: Spatial learning and memory in the Morris water maze. (A) Average swimming speed on the first learning day. (B) Acquisition along the 10 learning days, expressed as average distance travelled before reaching the target platform. (C) Probe trial on the last testing day, expressed as percentage of total time spent in each quadrant of the water maze. Quadrant 4 (Q4) is the quadrant that contained the platform during the learning days. (D) Working memory assesment in the Morris water maze showing the ratio of time to find the platform in the first trial over that of the fifth trial of each day.

To measure possible deficits in information processing, we used the PPI test and the conditioned taste aversion paradigm of latent inhibition. PPI represents a mechanism to filter out irrelevant stimuli and is assessed by measuring the startle response elicited by a loud sound (pulse) either or not preceded by a weaker sound (prepulse). Latent inhibition refers to the inhibition of a conditioned response to a stimulus when the individual has been repeatedly exposed to the stimulus before pairing with the unconditioned response. Here, latent inhibition was assessed by providing the animals with sucrose solution for several days before pairing it to an aversive stimulus (nausea). As represented in figure 4.6A, the percentage of startle inhibition by presentation of a prepulse was not different between different groups. Also latent inhibition, as represented by a similar water vs saccharine intake on the testing day of the conditioned

taste aversion test, is not altered (Figure 4.6B). These data suggest normal information processing in Poly IC exposed and CX3CR1$^{+/eGFP}$ animals.

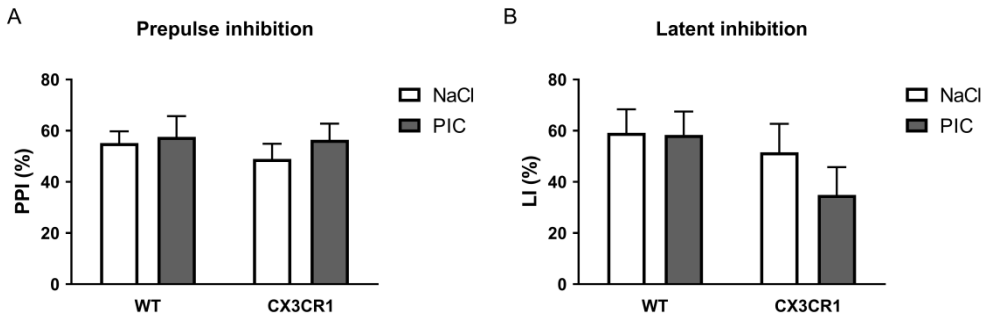


Figure 4.6: Measurement of information processing by the prepulse and latent inhibition test. (A) The percentage of prepulse inhibition as calculated by the startle response after a pulse preceded by a prepulse over the response after only a pulse is indicated. (B) The percentage of latent inhibition as calculated by the amount of water consumed over the total amount of fluid consumed (water + saccharine solution).

To test whether Poly IC exposed or CX3CR1$^{+/eGFP}$ animals exhibited alterations in the sensitivity of their dopamine system, we measured locomotor activity induced by systemic administration of amphetamine, a dopamine receptor agonist. With a more sensitive dopamine system, this would induce a higher locomotor response compared to controls. The first 60 minutes, locomotor activity upon a saline injection was measured. After 1 hour, an intraperitoneal injection of amphetamine was administered. All groups displayed a similar increase in locomotor activity after exposure to saline and amphetamine, indicating no change in dopamine sensitivity (Figure 4.7).

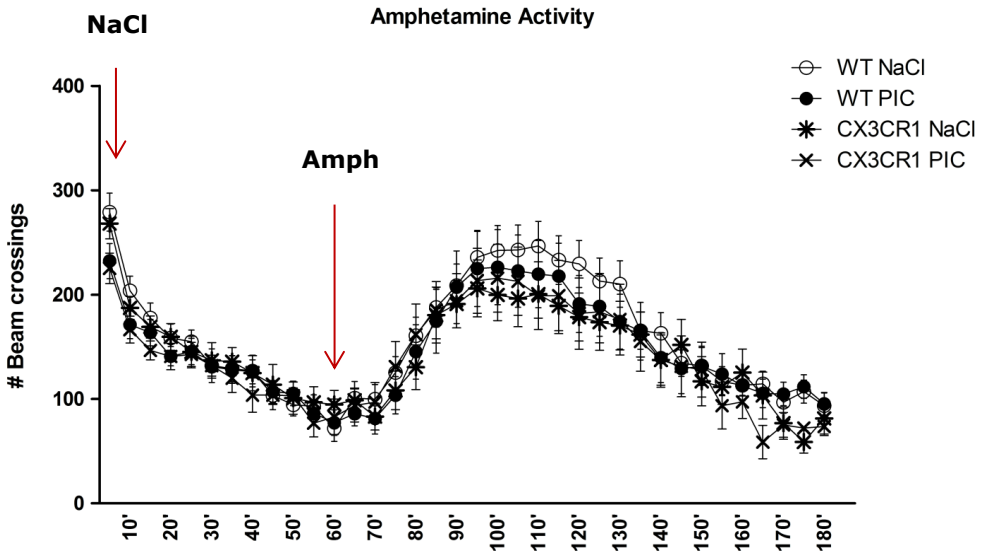


Figure 4.7: Locomotor response to amphetamine injection. At 0', animals were injected with NaCl and at 60' with Amph. The amount of beam crossings, as a measure for locomotor activity, are presented. Amph: amphetamine.

Finally, repetitive behavior was measured with the marble burying test. In this test, mice are placed in a cage containing glass marbles and allowed to explore for 30 minutes. The amount of marbles buried for more than 50% in the bedding are counted. As shown in figure 4.8, the amount of marbles buried was comparable in all groups, suggesting no increased repetitive behavior induced by either treatment or genotype.

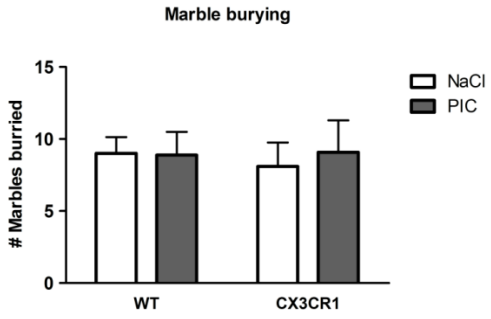


Figure 4.8: Repetitive behavior measured with the marble burying test. The amount of marbles buried for more than 50% in the bedding is shown.

4.5 Discussion

In this study, we induced MIA in wild type and CX3CR1$^{+/eGFP}$ heterozygous mice at E11.5 to describe the resulting behavioral phenotype. To ascertain that the maternal immune system was indeed activated by the administration of Poly IC, we measured serum levels of the pro-inflammatory cytokine IL6 5 hours after Poly IC injection as well as maternal body weight and temperature. Cunningham *et al.* 2007 described the body response to Poly IC in mice and found a peak increase in serum levels of IL1β, IL6, TNFα and IFNγ 3 hours after injection[327]. As IL6 has been described to be crucial in mediating the effects of MIA onto the offspring[140], we measured maternal serum IL6 5 hours after induction of MIA and found a significant increase, suggesting a systemic immune response to the Poly IC. Moreover, comparable to Cunningham *et al.* 2007, we found a transient decrease in body weight and temperature[327]. Although Missault *et al.* 2014 found some Poly IC treated mothers to gain weight 24 hours after MIA induction, in our experiments, all Poly IC treated mice lost weight over 24 hours[227]. Together with the fact that we did not find any difference in average litter size between Poly IC and NaCl injected mothers, these data indicate that Poly IC induced a transient stimulation of the immune system, severe enough to induce maternal sickness behavior but not too severe to induce embryo loss.

Despite a clear maternal response to the injection of Poly IC, no behavioral deficits were found in either of the experimental groups, except for a lack of preference for social novelty in CX3CR1 deficient mice. This impairment in social interaction is in line with the deficits found in CX3CR1 knock-out mice by Zhan *et al.* 2014[324]. While most studies employing an intraperitoneal administration of 20mg/kg Poly IC at either E9.5 or E12.5 describe deficits in PPI[161, 224, 250, 328], decreased social interaction[160, 161, 163, 250, 264, 267] and increased marble burying[162, 163, 250, 258], not all of them recapitulate these findings. In the study conducted by Ehninger *et al.* 2012, they found no difference in social interaction and open field exploration between wild type Poly IC and NaCl adult offspring[256]. Similarly, Wu *et al.* 2015 failed to demonstrate deficits in PPI and open field exploration while marble burying was increased in MIA offspring[258]. Most recently, Buschert *et al.* 2016 highlighted the importance of environmental enrichment to reveal the impact of MIA on offspring's behavior[153]. Under

standard housing conditions, MIA did not affect social interaction, a deficit that only became apparent under enriched housing conditions. In our experiments, animals were kept in standard housing conditions, which could possibly have masked behavioral differences between groups.

As described in chapter 2, mice homozygous for CX3CR1-eGFP do not express CX3CR1 protein which leads to altered microglial properties, a phenomenon that is not found in $CX3CR1^{+/eGFP}$. Similarly, behavioral alterations (either improvements or impairments) are evident in CX3CR1 knock-outs while these are more subtle or even absent in mice with one CX3CR1 allele[303, 323-325]. Based on the finding that blood and post mortem brain CX3CR1 levels in schizophrenia patients negatively correlate with symptom severity[322] and that acute LPS challenge caused a prolonged duration of social withdrawal in $CX3CR1^{-/-}$ mice[326], we hypothesized that a lack of one CX3CR1 allele could aggravate MIA outcome in the offspring. However, a recent publication by Rimmerman *et al.* 2017 described a protective effect of CX3CR1 deficiency on behavioral outcome upon stress[302]. Wild type mice exposed to short or chronic unpredictable stress displayed reduced sucrose preference and impaired novel object recognition memory while mice lacking CX3CR1 did not show these behavioral alterations. Thus, instead of interacting with MIA to induce behavioral changes in the offspring, the reduction in CX3CR1 might have protected these animals from developing autistic or schizophrenic features.

One drawback of this study is that we used more than 1 littermate per mother. As discussed by Meyer *et al.* 2009, littermates are exposed to a highly similar pre- and postnatal environment, and share genetic and epigenetic similarities[136]. Therefore, using littermates could possibly overestimate or in this case, mask behavioral deficits induced by MIA. Another disadvantage of the experiments described in this chapter is the acquisition of a blood sample to confirm an increase in maternal serum IL6 levels. While this measure gave valuable information on the immune activation status of the mother, it required an additional intervention that is likely to have induced maternal stress. Specifically, pregnant mice were briefly exposed to isoflurane, a small piece of the tail was cut and about 100µl of blood was taken. The combination of these interventions might have induced stress in the mother, a factor that by itself is

known to increase the risk for developing neuropsychiatric disorders in the offspring, and thus altered behavior also in offspring from NaCl injected mothers, thereby nullifying those induced by injection of Poly IC. Supporting this possibility, a recent publication by Mouihate and Mehdawi 2016 showed that both injection of NaCl or LPS in pregnant rats led to a high level of corticosterone in the fetal brain 4 hours after MIA[329]. To explore whether the experimental procedure accompanied by the blood sampling could cause behavioral alterations independent of Poly IC, one could expose pregnant mice to the different procedures separately (anesthesia, tissue damage, blood acquisition), and assess behavior in the offspring.

In conclusion, we found no difference in behavior between NaCl and Poly IC exposed animals while CX3CR1$^{+/eGFP}$ animals differed from wild type animals in that they did not show preference for social novelty in the social preference/novelty test.

Chapter 5

Influence of maternal immune activation on cortical development

5.1 Abstract

Several genes associated with neuropsychiatric disorders such as schizophrenia and ASD are involved in regulation of normal development of the brain and mutations in these genes have been shown to disrupt neurodevelopment. In the MIA model, it was recently shown that laminar positioning of interneurons and outgrowth of dopaminergic axons was altered in the offspring. To investigate whether prenatal immune challenge affects the normal trajectory of developing pyramidal neurons, we fluorescently labeled radial glia progenitors in the ventricular zone of the cortex by *in utero* electroporation and analyzed the distribution of the labeled cells at a later developmental stage. MIA was induced by 1 or 2 injections of Poly IC and different populations of neuronal progenitors were targeted by electroporation at different developmental stages. Although maternal serum IL6 levels were increased, positioning of labeled neurons was not affected by prenatal exposure to Poly IC. However, when comparing neuronal positioning between single and double injected mice, an increased percentage of cells in the upper region of the cortex was observed. These data suggest that prenatal stress, independent of infection, is sufficient to induce alterations in the development of pyramidal neurons.

5.2 Background

Although we showed in chapter 2 that embryonic microglia do not become activated after a maternal immune challenge with Poly IC, inflammatory mediators originating from the mother or the placenta could still affect normal brain development. For example, CXCL12 serves as a guidance molecule for migrating interneurons and is important for correct distribution of cajal retzius cells[330, 331]. Moreover, IL6 has been shown to play a role in neurogenesis, astrogliogenesis and oligodendrogliogensis[299, 332, 333].

Evidence for dysregulated neuronal development in neuropsychiatric disease comes from several studies investigating the role of genetic risk factors in the developing brain. As such, it was recently shown that a lack of FMRP, the gene that causes Fragile X Syndrome, impairs migration of neuronal progenitors by

interfering with their transition from the multipolar to the bipolar stage[334]. Absence of DISC1 impaired proper migration of radially migrating pyramidal neurons and tangentially migrating interneurons into the cortical plate[335, 336].

In the MIA model, reduced cell proliferation in the ventricular zone of the developing cortex and increased cell density in layers VI and V of the cortical plate were found[337]. Squarzoni *et al.* 2014 reported deficits in the laminar positioning of Lhx6-expressing interneurons and reduced outgrowth of dopaminergic axons after prenatal immune challenge[44].

Here we investigated whether the developmental trajectory of pyramidal neurons in the developing cortex could be affected by MIA. To this end, we fluorescently labeled developing neurons using *in utero* electroporation. This technique is widely used to examine the function of genes in the developing brain, as it allows labeling a specific pool of neurons in a specific time and place. Briefly, a DNA vector carrying a gene of interest is injected into the lateral ventricle of the developing brain at early embryonic stages. The vector is then incorporated into the cells lining the ventricular surface (radial glia) by the application of an electrical current which causes the formation of transient pores in cell membranes. By adjusting the direction of the electrodes and choosing a specific developmental stage for the electroporation, we can transfect radial glia cells in a spatially and temporally restricted manner and follow a specific subpopulation of their progeny. Besides analyzing the position of fluorescently labeled cells in fixed brain slices, this technique also allows to investigate their development in real time by imaging of live brain slices.

We electroporated a plasmid encoding the fluorescent molecule tdTomato in neuronal progenitors of the developing somatosensory cortex of MIA offspring at different developmental stages to investigate the formation of neuronal progenitors destined for different cortical layers. At the end of embryonic development, the distribution of tdTomato labeled cells in the cortex was compared between MIA and control animals.

5.3 Materials and methods

5.3.1 Animals

All experiments were conducted in accordance with the European Community guiding principles on the care and use of animals and with the approval of the Ethical Committee on Animal Research of Hasselt University. Mice were maintained in the animal facility of Hasselt University and Catholic University of Leuven in accordance with the guidelines of the Belgian Law and the European Council Directive. Animals were group-housed (unless otherwise stated) in a temperature and humidity controlled room with ad libitum access to food and water and a 12h/12h light dark cycle (lights on at 7.30AM).

For timed pregnancy mating, wild type C57Bl/6 females were mated with wild type males overnight. The morning when a vaginal plug was detected was designated as E0.5. In the morning of E11.5 and/or E14.5, E15.5 or E16.5, body temperature of pregnant mice was measured and weight was determined. Next, MIA was induced by an i.p. injection of Poly IC (Invivogen tlrl-picw, 20mg/kg, dissolved in NaCl, total volume of 100µl) between 9.00 AM and 9.30 AM. Control animals were given an injection of NaCl. Five hours after the injection, pregnant females were sedated with 2% isoflurane and blood was collected by cutting a small piece of the tail. Body weight was monitored 24 and 48 hours after injection. A timeline of experimental interventions is shown in figure 5.1.

5.3.2 *In utero* electroporation

Plasmid: tdTomato expressed under a chicken β actin promotor was used to visualize migrating neurons. The DNA was concentrated at 2 µg/µl. 1% of the non-toxic fast green dye was added to the DNA solution to monitor the location of the injected DNA solution.

Micropipettes: The micropipettes for injection were made from 1 mm diameter glass capillary tubes (Harvard Apparatus) with a micropipette puller (Sutter Instruments). The tip of the micropipettes was cut with a fine forceps at an outer diameter of 40µm and sharpened obliquely for 30 min using a microforge

(M830 Narashige). One micropipette was attached to a plastic tube to aspirate the DNA and make the injections.

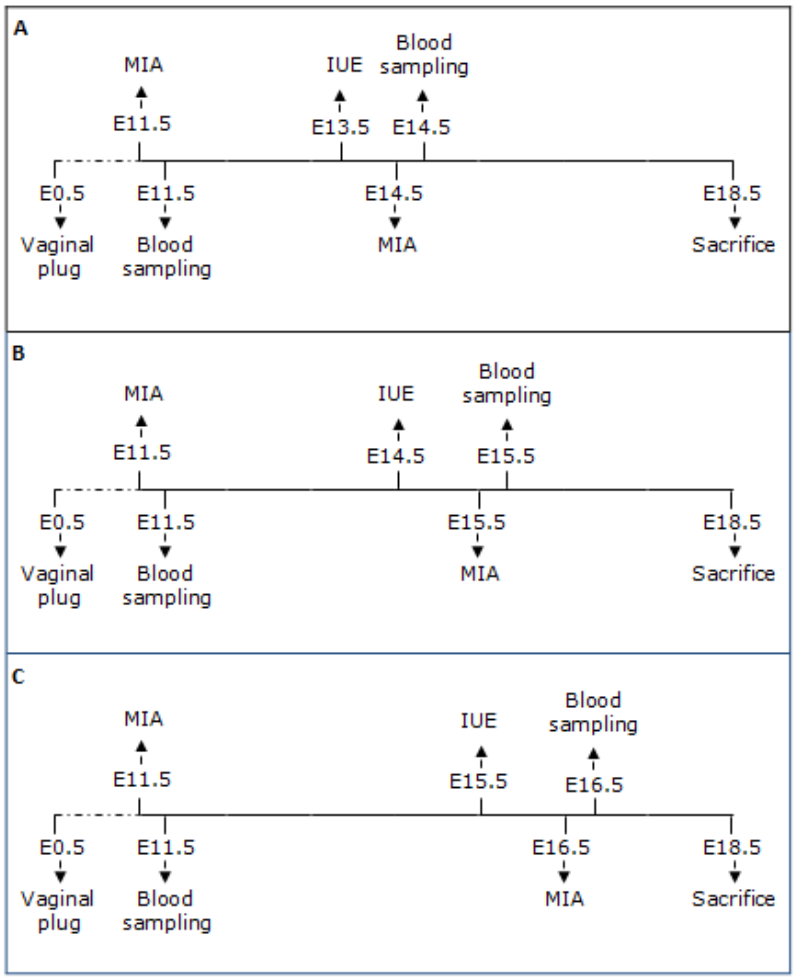


Figure 5.1: Timeline of experimental interventions with (A) IUE at E13,5, (B) IUE at E14,5 and (C) IUE at E15.5. IUE = *In utero* electroporation

Surgery: A pregnant C57Bl/6 wild type female at E13.5, E14.5 or E15.5 (around 2 PM) was anesthetized with 2% isoflurane while the mouse was placed on its back on a 37°C heated dissecting board. Eyes were moistened by covering them with a smear of Terra-Cortril®+polymyxine-B eyecream. Hair was shaved from the abdomen using a razor blade and 100% ethanol. An approximately 2 cm midline skin incision was made with fine scissors (Fine Science Tools). The skin

was detached from the abdominal wall with a razor blade to facilitate skin closure at the end of the procedure. A 2 cm midline incision parallel to the skin incision was made in the abdominal wall with fine scissors. A whole was cut in the center of sterile gauze that was placed over the incision. One uterine horn was exposed and placed on the sterile gauze. The uterus was kept moist with warm (37°C) saline at all times. After pressing the head of the embryo against the uterine wall to improve visualization, the micropipette was inserted in the lateral ventricle and 1-2 µl of DNA solution was injected. The fast green dye enabled the distribution of the DNA solution in the lateral ventricle to be seen through the uterine wall. The head of the embryo was pinched with a forceps-type electrode (5mm diameter) and electronic pulses were applied with a ECM 830 electroporator (Harvard apparatus) (37V, 50 ms pulse and 950 ms interval, 5 pulses). To introduce the plasmid into the cortex, the anode was placed on the center of the injected hemisphere and the cathode was placed on the chin. After the procedure was completed on the embryos from the first uterine horn, this horn was placed back in the abdominal cavity and the second uterine horn was exposed and subjected to the same procedure. When the procedure was completed on the second uterine horn, the abdominal cavity was filled with warm saline and the abdominal wall was closed with vicryl sutures (Ethicon). The skin was closed with 7 mm skin clips (Fine Science Tools) and the wound was cleaned with iso-Betadine. The mouse was kept warm until it recovered completely from anesthesia by placing it on a heating pad.

Embryo retrieval: At E18.5, the mother mouse was killed and the embryos were removed. Brains were dissected and fixed overnight in 4% PFA at 4°C.

5.3.3 Confocal microscopy and analysis

Fixed embryonic brains were cut in 100 µm coronal sections using a vibratome (Micron) and mounted on microscopy slides using vectashield (DAKO). Confocal images were obtained on a Nikon microscope (C2 Eclipse Ni-E) using Nis-Element software. From each tdTomato-positive brain slice, Z-stacks (10 µm/stack) were obtained from the tdTomato-positive region at a 10x magnification. Images were processed using the Fiji software (US National Institutes of Health).

For cortical positioning of tdTomato-labeled neurons, the total length of the cortex was divided in ten equal segments (BINs) and the amount of labeled cells in each BIN was manually counted. The amount of cells per BIN was summed up over all brain slices per embryo and divided by the total number of cells counted × 100 (as percentage).

5.3.4 Serum IL6 determination

100 µl maternal blood acquired via the tail was allowed to clot for 30 min at room temperature and centrifuged for 10 min, 8000 g at 4°C. Serum was aliquoted and stored at -80°C. IL6 concentration was determined using the mouse anti-IL6 ELISA kit (Affymetrix 88-7064-88) according to the manufacturer's instructions.

5.3.5 Genotyping

The KAPA mouse genotyping kit (KAPA Biosystems KK7352) was used according to the manufacturer's instructions.

For amplification of the SRY gene, the following primers were used:

Sry Fw: 5'-TTGTCTAGAGAGCATGGAGG-3' + Sry Rev: 5'-CTCCTCTGTGACACTTTAGC-3'

5.3.6 Statistics

Statistical analyses were performed with GraphPad Prism 5.0. For samples that passed the Kolmogarov-Smirnov test for normality test, a parametric test was performed while for those that were not normally distributed, a non-parametric test was performed. For body weight increase/loss 24 hours after injection, a student t-test was used. For serum IL6 levels, a two way ANOVA with Bonferroni posttest was used. For cortical positioning of neuronal progenitors, student t-test were performed on individual BINs. N represents the amount of mothers while n represents the amount of embryos. All values are presented as mean ± standard error mean.

5.4 Results

To test whether MIA could induce alterations in the positioning of developing neurons in the cortex, we labeled radial glia progenitors at E13.5, E14.5 or E15.5 by *in utero* electroporation of a tdTomato expressing plasmid. Corresponding to the inside-out formation of the cortex, electroporation at different embryonic ages targets neuronal progenitors destined for different cortical layers. Specifically, neuronal progenitors at E13.5 will mainly populate cortical layers V-IV, those born at E14.5 will make up layer IV-III and progenitors from E15.5 are destined to become layer III-II neurons. MIA was evoked by a double injection of Poly IC, the first injection administered at E11.5 and the second 24 hours after electroporation (so at E14.5, E15.5 or E16.5). Control animals were injected with NaCl. IL6 levels in maternal serum were significantly increased in Poly IC injected females compared to controls after the first injection, indicating that the injection of Poly IC induced systemic maternal inflammation (Figure 5.2A).

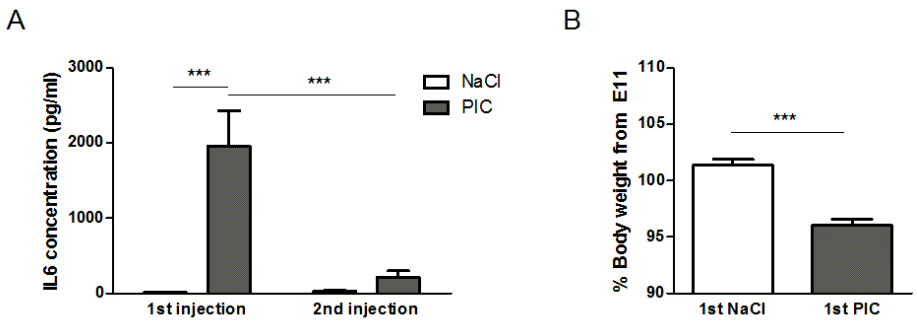


Figure 5.2: Maternal parameters after MIA. (A) IL6 concentration (pg/ml) in maternal serum 5 hours after the first and second injection of NaCl or PIC. (Two way ANOVA with Bonferroni posttest). *** = $p<0.001$. For 1st NaCl N=18. For 1st PIC N=17. For 2nd NaCl N=12. For 2nd PIC N=12. (B) Body weight increase/loss 24 hours after injection of NaCl or PIC, presented as percentage of body weight before injection. (Student T-Test) *** = $p<0.001$. For 1st NaCl n=14. For 1st PIC n=14. PIC = Poly IC

Strikingly, the increase in IL6 serum levels is significantly more modest after the second injection of Poly IC compared to the first injection, no longer reaching statistical significance compared to NaCl after Bonferroni post testing. As a

second control, weight gain 24 hours after injection was measured. Animals injected with Poly IC lost weight after 24 hours whereas control animals gained weight over 24 hours (Figure 5.2B). After the second injection, a decrease in body weight was no longer detectable (data not shown), likely because the relative contribution of the embryos to the total weight of the mother is dramatically increased.

At E18.5, embryos were harvested and brains cut into coronal vibratome sections. The total thickness of the developing cortex was divided in ten equal BINs and the percentage of tdTomato-labeled cells in each BIN calculated per embryonic brain. The mean percentage of cells in all BINs is shown in figure 5.3 for *in utero* electroporation at E13.5, E14.5 and E15.5.

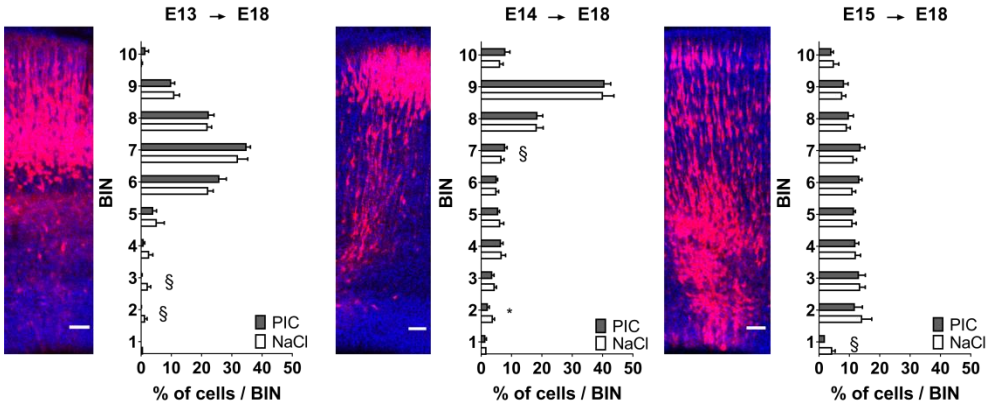


Figure 5.3: Cortical postitioning of neuronal progenitors labeled at E13.5, E14,5 or E15,5 in mice injected twice with eiter NaCl or PIC. The percentage of cells in each of the 10 equally divided BINs is shown. BIN 1 represents the ventricular zone of the neocortex, BIN 10 the marginal zone region. Values are reported as mean ± SEM. Left from the bar graph, a representative image from an NaCl injected mouse is shown. For IUE E13.5: n/N=5/3 for NaCl and n/N=4/2 for PIC. For IUE E14.5: n/N=9/4 for NaCl and n/N=15/5 for PIC. For IUE E15.5: n/N=5/3 for NaCl and n/N=7/4 for PIC. § = $p<0.1$, * $p<0.05$. Scale bars represent 25µm. PIC = Poly IC

The cortical plate roughly starts around BIN 6. With electroporation at E13.5, the highest percentage of cells was found in BINs 6, 7 and 8 which roughly corresponds to the lower cortical layers while only very few cells were found in the proliferative zones. This can be explained by the fact that tdTomato plasmid

that has largely been diluted out and/or the limited divisions radial glia have in mice.

Progeny of neuronal progenitors labeled at E14.5 were mainly located in BINs 8 and 9, corresponding to the middle to upper layers of the cortical plate. After electroporation at E15.5, the distribution of labeled cells appears to be more spread out. Compared to electroporation at E13.5 and E14.5, these progenitors did not have as much time to reach their final destination. In addition, onset of astrogenesis starts at the end of neurogenesis, possibly explaining the relatively large amount of cells in the lower BINs. Except for BIN 2 after electroporation at E14.5, the percentage of cells in no other BINs was significantly different, suggesting that immune stimulation with Poly IC does not substantially affect neuronal positioning. To exclude the possibility that female and male offspring would be differenially affected by MIA, we genotyped previously analysed samples to separate male from female embryos, but both sexes showed a similar distribution of labeled progenitors, independent of prenatal treatment (data not shown).

Aside from stimulation of the maternal immune system with an infectious agent, stress during pregnancy has also been shown to predispose to the development of schizophrenia and ASD and maternal stress has been described to exert effects on the developing brain[338]. Among others, prenatal stress affects morphological maturation of hippocampal neurons[339, 340], to down regulate expression of reelin[341] and to disturb the fetal serotonergic system[342]. To investigate whether an effect of immune stimulation with Poly IC on neuronal positioning could have been masked by the effect of additional stress from the second injection, we investigated neuronal positioning in mice receiving only a single injection at E11.5. As shown in figure 5.4A, except for the small decrease in BIN 1, no differences in the percentage of cells per BIN could be detected, suggesting that Poly IC has no effect on cortical postioning of neuronal progenitors.

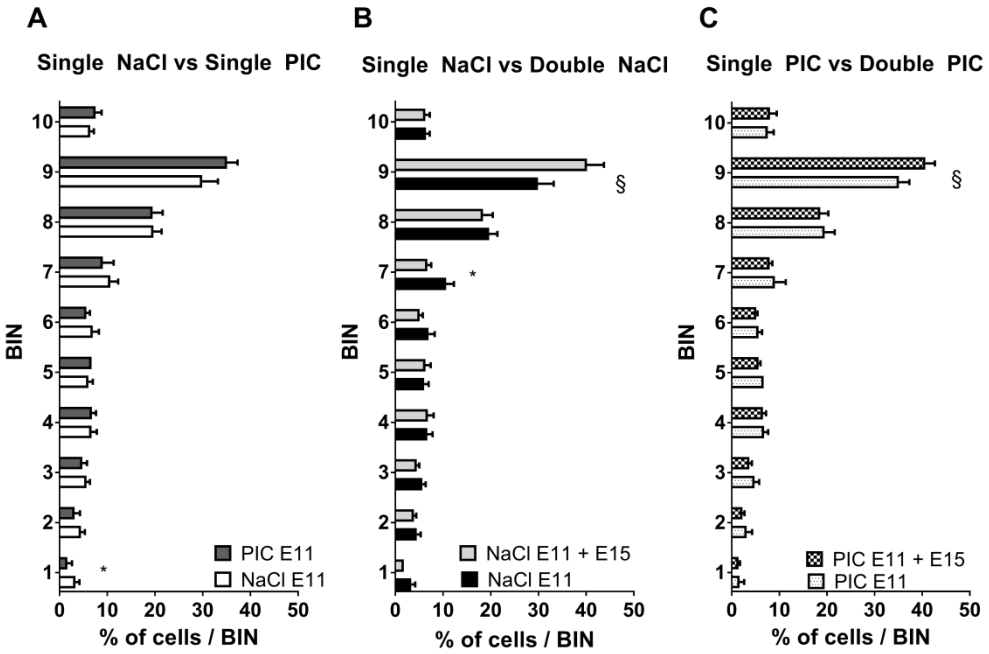


Figure 5.4: Cortical postitioning of neuronal progenitors labeled at E14.5 and NaCl or PIC at E11.5 (A) or NaCl at E11.5 and E15.5 (B) Percentage of cells in each of the 10 equally divided BINs is shown. BIN 1 represents the inner border of the neocortex, BIN 10 the outer border. Values are reported as mean ± SEM. * $p<0.05$. n/N=8/4 for NaCl E11 and n/N=9/5 for PIC E11. PIC = Poly IC

However, when we compare the percentage of cells in each BIN between the two control groups (i.e. single or double NaCl injection), we find a significant decrease in the percentage of cells in BIN 7 ($p=0.0274$) in double injected mice concomintant with a strong trend towards an increase in the percentage of cells in BIN 9 ($p=0.0532$). For the ease of interpretation, the data from figure 3 and 4A are shown together in figure 5.4B. These data suggest that the stress associated with the injection of NaCl and the blood sampling could lead to subtle deficits in neuronal positioning in the cortical plate. Off note, comparison of single and double Poly IC injection also yielded a trend toward a higher percentage of labeled cells in BIN 9 (Figure 5.4C). However, to make final conclusions on this, we would have to compare these data with those from mice that were only *in utero* electroporated but received no other injections.

5.5 Discussion

The goal of these experiments was to evaluate whether strong MIA by 2 injections of the viral mimic Poly IC could induce deficits in the cortical positioning of developing pyramidal neurons. We used *in utero* electroporation of a tdTomato expressing plasmid to label neuronal progenitors in the proliferative zone of the developing cortex. The DNA plasmid is taken up by cells lining the lateral ventricle under the influence of an electrical field and upon cellular division, the plasmid is divided among the mother and the daughter cell, thereby also labeling the progeny from originally electroporated cells. Depending on the embryonic age, neuronal progenitors in the proliferative zones are destined to make up a different cortical layer. Therefore, we electroporated at three different embryonic ages, E13.5, E14.4 or E15.5, to cover a wide range of neuronal precursors.

As a measure for immune stimulation in the mother, we determined IL6 values in maternal serum 5 hours after injection. At E11.5, IL6 was significantly increased in Poly IC injected mice compared to controls. Also the second injection of Poly IC resulted in a significantly increased amount of IL6 compared to injection with saline, but it was not as pronounced as that of the first injection. In 2005, Meyer *et al.* showed a dose dependent increase in maternal serum levels of IL10 upon Poly IC injection and dose dependent effects on the offspring deficits in PPI and sensitivity to amphetamine-induced hyperlocomotor activity[343]. Shi *et al.* 2003 found a similar dose dependent effect on PPI defects[284]. These data suggest that the second immune stimulation, although administered at the same dose as the first one, might not have been strong enough to induce deficits in neuronal migration. The reason for this reduced immune response is unclear. One possibility could be that the surgery to perform *in utero* electroporation increases glucocorticoid levels in the pregnant female. Glucocorticoids are potent immunosuppressants, as they attenuate signaling pathways downstream of many danger sensors, thereby suppressing the production of inflammatory mediators[344]. Furthermore, unlike Poly IC at E11.5, the second injection did not induce weight loss at 24 hours after injection. Whether this is due to a more modest immune response (as could be

concluded from lower IL6 levels) or to a relatively larger contribution of embryo weight to total maternal weight is not clear.

Another reason for not finding a difference in the neuronal positioning between NaCl and Poly IC exposed offspring (either with single or double injection) could be the stress accompanied by the protocol used. Stress is typically associated with stimulation of the HPA axis and increased production of glucocorticoid hormones such as cortisol in humans and corticosterone in rodents. Mouihate and Mehdawi 2016 investigated corticosterone levels in fetal brain upon maternal immune stimulation with LPS and found that 4 hours post-injection, both saline and LPS treated animals showed high levels of corticosterone that decreased drastically after 24 hours[329]. Importantly, corticosterone has been shown to influence migration and dendritic growth of developing purkinje cells in the cerebellum[345] and neuronal proliferation and differentiation in the developing hippocampus[346]. In addition to the effects of stress induced by handling and injection, a few studies also report on the effects of exposure to isoflurane on the developing brain. Wang *et al.* 2009 showed that general anesthesia in rats with isoflurane was able to induce neurodegeneration in the developing hippocampus and the retrosplenial cortex[347]. Similarly, Wei *et al.* 2005 described increased neuronal damage in primary neuronal cultures that were exposed to isoflurane[348]. As many general anesthetics, including isoflurane, affect GABA receptor signaling[349] and the fact that GABA receptors are involved in the regulation of neuronal migration (as reviewed by [350]), it is possible that isoflurane affects neuronal development in this way. Additionally, exposure to corticosterone during pregnancy can also affect placental properties, including altered amino acid supply and blood vessel density, which in turn could affect brain development[351, 352].

To elucidate whether the difference in neuronal positioning between single and double NaCl injected animals could be due to stimulation of the HPA axis by either *in utero* electroporation and/or injection handling, future experiments should include measurements of corticosterone levels in maternal blood and fetal brain after Poly IC injection and after *in utero* electroporation.

Chapter 6

The function of DISC1 in microglia

Based on:

This chapter is based on the master thesis by Sofie Kessels: "Functionally disturbed microglia: a risk factor for neurodevelopmental disorders?" and on the manuscript in preparation: "Efficient transfection of a microglia cell line using magnetofection"

Own contribution:

Supervision of the master thesis

6.1 Abstract

The role of microglia in neuropsychiatric disorders has gained much attention in recent years since evidence for increased microglia activation was found in patients with schizophrenia and ASD. While the function of several risk genes has been widely investigated in neurons, data on their function in microglia is lacking. Recently, the presence of DISC1, a risk gene associated with schizophrenia and other mental disorders, was suggested in brain cells other than neurons, including microglia. In neurons, DISC1 is involved in several cellular functions, including remodeling of the cytoskeleton. Since several hallmark functions of microglia, such as migration and phagocytosis, are highly dependent on the reorganization of the cytoskeleton, we aimed to characterize in more detail the expression of DISC1 in microglia and to evaluate the effects of DISC1 mutations on microglia phagocytosis. Additionally, in order to efficiently transfect BV2 cells, an immortalized microglial cell line, we compared different chemical transfection methods. We confirmed expression of DISC1 both on the mRNA and protein level in BV2 cells and primary microglia. While reduction of Disc1 mRNA levels by transfection with shRNA resulted in a decreased phagocytosis of latex beads, overexpression of full length or truncated DISC1 had no effect on this parameter. These data suggest that DISC1 is involved in the process of microglial phagocytosis and thus could contribute to disease pathogenesis by impaired functioning.

6.2 Background

In chapter 2, we found no evidence for the involvement of microglia in mediating the effects of MIA onto the developing brain. However, also several genetic mutations have been associated with neuropsychiatric disease and while the function of these genes is widely investigated in neurons, their function in microglia is barely explored. Among the many genes associated with schizophrenia, perhaps the best described is DISC1. A chromosomal translocation that caused a C-terminal truncation of DISC1 was originally found in a Scottish pedigree. Since its discovery, also other mutations in this gene have been found to be associated with disease. Among others, patients have

been described with a frameshift mutation in Disc1[353], with either a 1q42 deletion or a microduplication involving Disc1[354, 355], and DISC1 protein structural variants[356, 357].

As described in the general introduction, DISC1 has a plethora of interaction partners and it is thus not surprising that it is involved in many cellular functions[358]. Several studies have described the role of DISC1 in cytoskeletal rearrangements in neurons. For example, Kamiya *et al.* 2005 and Steinecke *et al.* 2012 showed an impaired neuronal migration of radially migrating pyramidal neurons and tangentially migrating interneurons[335, 336]. Moreover, Steinecke *et al.* 2014 showed that this impaired migration of GABAergic interneurons was correlated with deficits in cytoskeletal dynamics[359]. Specifically, knockdown of DISC1 reduced F-actin levels and the levels of phosphorylated Girdin, which cross-links F-actin. Moreover, acetylation of microtubules was decreased, which is believed to affect microtubule stabilization.

Microglia perform many tasks during development and in adulthood that are highly dependent on dynamic rearrangement of the cytoskeleton, including proliferation, migration and phagocytosis. Indeed, in 2016, Uhlemann *et al.* showed that phagocytosis of bacterial particles was impaired when actin turnover was disrupted pharmacologically[360]. This finding prompted us to investigate whether disruption of cytoskeletal dynamics by altered levels of DISC1 could also affect microglial phagocytosis. Alterations in this microglial function could alter the normal development of the brain by inducing changes in o.a. synaptic refinement and neuronal precursor pool maintenance and in this way contribute to neuropsychiatric disease pathogenesis. Firstly, we characterized in more detail the expression of DISC1 in primary microglia and in BV2 cells. Next, we compared several chemical transfection methods to identify the most efficient one for transfection of plasmid DNA in BV2 cells. Finally, we transfected BV2 cells with DNA constructs designed to interfere with normal DISC1 levels and assessed the effect on microglial phagocytosis of fluorescent latex beads.

6.3 Materials and methods

6.3.1 Animals

All experiments were conducted in accordance with the European Community guiding principles on the care and use of animals and with the approval of the Ethical Committee on Animal Research of Hasselt University. Mice were maintained in the animal facility of Hasselt University and Catholic University of Leuven in accordance with the guidelines of the Belgian Law and the European Council Directive. Animals were group-housed (unless otherwise stated) in a temperature and humidity controlled room with ad libitum access to food and water and a 12h/12h light dark cycle (lights on at 7.30AM).

6.3.2 Primary microglia isolation

Primary microglia were obtained from postnatal day 3 (P3) wild type pups using the shake-off method described by Mecha et al. Cerebra were isolated meninges removed in ice cold HBSS (Gibco) supplemented with 7mM HEPES (Gibco) and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich) and mechanically dissociated in ice cold DMEM D5796 (Sigma-Aldrich) with 1% P/S. The cell suspension was sieved through a 70µm cell strainer (Greiner) and centrifuged for 10 min, 300 g, 4°C. The pellet was resuspended in DMEM D5796 supplemented with 10% fetal bovine serum (FBS) (Gibco), 10% horse serum (Gibco) and 1% P/S and plated on Poly-D-Lysine coated (Sigma-Aldrich) culture flasks for 8-10 days. The next 5-6 days, medium was supplemented with one-third macrophage colony stimulating factor 1 conditioned medium (medium collected from cultured L929 cells). This microglia-enriched culture was then thoroughly agitated on an orbital shaker (3 h, 230 rpm, 37°C) to harvest the primary microglia.

6.3.3 Cell lines

The immortalized murine BV2 microglial cell line (kindly provided by dr. Jimmy Van den Eyden) was cultured in DMEM D5796 supplemented with 10% FBS and 1% P/S. The mouse fibroblast-like cell line L929 (kindly provided by dr. Kristiaan Wouters) was cultured in DMEM D5796 supplemented with 10% FBS, 1% P/S, 1% non-essential amino acids (Sigma-Aldrich) and 1% L-Glutamine (Sigma-

Aldrich). Cells were maintained in a humidified incubator at 37°C with a 5% CO2 atmosphere. Cells were split when 80-90% confluence was reached using trypsin/EDTA (Sigma-Aldrich) for 2-5 minutes at 37°C.

6.3.4 RT-PCR

BV2 cells and primary micorglia were lysed and homogenized with qiazol (Qiagen) and chloroform. After centrifugation (15 minutes, 12000 rpm, 4°C), total RNA was extracted using the RNeasy mini kit (Qiagen) and cDNA was synthesized using QScriptTM cDNA SuperMix (Quanta BioSciences) according to the manufacturer's instructions. Different primer pairs were used to detect different isoforms, as indicated in figure 6.1.

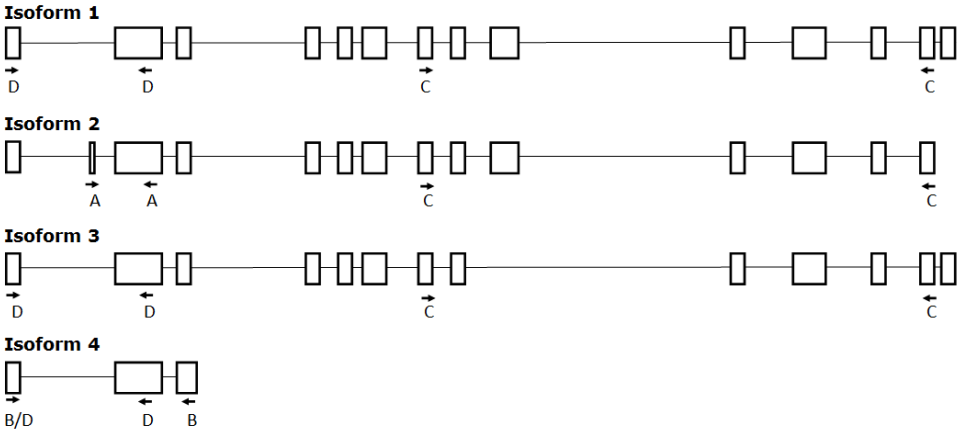


Figure 6.1: Schematic representation of different Disc1 isoforms and primer pairs used for RT-PCR.

The primer sequences were:

A forward: 5'-GAATTCAAGCTGGCTGACTC-3', A reverse: 5'-TGCCACATTCTGATTGCCTG-3',

B forward: 5'-ACTGTGAAACCTCTGGCATC-3', B reverse: 5'-ACGAACAGCTTTGGACTCAC-3',

C forward: 5'-AATCACTGAACCTGGCTGTC-3', C reverse: 5'-TCAGGCCTCGGTTTCCTGA-3',

D forward: 5'-ATGCAGGGCGGGGTCC-3', D reverse: 5'-TGCCACATTCTGATTGCCTG-3'

6.3.5 Western blot

BV2 cells and primary micorglia were lysed in cold RIPA buffer (50mM Tris pH 7,4; 150mM NaCl; 1mM EDTA; 1% NP-40; 0,25% Na-deoxycholate; protease inhibitor (Roche)). Samples were vortexed and centrifuged (15min, 12.500g, 4°C). Proteins (10-20 µg) were separated according to molecular weight using sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; resolving gel 12% and stacking gel 4%). Proteins were transferred to a PVDF membrane, which was first activated in methanol for 15min, at 350mA for 1,5 h. The membrane was blocked (5% milk in TBS-T) for 1 hour at room temperature and incubated overnight at 4°C with goat polyclonal primary anti-DISC1 antibody (200 µg/ml, Cat. Sc-47990, Santa Cruz Biotechnology). Next, the membrane was incubated for 2 hours at room temperature with a HRP conjugated secondary rabbit anti-goat antibody (1/2000 Cat. P0448, DAKO). Antibodies were diluted in blocking buffer. Enhanced chemiluminescence (ECL) using the Pierce ECL Plus Western Blotting Substrate (Thermo Scientific) was used before imaging with the ImageQuant LAS4000 mini (GE Healthcare Life Sciences).

6.3.6 Immunocytochemistry

BV2 cells were cultured on coverslips, fixed in 4% PFA for 15 min, washed three times with PBS containing 0.2% Triton X-100 and blocked for 1 hour with 5% Bovine serum albumin (BSA), 0.3% Tween-20 (Cat. No. 822184, Millipore, USA) in PBS containing 0.2% Triton X-100. Cells were incubated with goat polyclonal primary anti-DISC1 antibody (2000 µg/ml, Cat. Sc-47990, Santa Cruz Biotechnology) diluted in blocking buffer for 5 hours at room temperature. After washing, cells were incubated with Alexafluor 555-conjugated rabbit anti-goat IgG (1:1000, Life technologies) in blocking buffer for 1 hour at room temperature. Coverslips were mounted on microcopy slides using fluorescent mounting medium (DAKO). Images were obtained with the Nikon Eclipse 80i microscope.

6.3.7 Constructs

Ligations were performed with the rapid DNA dephosphorylation and ligation kit (Roche) and plasmids isolated with a plasmid DNA purification kit (Macherey-Nagel) according to the manufacturer's instructions. Restriction sites used for cloning are indicated in figure 6.2.

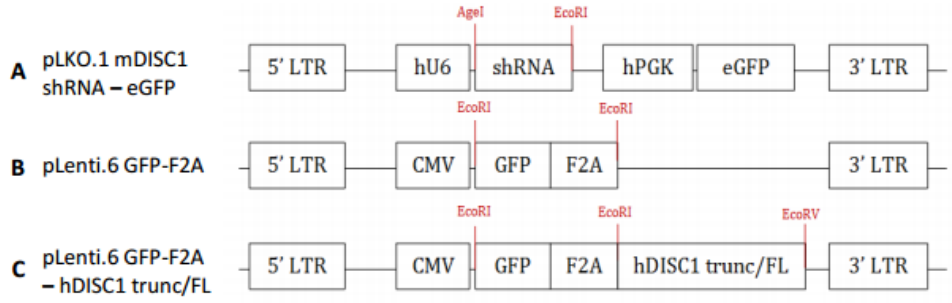


Figure 6.2: Schematic representation of genetic constructs used for transfection of BV2 cells. (A) shRNA against Disc1 was placed behind a hU6 promotor while the reporter gene eGFP was placed behind the hPKG promotor. (B) Control construct for overexpression, with only GFP-F2A behind the CMV promotor. (C) Overexpression construct for truncated or full length DISC1 in frame with GFP-F2A behind the CMV promotor.

Short hairpin RNA The pLKO.1 backbone (No. 10878, Addgene) was modified to generate our shRNA constructs. The puromycin resistance gene was replaced by an eGFP sequence and an shRNA directed against exon 2-3 of the Disc1 mRNA (sequence: 5'-GCTGGAGGTCACTTCCTTTAAT-3') (generously provided by Ines Royaux from Johnson & Johnson (USA) was ligated behind the hU6 promoter. A scrambled shRNA construct was also designed to use as a control condition.

Full length and truncated DISC1 The pLenti.6 backbone (No. V49610, Thermofisher) was used to generate full length and truncated human DISC1 constructs. The full length human Disc1 cDNA (generously provided by Johnson & Johnson) contained a G791A mutation which was first corrected by PCR splicing overlap extension (SOE) to obtain the human Disc1 Lv transcript variant (NM_001012957). A GFP-F2A sequence (obtained from plasmid 27437, Addgene) was ligated in between the CMV promoter and the Disc1 gene. To generate a truncated DISC1 plasmid, a stop codon (TGA) was inserted after

amino acid 597 of the full length human Disc1 cDNA to mimic the chromosomal translocation described in patients (36). The negative control vector contained only the GFP-F2A sequence.

6.3.8 Transfection

BV2 cells were transfected using magnetofection (Ozbiosciences). Cells were seeded on a Poly-L-Lysine coated 24-well plate at a density of 50.000 cells per well. For comparison of transfection efficiency of different transfection methods, cell were transfected after 24 hours with 100ng of plasmid DNA using either Calcium phosphate co-precipitation, X-tremeGENE (Sigma-Aldrich), Lipofectamine 2000 (Thermo Fischer Scientific) or Glial-Mag (Ozbiosciences) according to the manufacturer's instructions. For each transfection method, a negative control was included in which transfection reagent without DNA was applied to the cells.

For transfection with plasmids interfering with normal DISC1 expression, plasmid DNA (0,2µg in 100µl DMEM D5796) was added to 0,7µl magnetic bead solution (Glial-Mag) and incubated for 30 min at room temperature. 50µl was added to each well in a dropwise manner and incubated on a magnetic plate for 30 min at 37°C. Cells were incubated for another 2.5 hours in standard culture conditions after which the medium was changed. The reagents and the magnetic plate were obtained from Oz Biosciences (Marseilles, France) within the frame of a collaboration.

6.3.9 Flow cytometry to determine transfection efficiency

BV2 cells were transfected with an eGFP-expressing plasmid (pEGFP-N1, Clontech) using the four different transfection reagents described above. Twenty four hours after transfection, cells were harvested and washed in PBS. Afterwards, cells were dissolved in FACS buffer (PBS, 2% FCS, sodium azide) and data acquired using the FACS Fortessa and analyzed with FACS Diva 8.0.1 software (BD Biosciences). To determine cell viability, a subset of experiments was designed in which cells were stained with the Fixable Viability Stain 620 (BD Biosciences) before flow cytometry. Transfection efficiency was determined by measuring the percentage of eGFP-expressing cells among total cells.

6.3.10 Phagocytosis assay

The flow cytometric phagocytosis assay is based on the protocol 'Quantification of Microglial Phagocytosis by a Flow Cytometer-Based Assay' of Refik Pul et al.. 48 hours after magnetofection, cell were incubated with sky blue fluorescent carboxyl beads (Cat. CFP-0870-2, Spherotech) for 2 h at 37°C and 5% CO2. Afterwards, cells were washed 3 times in cold PBS and fixed for 20 minutes in 1% PFA. Cells were resuspended in filtered FACS buffer (PBS, 2% FCS, sodium azide), acquired using the FACS Aria II and analyzed with FACS Diva 6.1.3 software (BD Biosciences). Among transfected, and thus GFP-positive, cells the percentage of phagocytic cells was measured.

6.3.11 ELISA

BV2 cells were transfected with either an eGFP expressing plasmid (pEGFP-N1, Clontech) or with an IL6-eGFP expressing plasmid (#28088, Addgene). As a negative control, cells were incubated with magnetic nanoparticles alone. Culture medium was refreshed and collected 24 and 48 hours after transfection and IL6 concentrations were determined using a mouse IL6 ELISA (eBiosciences) according to the manufacturer's instructions.

6.3.12 Microscopy

Images were acquired with a Nikon Eclipse microscope and adjusted with FIJI software.

6.3.13 Statistics

Statistical analyses were performed with GraphPad Prism 5.0. For groups that passed the Kolmogarov-Smirnov test for normality test, parametric test were performed while for those that were not normally distributed, non-parametric tests were performed. The exact statistical test used is indicated in the figure legend. All values are presented as mean ± standard error mean.

6.4 Results

In 2010, Seshadri *et al.* used immunohistochemistry to provide the first evidence for the expression of DISC1 in microglia. As shown in figure 6.3, we confirmed the expression of DISC1 in the immortalized murine BV2 microglial cell line, with DISC1 protein mainly located in/around the nucleus and in lamellopodia.

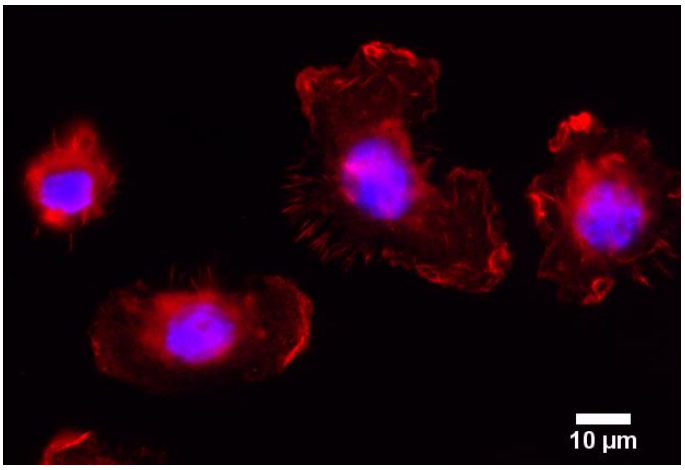


Figure 6.3: Immunocytochemical staining of cultured BV2 cells stained with DISC1 antibody, showing expression of DISC1 protein mainly around the nucleus and in cellular protrusions.

Presence of DISC1 was also determined on the mRNA level using RT-PCR and on the protein level using western blot. As shown in figure 6.4, DISC1 expression was confirmed both in BV2 cells and primary microglia. For RT-PCR, we used different sets of primers that could distinguish between different isoforms (see materials and methods). While we could not detect isoform 2 with the specific primer pair A, we detected isoform 4 with primer pair B and a mix of isoforms 1, 2, 3 and 4 with primer pairs C and D (Figure 6.4A). Using western blot, we show in BV2 cells the expression of the 2 most commonly described species of DISC1 protein, around a molecular weight of 75 and 100 kDa, which is predicted to roughly correspond to isoform 3 and 1 and 2 respectively. In primary microglia, we could only detect the smaller isoform (Figure 6.4B).

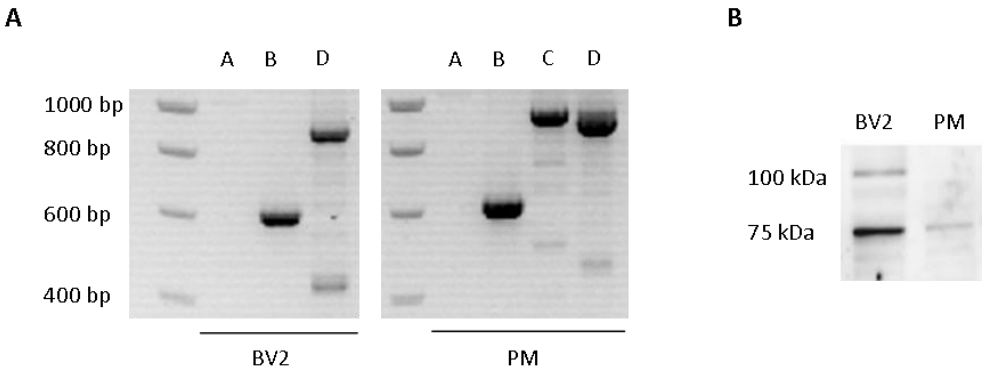


Figure 6.4: Expression of Disc1 mRNA and protein in BV2 cells and primary microglia (PM). (A) RT-PCR of the 4 isoforms of Disc1 in BV2 cells and primary microglia isolated from 3 day old mouse pups. Primer pair A amplifies isoform 2 (860 bp), primer pair B amplifies isoform 4 (594 bp), primer pair C amplifies isoform 1, 2 and 3 (917/917/728 bp) and primer pair D amplifies isoform 1, 3 and (4877 bp). (B) Western blot with antibody against DICS1 in BV2 cells and primary microglia isolated from 3 day old mouse pups. Bp = Base pairs

Having established the expression of DISC1 in microglia, we next aimed to interfere with endogenous DISC1 expression to determine whether DISC1 plays a role in microglia phagocytosis. Unfortunately, transfection of BV2 cells with the currently available chemical-based techniques yields only low transfection efficiencies. Recently a magnetofection kit was developed for optimal transfection of microglia cell lines (Glial-Mag; OZBiosciences). We compared the efficiency of plasmid gene delivery in BV2 cells using Glial-Mag to other commonly used chemical transfection methods in BV2 cells such as Calcium phosphate co-precipitation, X-tremeGENE and Lipofectamine 2000.

BV2 cells were transfected with an eGFP expressing plasmid (pEGFP-N1) and 24 hours after transfection, the transfection efficiency was determined by measuring the percentage of eGFP expressing cells among total cells using flow cytometry. We found that Glial-Mag magnetofection yielded significantly higher transfection efficiencies (34.95% ± 4.59, n=18) compared to Lipofectamine 2000 (12.51% ± 3.00, n=15, p=0.0269), X-tremeGene (3.30% ± 0.53, n=15, p=0.0005) and Calcium phosphate co-precipitation (0.34% ± 0.07, n=15, p<0.0001) (Figure 6.5A and B).

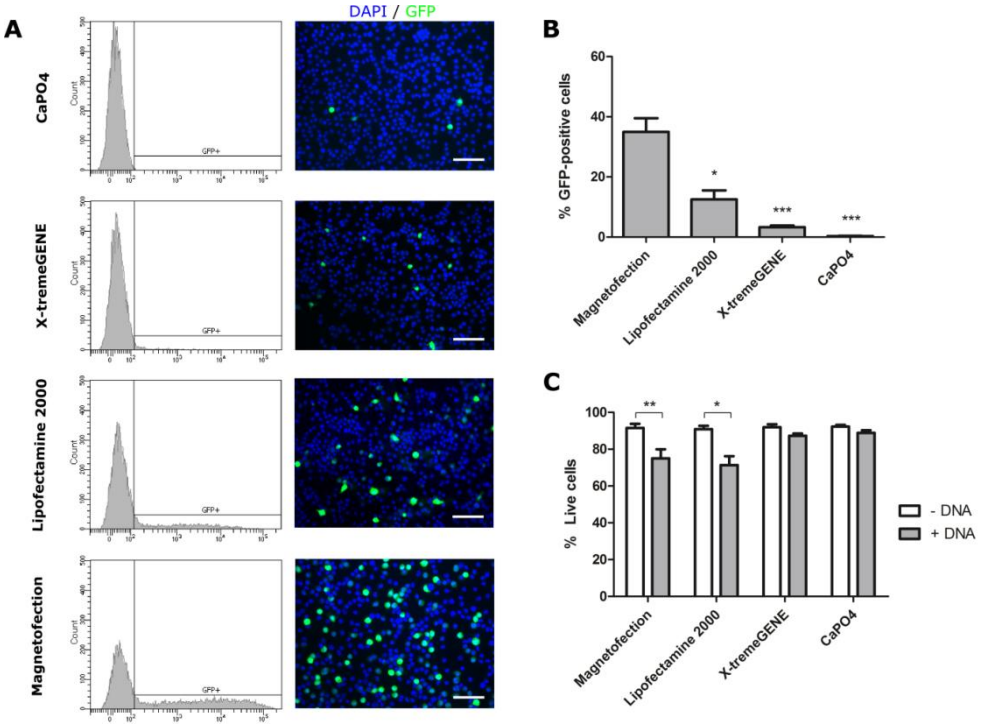


Figure 6.5: Glial-Mag magnetofection yields higher transfection efficiencies in BV2 cells compared to other chemical transfection methods. (A) Representative Flow Cytometry intensity histograms and fluorescence microscopy image (blue = DAPI, green = GFP) for each transfection method. (B) Transfection efficiency in BV2 cells expressed as percentage eGFP-positive cells among all cells for each transfection method (Kruskal-Wallis with Dunn's multiple comparison test to magnetofection). For magnetofection n=18, Lipofectamine 2000 n=15, X-tremeGENE n=15 and CaPO4 n=15. (C) Percentage of living cells after transfection with (+ DNA) or without (- DNA) plasmid DNA (Student T-test). For all groups n=5. Scale bars represent 100µm. Values are reported as mean ± SEM. * $p<0.05$,** $p<0.01$, *** $p<0.001$

To measure the toxicity induced by the different transfection methods we determined the percentage of living cells using Flow Cytometry. Application of transfection reagent alone yielded a similar amount of living cells for all transfection methods. Transfection with pEGFP-N1 did neither alter the percentage of living cells with Calcium phosphate co-precipitation nor with X-tremeGENE. In contrast, Lipofectamine 2000 and Glial-Mag magnetofection with pEGFP-N1 significantly decreased the percentage of viable cells compared to transfection reagent only (p=0.0052 and 0.016, n=5) (Figure 6.5C).

Transfection efficiency was also determined in primary microglia isolated from 3-day old wild type pups, but we did not achieve more than 2% transfected cells (data not shown).

Microglial cells are cytokine secreting immune cells of the brain and therefore magnetofection of a gene encoding a physiologically relevant and inflammatory cytokine, i.e. IL6, was tested. Cells were transfected with either an eGFP expressing plasmid (pEGFP-N1) or with an IL6-GFP expressing plasmid. As a negative control, cells were incubated with magnetic nanoparticles alone. Culture medium was refreshed and collected 24 and 48 hours after transfection and IL6 concentrations were determined using ELISA (Figure 6.6).

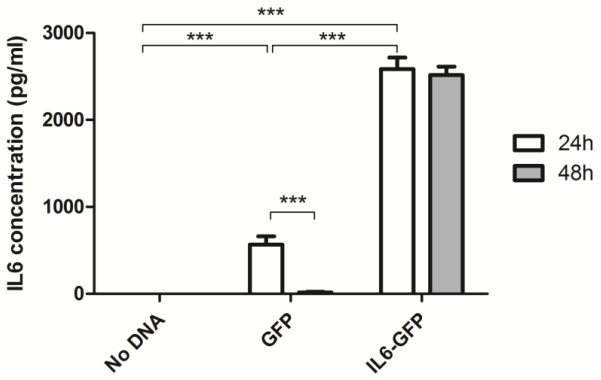


Figure 6.6: Glial-Mag magnetofection with IL6-GFP plasmid yields sustained secretion of IL6 after transfection. The amount of IL6 (pg/ml) was measured in culture medium 24 and 48 hours after transfection either with an eGFP expressing plasmid or with an IL6-GFP expressing plasmid. As a negative control, cells were incubated with magnetic nanoparticles alone (No DNA) (2-way repeated measures ANOVA with Bonferroni post hoc test). For all groups n=6. Values are reported as mean ± SEM. *** $p<0.001$

When solely magnetic nanoparticles were applied to the cells, Glial-Mag transfection reagent did not activate BV2 cells as demonstrated by the absence of IL6 in the culture medium. In contrast, magnetofection with plasmid DNA encoding eGFP resulted in IL6 production that was detectable in culture medium after 24 hours (565 pg/ml ± 94) but significantly decreased after 48 hours (17 pg/ml ± 6) ($p<0.0001$, n=6). This suggests that transfection with plasmid DNA induces a modest and short lived activation that ceases within 24 hours. On the

contrary, transfection with an IL6-eGFP expressing plasmid resulted in a sustained secretion of IL6 as it was present in the culture medium both at 24 (2585 pg/ml ± 132) and 48 hours (2516 pg/ml ± 98) after transfection.

Using magnetofection, we modulated endogenous DISC1 expression using three different approaches to mimic the pathological conditions described in patients[354, 355, 361].

Haploinsufficiency: a short hairpin RNA (shRNA) was used to obtain stable RNA interference and to knockdown the expression of the endogenous mouse Disc1 gene. Scrambled shRNA, which should not target any known mammalian mRNA sequence, was used as a control.

Dominant negative: a truncated Disc1 gene (Trunc) (with potential dominant negative effects) was expressed to interfere with the function of endogenous DISC1 protein.

Overexpression: the full length (FL) Disc1 coding sequence was used for overexpression of the DISC1 protein, thereby mimicking a genetic duplication. The negative control for experiments with the truncated protein or the overexpression is the backbone with only GFP2A.

All constructs were generated in a backbone that co-expresses eGFP, a feature that allowed us to discriminate cells that were successfully transfected from those that were not. In shRNA constructs, eGFP was placed behind a different promoter and consequently, the level of eGFP expression is not directly or stoichiometrically related to the shRNA expression level. In overexpression constructs, Disc1 sequences are fused to a GFP-F2A sequence which ensures a proportional relationship between GFP and DISC1 protein levels (either truncated or full length).

The constructs described in the materials and methods section were transfected in BV2 cells using magnetofection and 48 hours after transfection, cells were exposed to fluorescent latex beads. The percentage of transfected cells that phagocytosed at least one bead was measured using flow cytometry (Figure 6.7).

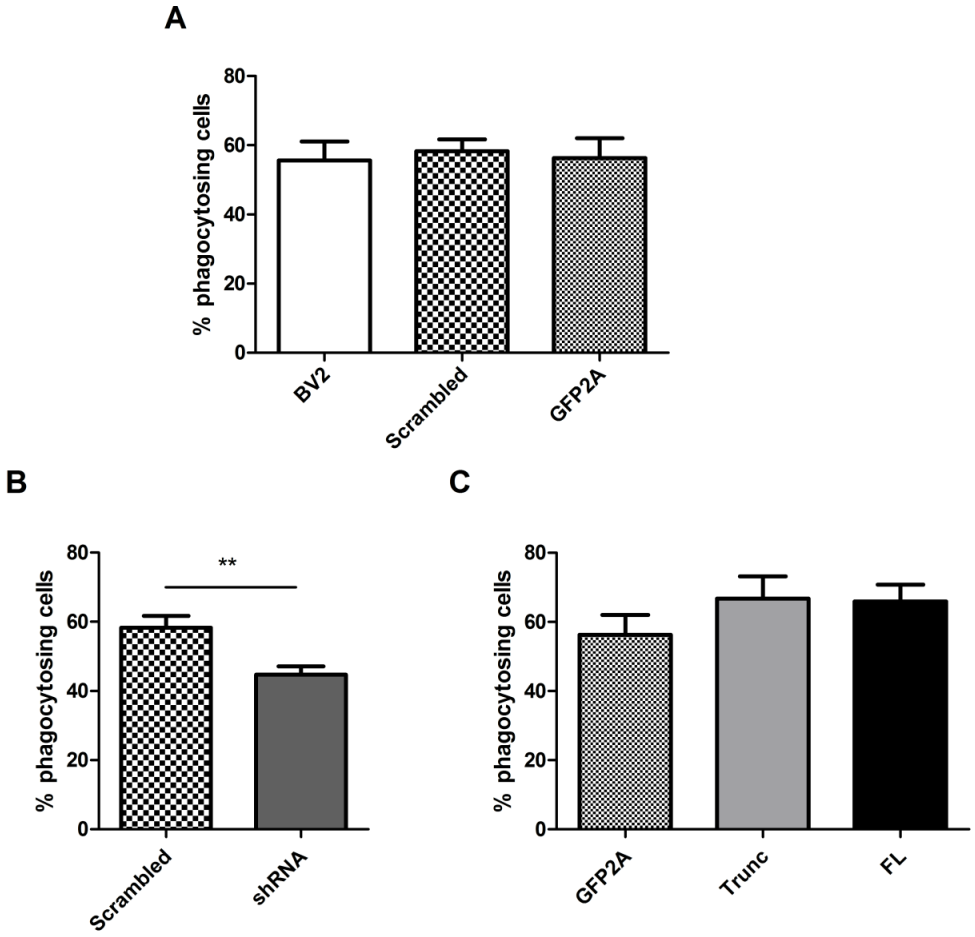


Figure 6.7: Phagocytosis assay in BV2 cells with modified endogenous DISC1 expression. Transfected BV2 cells were incubated with fluorescent latex beads and the percentage of phagocytosing cells (as measured by flow cytometry) is shown. (A) Non transfected BV2 cells and BV2 cells transfected with scrambled shRNA or GFP2A display the same percentage of phagocytosing cells. (1 way ANOVA) (B) BV2 cells tranfected with shRNA against Disc1 display a significantly reduced percentage of phagocytosing cells compared to those transfected with scrambled shRNA. (Student T-test) (C) BV2 cells transfected with truncated or full length DISC1 display the same percentage of phagocytosing cells as those transfected with GFP2A. (1 way ANOVA) For BV2 n=9, scrambled n=9, shRNA n=9, GFP2A n=6, Trunc n=5 and FL n=6. Trunc: trundated DISC1, FL: full length DISC1.

The percentage of phagocytosing cells was compared between untransfected BV2s and BV2s transfected with a scrambled shRNA or GFP2A construct, the 2 control conditions. No difference was found between the different conditions

(Figure 6.7A). When comparing BV2 cells transfected with shRNA against Disc1 to those transfected with scrambled shRNA, we found a significant reduction in the percentage of phagocytosing cells (Figure 6.7B). On the other hand, transfection with either truncated or full length DISC1 did not change the percentage of phagocytosing cells compared to control transfected cells (GFP2A) (Figure 6.7C). Of note, we also transfected BV2 cells with 3 other shRNAs and a pool of all 4 shRNAs together, but these conditions appeared to be not as efficient to reduce phagocytic activity (data not shown).

6.5 Discussion

As only little data exist on the presence and role of DISC1 in microglia, the aim of these experiments was to analyse the expression and role of DISC1 in both primary microglia and an immortalized murine microglial cell line, namely BV2 cells. We show that DISC1 can be detected mainly around the nucleus and in lamellipodia of cultured BV2 cells using immunohistochemistry. A similar subcellular localization of DISC1 protein has also been described in neuronal cells with DISC1 localized in dendrites of hippocampal neurons and around the nucleus and in the leading process tip of migrating interneurons[359, 362]. We used western blot to detect the molecular weight of the DISC1 protein expressed in microglia and found that BV2 cells expressed 2 DISC1 species with different molecular weight (75 and 100 kDa) while primary microglia expressed only 1 species (75 kDa). To identify different Disc1 isoforms on the mRNA level, we performed RT-PCR with different primer pairs that allow to distinguish between the 4 known isoforms in mice. (http://www.ensembl.org) Using this technique, we were able to detect expression of isoform 4 specifically and a combination of isoforms 1, 2, 3 and 4 both in BV2 cells and primary microglia. Although the results from the RT-PCR suggest the presence of isoform 1 in primary microglia, we were not able to detect the corresponding 100 kDa band on western blot. Also isoform 4, which is predicted to have a molecular weight of about 41 kDa, was detected by RT-PCR but not on western blot. As the antibody we used is directed against the N-terminal region of the protein (although the specific epitope is not known), it seems unlikely that it could not detect specific

isoforms, as most differences between isoforms are found in the C–terminal region. As we did not yet include a loading control in these preliminary experiments, it is possible that these isoforms were below the detection limit. It would be interesting to further characterize the DISC1 protein species found in BV2 cells and primary microglia using mass spectrometry and to compare them with species found in whole brain lysates.

To examine the functional consequence of DISC1 mutations described in humans, we generated several constructs to modify DISC1 expression in microglia. We used shRNA to reduce Disc1 mRNA levels, truncated DISC1 to investigate possible dominant negative effects and full length DISC1 to induce overexpression. After transfection of BV2 cells with these constructs, latex beads were administered to the cells and phagocytosis of the beads was measured after 2 hours. Although we did not yet confirm knockdown of Disc1 on the mRNA or protein level, there is a decrease in the percentage of phagocytosing cells upon transfection with Disc1 shRNA compared to scrambled RNA. On the other hand, overexpression of full length or truncated DISC1 does not change the amount of cells that phagocytosed the latex beads. The fact that overexpression of truncated DISC1 did not change the percentage of phagocytosing cells while transfection with shRNA caused a reduction suggests that truncated DISC1 protein does not exert dominant negative effects on this particular microglial function. As DISC1 protein requires to be dimerized to be functional, dominant negative effects would only apply to functions controlled by the C-terminal part of the protein which is lacking in the truncated protein (personal communication with Dr. Akira Sawa). Accordingly, the functional domain required for microglial phagocytosis is likely to be located N-terminal of the truncation, and dominant negative effects could still occur for functions requiring the C-terminal region of the DISC1 protein. Alternatively, truncated DISC1 protein could be degraded more easily with only endogenous protein expression dictating phagocytic function.

Importantly, future experiments should address the extent of Disc1 knockdown induced by the shRNA by measuring the reduction of mRNA using quantitative real time pcr and by quantifying protein levels using western blot. In addition, functional effects of interference with DISC1 expression should be confirmed in

primary microglia. Finally, it would be interesting to investigate the role of DISC1 in other microglial functions, such as migration and proliferation. In a next step, behavioral assessment of mice lacking DISC1 only in microglia (using the CX3CR1-CreER(T2) mice) could provide information on the contribution of microglia to schizophrenia symptoms.

In this chapter, we also aimed to evaluate whether higher transfection efficiencies could be achieved in BV2 cells using magnetofection compared to other commonly used chemical transfection methods. Chemical transfection is a relatively cheap and fast way to introduce plasmid DNA into cultured cells, compared to for example viral transduction. As reviewed by Zhang *et al.* 2009, macrophages, including microglia, are typically hard to transfect with the common chemical transfection reagents as they are equipped with many enzymes whose purpose is to degrade foreign nucleic acids[363]. Here, we show that Glial-Mag magnetofection of BV2 cells yields functional gene expression with a superior efficiency compared to commonly used transfection methods. Although magnetofection induces a higher amount of cell death compared to Calcium phosphate co-precipitation and X-tremeGENE, it is comparable to that induced by Lipofectamine 2000. Importantly, application of Glial-Mag reagent alone does neither induce cell death nor cell activation, indicating that the plasmid DNA and not the transfection method is responsible for these effects. In that light, it seems logical that there is elevated cell death using Lipofectamine 2000 or magnetofection since these methods yield higher transfection efficiencies. Importantly, magnetofection with eGFP plasmid DNA induced a modest release of the inflammatory cytokine IL6 in the culture medium, suggesting activation of the BV2 cells. BV2 cells exposed to the transfection reagent alone did not release IL6 into the culture medium, indicating that the activation is not induced by the reagent itself. 48 hours after magnetofection, IL6 was no longer detectable in the medium, suggesting that the inflammatory reaction was only short lived. This short lasting inflammatory response upon magnetofection should however be taken into account when designing experiments.

Chapter 7

General discussion

Microglia have long been considered to be the static immune cells of the brain that were only active in response to injury or disease. However, Nimmerjahn *et al.* showed in 2005 that so-called resting microglia are highly dynamic, extensively scanning their environment for threats[364]. Indeed, microglia have recently been shown to perform many more functions beside those in immune defense against pathogens, including the orchestration of the development of neuronal progenitors and modulation of synaptic connections. As such, the role of microglia in the pathogenesis of neuropsychiatric disorders such as schizophrenia and ASD is intensively investigated during recent years.

In this thesis, we tested the hypothesis that risk factors for neuropsychiatric disorders such as prenatal infection and mutations in the Disc1 gene affect normal development of the brain through alteration of microglia function and thereby contribute to the increased disease risk. In this chapter, I will place the obtained results in a broader context, focusing on the current knowledge obtained by the MIA model (7.1), the described neurodevelopmental defects in neuropsychiatric disorders and animal models thereof (7.2) and the evidence for a role for microglia in these disorders (7.3).

7.1 Maternal immune activation as an environmental risk factor for neuropsychiatric disease

With the latest outbreak of the ZIKA virus, awareness about the consequences of maternal infection on the developing brain has been raised. ZIKA virus infection during pregnancy causes severe defects in neurological development, such as microcephaly, hearing loss, seizures etc[365]. As reviewed by Walter *et al.* 2017, ZIKA virus has been shown to be able to infect placental and endothelial cells, thereby possibly affecting placental perfusion and production of developmental factors by the placenta, compromising normal brain development. Moreover, ZIKA virus has been shown to cross the placenta and directly infect the fetal nervous tissue. After infection of neural cells, ZIKA virus can affect proper regulation of cell death, disrupt neurogenesis and interfere with neuronal migration[366].

Although ZIKA virus infection exerts its detrimental effects, at least in part, by the vertical transmission from the mother to the child, many other infections are not transferred to the developing child but still retain the capacity to affect normal brain development and predispose the child to developing neuropsychiatric disorders later in life. It is believed that the response of the maternal immune system to the infectious agent is the main culprit in this regard.

7.1.1 Factors influencing the outcome of MIA on the offspring

A wealth of studies has been dedicated to elucidating the effects of MIA on the developing fetus. Comparison of individual studies is often complicated by the different paradigms used to induce MIA and to evaluate the effects on the offspring. Variability can arise from differences in the immune activating agents used, the dose and administration route and the age of MIA induction. In this thesis (**Chapter 2, 3, 4 and 5**), we used an intraperitoneal injection of 20mg/kg Poly IC to induce MIA as this protocol was shown to induce behavioral deficits in the offspring when administered at E12.5 by several groups. We injected Poly IC at E11.5 and/or E15.5 since these represent important developmental time points for microglia. However, several different protocols exist in literature, which I will comment on in the following sections.

Immune activating agent

To imitate a viral or bacterial infection during pregnancy in animal models, infectious mimicking agents such as Poly IC and LPS have been used widely. Poly IC is a synthetic double stranded RNA that activates TLR3 and thereby mimics a viral infection. LPS is gram negative bacterial cell wall component that activates TLR4 and thereby mimics a bacterial infection. In this thesis, we used Poly IC as this agent is most often used in mice, however, it is possible that the use of LPS in our study would have led to different outcomes. For example, as will be discussed further (7.3.1), embryonic microglia activation upon MIA was mainly described in studies using LPS as immune activating agent[57, 167, 265]. This MIA-induced activation of embryonic microglia could have influenced the developmental trajectory of pyramidal neurons more than with an unaltered activation status of embryonic microglia. Indeed, Squarzoni *et al.* showed a

disturbed localization of Lhx6-expressing neocortical interneurons upon LPS-induced MIA[44]. On the other hand, behavioral alterations have been widely described using Poly IC to stimulate the maternal immune system, so the fact that we could not detect behavioral abnormalities in MIA offspring is unlikely to be due to the use of Poly IC instead of LPS.

While most studies use either LPS or Poly IC to induce MIA, only a handful of studies directly compared the effects of both agents. Harvey *et al.* 2012 showed an increased body weight of offspring exposed to LPS *in utero* (0.2mg/kg) at P14 while that of Poly IC (20mg/kg) exposed offspring was not different from controls[367]. At P28, body weight of both LPS and Poly IC exposed offspring was lower compared to controls. At P14, neuronal density in the hippocampus was increased in LPS but not Poly IC offspring compared to controls, an effect that disappeared by P28. In contrast, the density of reelin positive cells in the hippocampus was only affected in Poly IC treated offspring[367]. Fortier *et al.* 2007 reported a decrease in PPI in male offspring of mothers treated with 50µg/kg LPS but not in offspring of mothers treated with 750µg/kg Poly IC[368]. Arsenault *et al.* 2014 found a decreased body weight of Poly IC (5mg/kg) exposed pups until P10 while those exposed to LPS (120µg/kg) were comparable to controls[369]. Poly IC treated pups also displayed a delay in the righting, geotaxis and grasping reflex, which was not found in LPS offspring. Finally, Xuan *et al.* 2014 described deficits in locomotor activity and social interaction in adult males prenatally exposed to Poly IC (20mg/kg) but not in LPS offspring (75µg/kg)[370].

While mimicking agents such as Poly IC and LPS are advantageous as they avoid the use of live viruses and bacteria in the lab and allow standardization of methodology, the maternal immune response to live pathogens is still different. Unlike exposure to Poly IC, a real virus activates both the innate and the acquired immune system leading to a broader response[371]. In addition, while Poly IC and LPS are usually injected intravenously or intraperitoneally, viruses and bacteria usually infect epithelial cells of the respiratory and digestive tracts instead of the blood stream or the peritoneum. Activation of immune cells in different tissues may lead to the generation of an innate immune response with different profiles of cytokine production[372].

The combination of differences and similarities in the outcome of LPS versus Poly IC exposure could be explained by the fact that the mimicking agents activate a different TLR and can thus induce different combinations of inflammatory mediators. Beside TLR3 and 4, several other TLRs exist and thus also other models of maternal immune stimulation have been used. For example, maternal infection with Group B Streptococcus, which mainly activates the TLR2, was shown to induce ASD-like behavior, such as social and communicative impairments and deficits in sensorimotor gating, in male offspring[373]. Morphological brain alterations induced by the infection included enlargement of the lateral ventricles, oligodendrocyte loss and disorganization of subcortical tissue[373, 374]. Also maternal allergic asthma, which is characterized by elevated levels of IL4 and IL5 in maternal blood, resulted in decreased social interaction and increased repetitive behavior in the offspring[145].

Dose and administration route

A range of different doses to induce MIA are being used, both for LPS and Poly IC, injected either intravenously, intraperitoneally or subcutaneously and injected only once or repeatedly. Shi *et al.* 2003 and Meyer *et al.* 2005 described dose dependent effects of Poly IC on maternal serum levels of IL10 and on the offspring's deficits in PPI and sensitivity to amphetamine-induced hyperlocomotor activity[284, 343]. By comparing the results of these studies, it is also apparent that lower doses of Poly IC are needed to induce deficits in PPI when administered intravenously compared to intraperitoneally. While PPI deficits were already detectable at a dose of 5mk/kg IV in the study of Meyer *et al.* 2015, these deficits only emerged with 20mg/kg IP (and not at 2.5, 5 or 10 mg/kg) in the study of Shi *et al.* 2013[284, 343]. Further complicating the picture is the fact that LPS is extracted from live E Coli and the bioactivity per amount is dependent on the lot and the serotype of the E Coli Strain[375]. Similarly, while Poly IC is a synthetic product, Harvey *et al.* 2012 showed that different batches from the same supplier induced varying amounts of IL6 in maternal plasma, ranging from 495.33±164.93 pg/ml to 8753.04±2522.80 pg/ml three hours after injection of Poly IC in non-pregnant female mice[367]. We found the same discrepancy in maternal IL6 values depending on the batch of Poly IC used. For this reason, we measured serum IL6 levels in all pregnant females to ensure

that immune stimulation occurred. Indeed, in **chapter 2, 4 and 5** we show that induction of MIA was accompanied with a significant increase in maternal IL6 levels after 5 hours. Although labor intensive, a new batch of Poly IC should ideally be tested for its potency to induce IL6 upregulation in the serum. As an extra control of sickness behavior, in **chapter 4 and 5** we also assessed changes in body weight and/or temperature. In contrast to Missault *et al.* 2014 who described behavioral deficits in MIA offspring when the mother lost weight after MIA but not when the mother gained weight, in our experiments we found that all Poly IC injected mice lost weight 24 hours after treatment[227]. The fact that we found increased IL6 levels in maternal serum and decreased maternal body weight in all animals upon induction of MIA suggests that the MIA protocol succeeded in inducing an inflammatory response and thus that the lack of behavioral changes, microglial alterations and changes in the development of pyramidal neurons cannot be attributed to dose or administration route we used. Moreover, Pratt *et al.* also induced MIA by an intraperitoneal injection of Poly IC at 20mg/kg and found upregulation of a few inflammatory mediators in isolated embryonic microglia, although not the ones that we investigated. So we might have also detected changes in embryonic microglia if we used a bigger set of markers. Nevertheless, intravenous administration instead of intraperitoneal Poly IC might have generated more consistent inflammatory responses between animals.

Although these differences in immune stimulation depending on the exact protocol or product used make it hard to compare results from different studies, it raises the interesting question whether there is a threshold of inflammatory mediators in maternal blood that determines whether offspring is affected or not. Similarly, it is important to know whether the level of inflammatory mediators in maternal blood correlates with the severity or amount of behavioral deficits.

Age of MIA induction

We induced MIA at E11.5 and/or E15.5, as these embryonic ages represent important developmental stages of microglia development[38]. While we did not assess behavioral deficits induced by MIA at both E11.5 and E15.5, we could not detect changes in behavior in the male offspring of pregnant females injected

with Poly IC at E11.5 (**Chapter 4**). We were also not able to detect changes in the activation status of embryonic microglia (**Chapter 2**) or in the development of pyramidal neurons (**Chapter 5**). Whether we would have found different results if we used a different embryonic age to induce MIA seem unlikely. At E11.5, microglia are already present in the brain parenchyma and could thus have been affected by the maternal immune response. In addition, a wide range of embryonic ages has been used by other groups to induce MIA and describe all kinds of changes in the offspring, so it seems unlikely that the developing embryo would not be affected only at E11.5. In chapter 4, we administered the second immune stimulus 24 hours after labeling of neuronal progenitors to assure that these cells would be exposed to the inflammatory environment at a time point when they would not have reached their final destination yet.

Meyer *et al.* 2006 was the first to compare the influence of gestational age of MIA induction on the precise behavioral outcome in the offspring[181]. Specifically, offspring of mothers injected with Poly IC at E9 showed anxiety-like behavior in the open field while those injected at E17 did not. Conversely, E17 MIA offspring displayed decreased reversal learning but E9 exposed animals did not[181]. These data were expanded in Meyer *et al.* 2008 showing that MIA at E9 caused deficits in PPI while MIA at E17 resulted in working memory deficits[148]. The latter findings were confirmed by Li *et al.* 2009 and Connor *et al.* 2012[166, 376]. Similarly, Richetto *et al.* 2016 found deficits in PPI after prenatal immune challenge at E9, deficits in spatial recognition after MIA at E17 and social interaction deficits in both conditions[175]. Although Meehan *et al.* 2016 also reported working memory deficits only after late gestation immune activation, PPI deficits were detected in both early and late gestation MIA offspring[156]. In contrast, Waterhouse *et al.* 2016 described deficits in sensory motor gating and working memory only in offspring of rat dams injected with LPS at E10, not E15 or E18[158]. Finally, MIA offspring of mothers injected with LPS at E19 displayed impairments in motor performance while reward seeking behavior was decreased after MIA at E12[154].

As discussed by Meehan *et al.* 2016, there are a few factors that could explain the divergent outcomes of MIA at different gestational ages. First, the maternal immune response differs throughout pregnancy, which can influence which

inflammatory mediators are released. Secondly, placental properties, such as permeability, change over the course of pregnancy. Finally, the developmental stage of the fetus itself and the precise ongoing developmental process at the time of infection might dictate which neural circuit is most affected[156].

Importantly, the rodent brain at birth resembles the developmental stage of the start of the second trimester in humans. Therefore, mimicking human infections in the third trimester requires infection of the new born neonate in rodents. Indeed, several studies have used this paradigm to investigate the effects of MIA on the developing brain[377]. It should be noted however that in this model, 'developing' mice are directly exposed to the infectious agent with effects relying on the response of the immune system of the neonate, while the role of the immune system of the mother and the placental interface are abolished[378].

Age of behavioral assessment

The MIA model is mostly used to induce schizophrenia and/or ASD-like behavior. While both diseases share overlapping symptomatology, they differ greatly from each other in the age of onset. ASD is usually diagnosed around the age of 3 where for schizophrenia this is the case only around early adulthood. Nevertheless, negative and cognitive symptoms associated with schizophrenia appear to emerge at an earlier age, before diagnosis. In our experiments, we started behavioral testing at early adulthood (P60) as most behavioral deficits are clearly present at this age

Several groups investigated the time dependent emergence of behavioral deficits upon prenatal exposure to LPS or Poly IC and find them to appear either around pubescence (P30) or around early adulthood (P60)[149, 205]. Han *et al.* 2016 and Fujita *et al.* 2016 reported PPI deficits in early adulthood (P70) but not pubescence (P28) when prenatally exposed to Poly IC[150, 266]. On the other hand, Romero *et al.* 2010, Zuckerman *et al.* 2003 and Wolff *et al.* 2008 detected deficits in PPI already in pubescence (P35)[197, 379, 380]. Similarly, (working) memory deficits were found already in pubescence (P28) by Han *et al.* 2016 and Fujita *et al.* 2016 but only emerged in adulthood (P60-P95) in studies by Batinic *et al.* 2016, Ozawa *et al.* 2006 and Richetto *et al.* 2013[150, 151, 205, 266, 381]. Straley *et al.* 2016 also found impairments in motor performance and reward

seeking behavior to emerge in adulthood (P60) but not in pubescence (P30)[154]. Deficits in behaviors reminiscent of ASD have also been described in varying degrees at several postnatal ages, including alteration in ultrasonic vocalizations, deficits in social interaction and increased repetitive behaviors[159, 163, 167, 250, 264]. In **chapter 4**, we started behavioral testing of male MIA offspring at 9 weeks of age with open field and social exploration and ended at 22 weeks of age with amphetamine induced locomotor activity. Giovanoli *e al.* 2015, Hadar *et al.* 2015 and Han *et al.* 2016 showed that PPI deficits were not detectable before 9 weeks of age but presented starting from 10 weeks of age[149, 150, 382]. We assessed PPI deficits when MIA offspring was 16 weeks old and found no difference between Poly IC and NaCl exposed offspring. Similarly, Richetto *et al.* 2013 found working memory deficits in MIA offspring at 14 weeks of age but not at 4 weeks[381]. While we also assessed working memory deficits in the Morris water maze with 14 week old MIA offspring, we found no difference in performance compared to the control group. These data suggest that the timing of behavioral testing is not likely to explain why we could not detect differences in behavior.

Whether MIA induces distinctly ASD or schizophrenia-like behavior is doubtful. Probably, MIA induces deficits in behavior that are on a continuum and interaction with other genetic and environmental factors determines the exact behavioral outcome. Indeed, as described in the general introduction, a few studies have addressed the interaction between MIA and genetic risk factors for neuropsychiatric disorders and found mostly additive or synergistic effects on aberrant behavior. Moreover, Schwartzer *et al.* 2013 and Babri *et al.* 2014 reported a different vulnerability of inbred mouse strains, with different genetic background, to the effects of MIA[383, 384]. Alterations in ultrasonic vocalizations, social interaction and repetitive behavior were more pronounced in BTBR mice compared to C57Bl/6 mice[384]. Similarly, maternal inflammation in NMRI mice induced anxiety and depression like behavior but this could not be detected in C57Bl/6 mice[383].

Other factors influencing MIA outcome

Besides factors directly related to the paradigm used to induce maternal immune activation, other factors can influence the precise behavioral outcome in the offspring.

One contributing factor for the increased incidence of ASDs is thought to be the dramatic decreased consumption of anti-inflammatory dietary omega 3 polyunsaturated fatty acids (PUFA) relative to the amount of pro-inflammatory omega 6 PUFAs and saturated fats in the western diet. Beside their anti-inflammatory properties, omega 3 PUFAs are also important for optimal brain development. As vertebrates are not capable of *de novo* synthesis of omega 3 PUFA, they must obtain these fatty acids from the diet. The importance of dietary omega 3 PUFA for brain development has been documented in rodents: deficiency during development in rodents leads to deficits in cognition, vision and a wide array of behaviors[385-387] while feeding mice diets high in omega 6 PUFA during gestation produces autistic-like features in offspring[388].

To address the role of omega 3 PUFAs in the effects of MIA on the offspring, 2 recent studies compared behavioral deficits induced by MIA between diets with different omega 3 PUFA contents. In Li *et al.* 2015, MIA was induced at E9 with Poly IC and offspring was fed with an omega 3 PUFA enriched diet from weaning on. While MIA offspring fed with a regular diet showed anxiety-like behavior and deficits in PPI in adulthood, the behavioral alterations were not detected in MIA offspring that received the enriched diet[263]. Similarly, Weiser *et al.* 2016 compared the effects of diet either or not supplemented with docosahexaenoic acid (DHA) on behavioral alterations in offspring prenatally exposed to Poly IC at E12.5. Unlike Li *et al.* 2015, this study already started feeding the enriched diet during pregnancy and lactation and continued to provide it to the offspring after weaning. While MIA offspring fed with low DHA diet displayed reduced sociability and increased repetitive behavior, the opposite was true of high DHA fed MIA offspring[264].

In the study of Weiser *et al.* 2016, low omega 3 PUFA diet had a ratio of omega 6/omega3 of 20/1 while supplemented diet had a ratio of 5/1. Similarly, control diet of Li *et al.* 2015 had an omega 6/omega 3 of 13/1 while the enriched diet

had a ratio of 1/1[263, 264]. The diet used in our lab had a ratio of 10/1, which is relatively low compared to the control diets used by Weiser *et al.* 2016 and Li *et al.* 2015, which could have masked behavioral deficits in MIA offspring. (**chapter 2**) While differences in rodent diet are not typically considered as variables in experiments, they often differ in the content of omega 3 PUFAs. Future experiments should take into account the fatty acid content of the diet supplied to their lab animals

Secondly, adverse environmental factors (such as perinatal stress) have been shown to enhance the risk to develop schizophrenia[259, 389]. In contrast, environmental enrichment influenced emotional behavior and improved cognitive function in wild type mice and improved brain pathology in rodent models of Huntington's disease, Alzheimer's disease and depression[390-393]. Buschert *et al.* 2016 therefore asked whether environmental enrichment could influence the behavioral outcome of mice prenatally exposed to Poly IC[153]. To this end, MIA offspring was either housed in standard conditions or in a cage with different objects, including a running wheel, plastic tubes, a playing ball etc, which were exchanged every week during cage cleaning. Strikingly, while prenatal exposure to Poly IC at E9 induced deficits in social interaction in the environmentally enriched group, it failed to do so in the standard housed animals. Moreover, social interaction was comparable between standard housed animals and environmentally enriched animals prenatally exposed to Poly IC, suggesting that standard housing conditions can mask behavioral deficits induced by MIA. Problem solving behavior on the other hand was similar in all groups, suggesting that environmental enrichment does not influence cognitive brain function[153]. In our experiments, animals were kept in standard housing conditions which could have masked possible differences in behavior caused by the MIA. (**chapter 4**)

7.1.2 The gender bias in autism and schizophrenia reflected in the MIA model

In ASD, there is a clear bias towards a higher incidence in males. Also in schizophrenia, males have been shown to have an earlier disease onset, a wider range of symptoms and a lesser response to treatment. Giving this strong male

bias, many studies investigating consequences of MIA on the offspring focused on male offspring. Nevertheless, a handful of studies addressed the discrepancy in behavioral deficits between sexes.

After exposure to late gestation LPS, adult female offspring displayed locomotor abnormalities (i.e. baseline hypolocomotion and decreased reactivity to amphetamine) while males were impaired in the Morris water maze task (both spatial learning (acquisition trials) and memory (probe trial). Social interaction was not affected in either of the sexes[151]. On the other hand, LeBelle *et al.* 2014 found behavioral deficits in social communication and interaction, anxiety and repetitive behavior in both males and females prenatally exposed to LPS[167]. Similarly, Fernandez De Cossio *et al.* 2016 found impairments in social communication and interaction and repetitive behavior in both genders[159]. In Naviaux *et al.* 2013, male offspring of Poly IC treated dams showed deficits in social interaction and locomotor coordination (on the rotarod) while females displayed only mild social interaction impairments[267]. Meehan *et al.* 2016 showed that late gestation exposure to Poly IC resulted in working memory deficits in both sexes, but PPI deficits were only detectable in males. Response to an amphetamine challenge was not affected[156]. Taylor *et al.* 2012 showed decreased play behavior in males prenatally exposed to LPS but not in females[394]. Xuan *et al.* 2014 showed decreased social interaction and locomotor activity in both sexes and increased repetitive behavior in males prenatally exposed to Poly IC. Curiously, when MIA was induced by LPS, social interaction was only impaired in females (not in males) and repetitive behavior was increased in males (but not in females) [370]. While in **chapter 2** we did not distinguish between male and female embryos, we took sex into account in **chapter 5.** However, since we did not find a difference in neuronal positioning between male and female offspring, we pooled data from both sexes. In **chapter 4**, we assessed behavior only in male offspring, as we expected to pick up behavioral differences more readily in this sex, given the sex bias in ASD and schizophrenia.

Strikingly, differences in autistic like behavior between males and females was also found in a genetic model for autism. While social alterations are found in both males and females lacking Neurexin 1, increased response to cage novelty

and anxiety was only present in males while locomotor activity decreased only in females[395, 396]. The exact reason for the higher vulnerability of males to develop these disorders is unclear, but several developmental processes appear to be sex specific and could thus provide clues to the origins of sex bias in neurodevelopmental disorders.

The placenta represents the interface between the mother and the fetus, regulating exchange of nutrients and waste, and is mainly of fetal origin and thus has sex dependent characteristics. Placentas of different sexes have been shown to respond differently to environmental changes (as reviewed by [397]). Differences in nutrition affected gene expression in the placenta differentially in males versus females[398, 399]. In addition, high fat and/or salt diet caused a decreased placental size in males together with an increased expression of pro-inflammatory mediators[400]. Similarly, maternal obesity, which in itself is associated with increased risk for neuropsychiatric disorder in the offspring, induced by a high-fat diet resulted in placental macrophage activation and cytokine expression in males[401]. Conversely, maternal nutrient restriction in primates reduced expression of genes involved in programmed cell death and increased expression of those involved in cell proliferation in female but not in male placentas[402]. Finally, Bronson *et al.* 2014 showed that male placentas had upregulated gene expression of pro-inflammatory cytokines upon early prenatal stress induction while female placentas did not[235].

Secondly, brain development is influenced by sex hormones[403]. Early in development, the SRY gene on the Y-chromosome induces formation of the male testicles that produce androgens such as testosterone and dihydrotestosterone. After entering the brain, testosterone is converted to estradiol which can bind to and exert effects via the estrogen receptor. Dihydrotestosterone can function directly via the androgen receptor. Several brain regions differ in size, such as the sexually dimorphic nucleus of the preoptic area and the anteroventral periventricular nucleus, which is believed to be induced by sex hormones that modulate either cell survival or apoptosis[404]. Also the process of microglial development is influenced by the sex of the fetus. Schwartz *et al.* 2012 demonstrated that males have more microglia in total and more with an amoeboid or stouted morphology in early postnatal development in several brain

regions[405]. These morphological differences reversed later in development, with females having more microglia with thick, long processes compared to males. Microglial gene expression of a large number of cytokines, chemokines and their receptors is also highly dependent on fetal sex[405]. Matcovitch-Natan *et al.* 2016 recently described that MIA altered gene expression in developing microglia, shifting it towards the expression profile of a more advanced developmental stage and possibly disturbing their normal function in the developing brain[406]. Whether this effect was sex dependent was not investigated. Steroid hormone receptors are expressed on several immune cells of the brain and the immune response is thus likely to be different between sexes. Cultured microglia and astrocytes isolated from males produced more IL1β in response to LPS that those isolated from females[407, 408].

Few studies have addressed the sex dependent effects of MIA on the developing brain aside from behavior. While Van den Eynde *et al.* 2014 and Zhu *et al.* 2014 showed no difference in microglia activation in adulthood between male and female MIA offspring, Manitz *et al.* 2016 showed decreased microglial CD45 expression that was only evident in males[161, 409, 410]. De Souza *et al.* 2015 found increased expression of GFAP in frontal cortex of both male and female MIA offspring but higher GFAP levels in the hippocampus were found only in males[411]. Future studies would benefit from describing in more detail the discrepancies and similarities in early life infection between male and female offspring.

7.1.3 Cytokines as main responsibles for conveying the effects of MIA on the offspring

The first indication of the importance of single inflammatory mediators in conveying the effects of maternal immune stimulation onto the developing embryo came from Smith *et al.* in 2007. Using different approaches, Smith *et al.* demonstrated the critical role of IL6 in MIA induced behavioral deficits[140]. Specifically, injection of pregnant mothers with recombinant IL6, in the absence of infection, caused deficits in PPI and latent inhibition in the offspring. The same was not true for injection with recombinant IFNγ. In addition, co-administration of an IL6 antibody after Poly IC induced MIA was able to prevent

the onset of cognitive processing, exploratory and social interaction deficits. Finally, MIA offspring born to IL6 knock-out mice did not exhibit behavioral deficits[140].

Only recently, Choi *et al.* 2016 pinpointed IL17a as the intermediate cytokine through which IL6 mediates its effects on the offspring[412]. In this study, MIA was induced with Poly IC at E12, which resulted in patches of disorganized cortex cytoarchitecture (immunohistochemical staining against transcription factors Satb2 and Tbr1) in male offspring at E18, suggesting defects in neuronal positioning. Adult MIA offspring showed deficits in ultrasonic vocalization, social interaction and repetitive behavior, all of which could be prevented by co-administration of an IL17a blocking antibody. Moreover, disorganized cortical patches and ASD related behavior could also be elicited by an intracerebroventricular injection of recombinant IL17a, in the absence of maternal infection. Strikingly, while recombinant IL6 injection in the mother induced cortical layering deficits in the embryo and behavioral deficits in adult offspring, intracerebroventricular injection of IL6 failed to do the same. In addition, co-administration of IL17a blocking antibody in the mother prevented the effects of systemic IL6 administration. Therefore, the authors propose that IL6 promotes the production of IL17a by Th17 cells, which in turn is responsible for the detrimental effects of MIA on the offspring[412].

In investigating the link between MIA and epilepsy, Washington *et al.* 2015 and Pineda *et al.* 2013 confirmed that injection of recombinant IL6 in the mother caused reduced sociability in the adult offspring while IL1β failed to do so[247, 248]. On the contrary, the presence of both IL6 and IL1β was necessary to increase susceptibility to seizures[247, 248]. Importantly, the placenta appears to be a crucial mediator in the IL6 signaling pathway. Wu *et al.* 2016 recently showed that IL6Ra deletion specifically in placental trophoblasts was able to prevent the induction of the inflammatory cascade, behavioral deficits (social interaction and marble burying) and loss of purkinje cells in the cerebellum of the offspring[160].

As IL6 has been described to be an important mediator of the effects of MIA onto the developing offspring, we determined IL6 levels in maternal serum 5 hours after injection of Poly IC. (**Chapter 2, 4 and 5**) Despite a significant

increase in serum IL6 levels upon induction of MIA, we found no difference in the activation status of embryonic microglia (**chapter 2**), positioning of developing pyramidal neurons (**chapter 5**) or behavior (**chapter 4**) in the offspring.

7.1.4 MIA and other neurodegenerative disorders

As described in **chapter 4**, we performed a battery of behavioral test to detect ASD and schizophrenia-like behavior in MIA offspring. Although the vast majority of research has focused on the association between MIA and neurodevelopmental disorders such as schizophrenia and ASD, recent research also suggests a link with neurodegenerative disorders, such as Alzheimer's and Parkinson's Disease, which might not be too surprising as, like schizophrenia and ASD, Alzheimer's Disease (AD) is also characterized by synaptic deficits[79].

In 2010, Hao *et al.* showed that prenatal exposure to LPS caused spatial learning and memory deficits in aged offspring together with neuron loss, decreased expression of synaptophysin and increased expression of GFAP in the hippocampus[413]. Similarly, Krstic *et al.* 2012 showed that MIA induced by a single injection of Poly IC resulted in deficits in working memory and food hoarding in aged offspring[414]. While hippocampal Amyloid precursor protein was increased, synaptophysin and BDNF was decreased[382, 414]. Prenatal immune challenge during late gestation resulted in acceleration of aging-associated neuropathological alterations in the hippocampus such as reelin protein deposition[415, 416]. Supporting the idea of MIA predisposing to AD in humans, only recently, Dean *et al.* 2014 and Knickmeyer *et al.* 2014 showed grey and white matter changes in areas usually affected in AD in infants carrying a genetic risk factor for AD (the ApoE4 allele)[417, 418].

A few studies in animals also suggest a potential role for MIA in Parkinson's Disease (PD). PD is mainly characterized by loss of dopaminergic neurons in the substantia nigra which leads to decreased dopaminergic innervation in the striatum. In organotypic brain slice cultures from embryos exposed to prenatal infection, dopaminergic neurons were lost at a higher rate in the substantia nigra with time and striatal innervation was reduced[198]. In addition, prenatal exposure to LPS resulted in the birth of animals with fewer than normal

dopaminergic neurons which is still apparent and even progressed in aged offspring. Loss of dopaminergic neurons was accompanied by reductions in striatal dopamine and increases in dopamine activity[419]. MIA caused a reduced outgrowth of dopaminergic axons in the embryo and imbalance in the dopaminergic innervation of the striatum at birth[44].

7.2 Neuronal development deficits in MIA and other ASD and schizophrenia models

Embryonic brain development involves the integration of a series of tightly regulated processes, including proliferation, migration and differentiation of neuronal progenitors. ASD and schizophrenia are considered to be neurodevelopmental disorders implying that deficits or alterations in key processes of brain development can increase the risk for developing such disorders. In schizophrenia, decreased neuronal density in superficial white mater and increased density in deep white matter has been reported[420-425]. In addition, periventricular heterotopia, a neuronal migration disorder, has been associated with the occurrence of neuropsychiatric symptoms[426]. Heterotopias were also detected in idiopathic ASD cases and in individuals with a duplication of chromosome 15q11.2-q13, which is highly associated with intellectual disability and ASD[427].

Using genetic mouse models of ASD and schizophrenia, deficits in many neurodevelopmental processes have been found to at least contribute to the development of the disease. So although the risk for neuropsychiatric disorders is determined by many different genetic and environmental factors, they might all converge on only a few pathways. Although a wealth of studies have been conducted on the role of different risk genes in neuronal positioning, I here only highlight two studies that suggest ectopic positioning of neuronal cells, as was the case in our experiments. Orosco *et al.* 2014 found that loss of Wdfy3, a phosphatidylinositol 3-phosphate-binding protein of which the coding gene was recently found to be mutated in an ASD patient[428], resulted in regionally enlarged cortical patches[429]. They found that there was an increase in proliferative divisions on the expense of differentiative divisions together with a

more rapid turnover of intermediate progenitors into neurons. They also found heterotopic clusters of cells that migrated to superficial laminar positions, a feature that is also described in autism patients[430, 431]. Likewise, Penagarikano *et al.* 2011 described neuronal migration abnormalities in mice lacking the ASD gene CNTNAP2 (a cell adhesion molecule)[432]. However, instead of migration of deeper layer neurons into superficial layers, here they show ectopic neurons in the corpus callosum and higher numbers of Cux1 positive (upper layer) cells in the deeper cortical layers. In addition, CNTNAP2 knock-out mice had a reduced number of interneurons in all laminae. Concomitant with these deficits in neuronal positioning, they found an asynchronous firing in later II/III neurons[432].

While the studies described above employed conventional immunohistochemical techniques to detect ectopic positioning of neurons, Lang *et al.* 2016 used *in utero* electroporation to more thoroughly address the cause of laminar positioning defects caused by knock-down of Ulk4 (a serine/threonine kinase), a rare risk factor for mental disorders[433]. Tracing cells labeled with GFP at E15.5 in P7 brains, they found many neurons to be scattered along the migratory route or trapped in the deeper cortical layers. Curiously, these ectopically positioned GFP-cells retained expression of upper layer marker Satb2, indicating that fate specification was not changed[433]. In a mouse model of Fragile X Syndrome, genetic ablation of the FMRP gene caused a delay in the migration of GFP labeled neurons which was attributed to an impaired transition from the multipolar to the bipolar stage[334].

Although evidence for neuronal development deficits is compelling in genetic mouse models of ASD and schizophrenia, it is less well established whether environmental stressors such as housing stress or MIA mediate their detrimental effects via similar mechanisms. In 2015, Palacios-Garcia *et al.* reported on a decreased number of reelin-positive neurons and decreased reelin expression in layer I of the cortex after prenatal stress[341]. Reelin is essential for the inside-out layering of the cortex, so it seems reasonable that alterations in this pathway could affect neuronal positioning. The inflammatory mediator IL1β has also been described to influence neuronal development. Ma *et al.* 2014 showed an increase in the number of migrating neurons with an increasing concentration

gradient of IL1β *in vitro*. Using *in utero* electroporation of IL1 receptor shRNA in rats they described a severe retardation in the radial migration of neuronal progenitors[434]. In addition, Crampton *et al.* 2012 showed a role for IL1β in promoting neuronal progenitor differentiation towards glial cells at the expense of neuronal differentiation *in vitro*[435]. Besides the effects described on pyramidal neurons, also migration/positioning of interneurons appear to be affected. Squarzoni *et al.* 2014 described an abnormal distribution of Lhx6 interneurons with a less focal distribution around layer V after LPS-induced MIA[44]. Similarly, Ducharme *et al.* 2012 showed a decreased density in parvalbumin interneurons in the hippocampus after prenatal immune challenge[185].

Despite these studies suggesting a role for neuronal migration deficits in MIA, we found no change in laminar positioning of tdTomato labeled progenitors after a maternal immune challenge with Poly IC. (**Chapter 5**) Nevertheless, the induction of a second stimulus (independent of whether it was with NaCl or Poly IC), along with the stress it brings, appears to shift developing neurons upwards in the cortical plate compared to single injected mice. As prenatal stress was able to affect expression of reelin[341], it seems reasonable to believe that the stress induced by the second injection affects neuronal development. Indeed, interference with the reelin signaling pathway by deficiency of Cul5 (a protein that serves as a scaffold for ubiquitin ligases) caused an overmigration phenotype of labeled neurons, similar to what we have found[436]. Future studies should address whether single and/or double injection induces a neurodevelopmental phenotype that is different from non-injected mice and which stressor is responsible for this effect (handling, isoflurane exposure or blood taking). In addition, it would be of interest to subject the offspring of non-injected, single injected an double injected mothers to behavioral testing.

7.3 Microglia as mediators of neuropsychiatric disease

The role of microglia in the pathogenesis of neuropsychiatric disorders has received much attention since several post mortem immunohistochemical studies and *in vivo* PET imaging studies suggested an activated microglia

phenotype in ASD and schizophrenia patients. In **chapter 2**, we assessed whether embryonic microglia could mediate the effects of MIA onto the developing offspring, while in **chapter 6**, we explored the role of a risk gene associated with schizophrenia, i.e. DISC1, in microglial functioning.

7.3.1 The role of microglia in MIA

The role of microglia in neuropsychiatric disorders has recently gained a lot of attention. As the resident immune cells of the brain, microglia are equipped to fight of infections and clear damaged tissue by secreting a plethora of inflammatory mediators and phagocytosing cellular debris. Several imaging and post mortem studies reported on an activated microglial phenotype in the brains of autistic and schizophrenic patients[109, 110, 112, 114, 293]. In addition, levels of inflammatory cytokines were shown to be upregulated in cerebrospinal fluid and/or blood of patients, suggesting an ongoing inflammatory reaction in the brain. Supporting this idea, treatment of schizophrenia patients with the anti-inflammatory antibiotic minocycline is capable of improving negative symptoms (as reviewed by [437, 438]). Beside their established role in neuroinflammation, microglia are also important mediators of synaptic remodeling. While ASD is usually characterized by an increased synaptic density, schizophrenia is mainly associated with a decreased amount of synaptic spines[79].

To address the contribution of microglia in the MIA model of neuropsychiatric disorders, several groups aimed to analyze the activation state of these cells. Results from early postnatal to adult MIA offspring have been very contradictory, with several studies suggesting an activated phenotype of microglia while others find no difference compared to control animals. An increased density of microglia has been reported in several brain regions including hippocampus, striatum, thalamus, nucleus accumbens and cortex in adolescent, adult or aged MIA offspring[149, 161, 225, 226, 261, 410]. Morphology of microglia was also found to be changed, with a more amoeboid shape and less branches[161, 410]. In early postnatal MIA offspring, Girard *et al.* 2010 and Li *et al.* 2014 detected an increased microglial density and an increase in proliferating microglia[177, 234]. Finally, increased expression of MHC II, IL1β and TNFα were detected in isolated microglia from aged offspring[149, 261]. In contrast, no evidence for microglial

activation in early postnatal, adolescent, adult or aged MIA offspring was found by Giovanoli *et al.* 2015 and 2016, Paylor *et al.* 2016, Pineda *et al.* 2013, Willi *et al.* 2013, Missault *et al.* 2014, Mouihate *et al.* 2016, Garay *et al.* 2013, Antonson *et al.* 2017 or Hsiao *et al.* 2012[138, 178, 182, 183, 224, 227, 246, 247, 382, 439].

Whether increased microglia activation in MIA offspring would be causal for or merely a consequence of neuropsychiatric disease is not clear. To this end, a handful of studies investigated the microglial phenotype upon MIA in the offspring *in utero*. While in adult animals microglia activation is often deduced from a more amoeboid cell shape, embryonic microglia are by default more amoeboid, making morphology an inappropriate parameter. Nevertheless, results on embryonic microglia activation are divergent. LeBelle *et al.* 2014 and Tronnes *et al.* 2016 found an increased microglial density with microglia appearing with larger cell bodies and fewer/shorter processes at E18 after MIA and Schmidt *et al.* 2016 found an increased percentage of proliferating microglia after intra-amniotic LPS administration[167, 265, 440]. While Pratt *et al.* 2013, Pont-Lezica *et al.* 2014 and Cunningham *et al.* 2013 did not find these alterations in microglia density, they were able to demonstrate an increased proportion of microglia expressing iNOS or IL1β and altered gene and protein expression in isolated microglia[57, 64, 211]. In **chapter 2**, we did not find altered expression of inflammatory markers iNOS, IL1β or Mac2 upon induction of MIA, nor could we detect alterations in microglial density. Although we detected an increased amount of microglia expressing iNOS or IL1β upon LPS treatment of acute brain slices, stimulation with Poly IC or recombinant IL6 failed to do the same.

Interestingly, except for Pratt *et al.* 2013, all other studies described above suggesting microglial activation in embryos exposed to MIA used LPS to stimulate the immune system of the mother. As we were also able to demonstrate microglia activation upon LPS in acute brain slices, this might indicate that LPS is a more potent activator. In line with this, using LPS in mice is more potent at inducing embryonic lethality compared to Poly IC[368, 369]. While Pratt *et al.* 2013 used a paradigm to induce MIA highly similar to the one we used in **chapter 2** (intraperitoneal Poly IC (20mg/kg) at E12.5), they also found no upregulation of IL1β mRNA or protein in isolated microglia. Taken together, these data suggest that microglia activation upon MIA might depend on the

exact immune stimulant used and a comparative study between LPS and Poly IC would be of interest to address these differences.

While embryonic microglia have not yet invaded the brain parenchyma at E9 in the mouse embryo[38, 285], several studies found behavioral deficits reminiscent of ASD and schizophrenia in offspring of mothers in which MIA was induced at this age, including decreased social interaction, deficits in PPI, increased locomotor response to amphetamine and reduced exploration in the open field and elevated plus maze[152, 161, 166, 175, 181, 206, 212, 229, 232, 263, 328, 343]. Although it cannot be excluded that microglial precursors that originate from the yolk sack starting from E7.5 are affected by MIA, even before they reach the brain, this indicates that microglia are unlikely to be the sole mediators causing the detrimental effects of MIA in the offspring.

However, it is possible that, when MIA is induced at an age where microglia have populated the embryonic brain, they could aggravate the behavioral phenotype. Although not consistently reported, several studies showed increased levels of inflammatory cytokines in the embryonic brain when exposed to prenatal infection. Regardless of whether these cytokines originate from the mother or from the fetus itself, we expected (but did not find) these cytokines to affect microglia and cause alterations in their activation state or in their normal physiological function during brain development. Alternatively, prenatal immune stimulation might prime embryonic microglia, causing them to respond more vigorously upon an adverse event during childhood or adolescence. Although we did not confirm changes in microglial activation (**Chapter 2**), Krstic *et al.* 2012 found an increased CD68 reactivity and microglial morphology in aged offspring of late gestation MIA, but only when challenged postnatally with LPS[414]. Similarly, Giovanoli *et al.* 2013 and 2016 described increased expression of CD68 and CD11b together with an enlargement of microglia cell soma area in adolescent MIA offspring that underwent postnatal stress[183, 259]. In the study of Cao *et al.* 2015, embryonic microglia were 'primed' by LPS administration to sheep fetuses and isolated and cultured 3 days later[441]. Microglia *in utero* exposed to LPS had higher baseline IL1β secretion and increased IL1β secretion much more after *in vitro* exposure to LPS (second hit) compared to non-primed microglia. Comparing microglia that were *in utero* exposed to LPS with those

that were not, there were 258 differentially expressed genes. Non-primed microglia that did or did not receive LPS *in vitro* had 6642 differentially expressed genes. On the other hand, microglia exposed to LPS *in utero* that did or did not receive LPS *in vitro* had only 6 differentially expressed genes[441].

7.3.2 The role of genetic risk factors in microglia

Studies on genetic moues models of neuropsychiatric disorders have mainly focused on the functional deficit caused by a particular genetic disruption in neurons. Whether the disrupted gene is also expressed in microglia and whether its disruption in this cell type could contribute to the disease is poorly investigated. DAP12 and TREM2 (a membrane receptor) are 2 genes that, when disrupted, lead to the development of Nasu-Hakola disease, a severe disorder characterized by bone cysts and fractures during adolescence, accompanied by early-onset frontal lobe syndrome and progressive presenile dementia. DAP12 and TREM2 are expressed on the plasma membrane of microglia and can activate several downstream effector molecules that are important for cellular differentiation, activation, migration, cytokine production, and phagocytosis[442].

Disruption of the MecP2 gene causes Rett syndrome, a severe neurological disorder that can present with autistic features. MecP2 is an epigenetic regulator that has been shown to regulate glucocorticoid and hypoxia induced gene expression in microglia[443]. In the same study, the investigators crossed CX3CR1-CreER(T2) mice with MecP2-LoxStop mice. These mice are deficient of MecP2 but upon injection of tamoxifen, MecP2 expression is restored in CX3CR1-expressing cells, and thus in microglia. When microglial MecP2 expression was reinstated at 9 weeks of age, the lifespan of affected animals was significantly increased[443].These studies suggest that microglia may cause, or at least contribute to the development of these disorders.

Recently, expression of 2 other risk genes was described in microglia. Gholizadeh *et al.* 2015 showed expression of FMRP in more than 75% of microglia at P0[444]. FMRP is the gene causing Fragile X Syndrome, a disorder with many autistic features. The role of FMRP in microglia is not yet investigated, but as FMRP is important in local mRNA translation, it might be of importance for local protein expression in microglial processes. Seshadri *et al.*

2010 on the other hand showed expression of DISC1 in microglia, a gene that is highly correlated with schizophrenia and other psychiatric disorders[273]. As described in the general introduction, DISC1 interacts with a great amount of other proteins, including those regulating cytoskeletal remodeling. In **chapter 6**, we investigated in more detail the expression of this protein in microglia and explored its function in microglial phagocytosis, a process that is highly dependent on rearrangement of the cytoskeleton. We found that transfection of shRNA directed against Disc1 mRNA caused a decrease in phagocytosis of latex beads. These preliminary results suggest that typical risk genes for ASD or schizophrenia might partly exert their detrimental effect through microglial dysfunction in the developing/adult brain. To further characterize the impact of DISC1 deficiency, it would be of interest to investigate this proteins role in other physiological functions of microglia such as migration and proliferation *in vitro* and to expand these findings in an *in vivo* model where DISC1 expression is specifically altered in microglia. In addition, it would be of interest to determine whether also other genetic risk factors, such as FMRP, could play an important role in the normal functioning of microglia.

In **conclusion**, our results indicate that although microglia are not likely to be the main effectors of MIA during development, they could contribute to the pathogenesis of neuropsychiatric disorders in the context of genetic risk factors. In preliminary experiments we found that modulation of DISC1 expression affected microglial phagocytosis *in vitro*, but investigation of its role in other microglial functions and in an *in vivo* setting is warranted. Unexpectedly, we also found no effect of MIA on the developmental trajectory of pyramidal neurons, as evidenced by a similar distribution in the developing cortex of neuronal progenitors labeled by *in utero* electroporation. However, a shift in neuronal positioning was observed between offspring exposed to 1 or 2 injections with NaCl, which served as control conditions for injection with Poly IC. Although these data might suggest that maternal stress associated with the second injection could have caused this phenotype, a comparison should be made with non-injected animals. In addition, the exact stressor responsible for this effect should be identified. These could be either the stress induced by animal handling, the exposure to isoflurane or the acquisition of maternal blood via the tail.

English summary

Microglia are the immune cells of the brain and thus protect it against damage and infections. In recent years, extensive research is conducted in order to examine the role of these cells in the development of the brain. It was found that microglia are important for amongst others regulation of the neuronal precursor pool and modulation of synaptic connections. Proper development of the brain is crucial since disturbances can increase the risk for developing neuropsychiatric disorders such as schizophrenia and autism spectrum disorder. This disturbance in neurodevelopment can be caused by both genetic and environmental risk factors that influence the developing brain.

One of these environmental risk factors that received much attention in recent years is the activation of the maternal immune system during pregnancy by for example an infection. In this thesis, we investigated whether maternal infection would influence the activation status of the embryonic microglia and whether it could disturb the normal development of the brain. The immune system of pregnant mice was stimulated by a single or double injection of the synthetic double stranded RNA Poly IC, which mimics a viral infection. Next, the activation status of embryonic microglia was assessed by the expression of several activation markers (**Chapter 2**). Despite the fact that the immune system of the pregnant mouse was indeed activated (as evidenced by an increase in the pro-inflammatory cytokine IL6 in the maternal serum) we found no difference in the expression of the different activation markers between immune challenged and control mice. In contrast to other research groups, we also found no schizophrenia- or autism like behavior in the offspring of mothers that received one injection of Poly IC during pregnancy (**Chapter 4**). To more thoroughly study the role of embryonic microglia in maternal infection, we aimed at creating an inducible genetic mouse model in which microglia could be eliminated by deletion of the PU.1 gene, an important gene in the development of microglia (**Chapter 3**). Despite the fact that we confirmed genetic recombination of the PU.1 gene, microglia were still present in embryonic and adult brain. The proper development of the brain was not affected by embryonic exposure to Poly IC (**Chapter 5**). We labeled neuronal precursors with the red fluorescent protein tdTomato by means of *in utero* electroporation to assess the distribution of labeled cells in the cortex at a later developmental stage. We found that nor one, nor two injections of Poly IC changed the distribution of these labeled cells

compared to their respective single or double saline injection controls. However, the distribution of labeled cells differed between animals receiving only one injection compared to those receiving two injections. Whether this effect could be caused by the stress associated with the second injection remains to be investigated.

Beside environmental factors, also genetic factors play an important role in neuropsychiatric disease. One of these genes that is associated with schizophrenia is DISC1, a gene in which several different mutations increase the risk for developing a neuropsychiatric disorder. The function of the DISC1 protein is already extensively investigated in neurons but their role in the function of microglia did not receive any attention. In **chapter 6** we found that, both at the mRNA and protein level, DISC1 is expressed in primary microglia isolated from young mice and in BV2 cells, an immortalized microglial cell line. To investigate whether DISC1 plays a role in phagocytosis, an important function of microglia, we modified the endogenous expression of this protein in BV2 cells by transfection with different plasmids. We showed that reduction of Disc1 mRNA levels resulted in reduced phagocytosis of latex beads while overexpression of DISC1 did not influence this particular function. Whether DISC1 plays a role in other microglial functions remains to be investigated.

Nederlandse samenvatting

Microglia zijn de immuuncellen van de hersenen en staan daarbij in voor de bescherming tegen beschadiging en infecties. De laatste jaren is echter ook veel onderzoek verricht naar de rol van deze cellen in de ontwikkeling van de hersenen. Microglia zijn van belang voor onder andere de regulatie van de neuronale precursoren en de modulatie van synaptische verbindingen tussen de zenuwcellen. Correcte ontwikkeling van de hersenen is van groot belang aangezien verstoringen hierin het risico vergroten op de ontwikkeling van neuropsychiatrische stoornissen zoals schizofrenie en autisme spectrum stoornis. Een verstoorde hersenontwikkeling kan veroorzaakt worden door zowel genetische als omgevingsfactoren die de hersenontwikkeling beïnvloeden.

Eén van deze omgevingsfactoren waaraan de laatste jaren veel aandacht aan besteed is, is de activatie van het maternale immuunsysteem tijdens de zwangerschap door bijvoorbeeld een infectie. In deze thesis onderzochten we of maternale infectie een invloed had op de activatie van de embryonale microglia en of hierdoor de normale ontwikkeling van de hersenen verstoord werd. Het immuunsysteem van zwangere muizen werd gestimuleerd door één of twee injecties met het synthetisch dubbelstrengige RNA Poly IC, waarmee een virale infectie gesimuleerd wordt. Vervolgens werd de activatiestatus van de embryonale microglia bestudeerd aan de hand van de expressie van klassieke activatiemerkers (**Hoofdstuk 2**). Ondanks het feit dat het immuunsysteem van de moedermuis wel degelijk gestimuleerd werd door de injectie met Poly IC (zoals bepaald door een stijging van het pro-inflammatoire cytokine IL6 in het maternale serum) vonden we geen verschil in de expressie van de verschillende activatiemerkers tussen immuungestimuleerde en controle muizen. In tegenstelling tot andere onderzoeksgroepen vonden we ook geen schizofrenie- of autismeachtig gedrag in nakomelingen van moeders die een injectie Poly IC kregen tijdens de zwangerschap (**Hoofdstuk 4**). Om de rol van embryonale microglia bij maternale infectie beter te bestuderen trachtten we een induceerbaar genetisch muismodel te creëren waarbij microglia geëlimineerd konden worden door uitschakeling van het PU.1 gen, een belangrijk gen in de ontwikkeling van microglia (**Hoofdstuk 3**). Ondanks het feit dat we genetische recombinatie van het PU.1 gen konden vaststellen bleven microglia aanwezig in embryonale en volwassen hersenen. Ook in de cellulaire ontwikkeling van de hersenen konden we geen effect detecteren van embryonale blootstelling aan

Poly IC (**Hoofdstuk 5**). Om dit te onderzoeken markeerden we neuronale precursorcellen met het rood fluorescente eiwit tdTomato door middel van *in utero* electroporatie. Op deze manier konden we op een later ontwikkelingsstadium de verdeling van deze cellen in de cortex bepalen. We vonden dat noch één, noch twee injecties met Poly IC de verdeling van deze cellen veranderde ten opzichte van hun respectievelijke enkele en dubbele controleïnjectie. Echter, de verdeling van gemarkeerde cellen verschilde van dieren die slechts één injectie kregen ten opzichte van dieren die twee injecties kregen. Het dient verder onderzocht te worden of dit effect veroorzaakt kon worden door de stress die gepaard ging met de tweede injectie.

Behalve omgevingsfactoren spelen ook genetische factoren een belangrijke rol in neuropsychiatrische stoornissen. Een van deze genen die geassocieerd wordt met schizofrenie is DISC1, een gen waarbij verschillende mutaties het risico vergroten op de ontwikkeling van neuropsychiatrische aandoeningen. De functie van het DISC1 eiwit is reeds uitgebreid bestudeerd in neuronen maar onderzoek naar hun rol in de functies van microglia is tot dusver niet onderzocht. In **hoofdstuk 6** beschrijven we dat op zowel het mRNA als het eiwit niveau DISC1 effectief tot expressie wordt gebracht in primaire microglia geïsoleerd van jongen muizen en in BV2 cellen, een geïmmortaliseerde micrgoliale cellijn. Om te onderzoeken of DISC1 een rol speelde in fagocytose, een belangrijke functie van microglia, veranderden we de endogene expressie van dit eiwit in BV2 cellen door middel van transfectie met verschillende plasmides. Op deze manier konden we aantonen dat vermindering van Disc1 mRNA leidde tot een verminderde fagocytose van latex partikels terwijl overexpressie van DISC1 hier geen invloed op had. Of DISC1 ook een rol speelt in andere microgliale functies dient nog onderzocht te worden.

References

1. Eric R. Kandel, J.H.S., Thomas M. Jessel, Steven A. Siegelbaum, A.J. Hudspeth, *Principles of neural science*. Fift Edition ed. 2013: The McGraw-Hill Companies.
2. Valenzuela, C.F., M.P. Puglia, and S. Zucca, *Focus on: neurotransmitter systems.* Alcohol Res Health, 2011. **34**(1): p. 106-20.
3. Moffat, J.J., et al., *Genes and brain malformations associated with abnormal neuron positioning.* Mol Brain, 2015. **8**(1): p. 72.
4. Malatesta, P., E. Hartfuss, and M. Gotz, *Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage.* Development, 2000. **127**(24): p. 5253-63.
5. Miyata, T., et al., *Asymmetric inheritance of radial glial fibers by cortical neurons.* Neuron, 2001. **31**(5): p. 727-41.
6. Noctor, S.C., et al., *Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia.* J Neurosci, 2002. **22**(8): p. 3161-73.
7. Tamamaki, N., et al., *Radial glia is a progenitor of neocortical neurons in the developing cerebral cortex.* Neurosci Res, 2001. **41**(1): p. 51-60.
8. Haubensak, W., et al., *Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 3196-201.
9. Kowalczyk, T., et al., *Intermediate neuronal progenitors (basal progenitors) produce pyramidal-projection neurons for all layers of cerebral cortex.* Cereb Cortex, 2009. **19**(10): p. 2439-50.
10. Noctor, S.C., et al., *Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases.* Nat Neurosci, 2004. **7**(2): p. 136-44.
11. Tabata, H. and K. Nakajima, *Multipolar migration: the third mode of radial neuronal migration in the developing cerebral cortex.* J Neurosci, 2003. **23**(31): p. 9996-10001.
12. Tabata, H. and K. Nagata, *Decoding the molecular mechanisms of neuronal migration using in utero electroporation.* Med Mol Morphol, 2016. **49**(2): p. 63-75.

13. Ohtaka-Maruyama, C. and H. Okado, *Molecular Pathways Underlying Projection Neuron Production and Migration during Cerebral Cortical Development.* Front Neurosci, 2015. **9**: p. 447.
14. Hur, E.M. and F.Q. Zhou, *GSK3 signalling in neural development.* Nat Rev Neurosci, 2010. **11**(8): p. 539-51.
15. van den Ameele, J., et al., *Thinking out of the dish: what to learn about cortical development using pluripotent stem cells.* Trends Neurosci, 2014. **37**(6): p. 334-42.
16. Elias, L.A., D.D. Wang, and A.R. Kriegstein, *Gap junction adhesion is necessary for radial migration in the neocortex.* Nature, 2007. **448**(7156): p. 901-7.
17. Jossin, Y. and J.A. Cooper, *Reelin, Rap1 and N-cadherin orient the migration of multipolar neurons in the developing neocortex.* Nat Neurosci, 2011. **14**(6): p. 697-703.
18. Kawauchi, T., et al., *Rab GTPases-dependent endocytic pathways regulate neuronal migration and maturation through N-cadherin trafficking.* Neuron, 2010. **67**(4): p. 588-602.
19. Xie, Z., et al., *Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration.* Cell, 2003. **114**(4): p. 469-82.
20. Shu, T., et al., *Ndel1 operates in a common pathway with LIS1 and cytoplasmic dynein to regulate cortical neuronal positioning.* Neuron, 2004. **44**(2): p. 263-77.
21. Tanaka, T., et al., *Lis1 and doublecortin function with dynein to mediate coupling of the nucleus to the centrosome in neuronal migration.* J Cell Biol, 2004. **165**(5): p. 709-21.
22. Tsai, J.W., K.H. Bremner, and R.B. Vallee, *Dual subcellular roles for LIS1 and dynein in radial neuronal migration in live brain tissue.* Nat Neurosci, 2007. **10**(8): p. 970-9.
23. Schaar, B.T. and S.K. McConnell, *Cytoskeletal coordination during neuronal migration.* Proc Natl Acad Sci U S A, 2005. **102**(38): p. 13652-7.

24. Costa, C., B. Harding, and A.J. Copp, *Neuronal migration defects in the Dreher (Lmx1a) mutant mouse: role of disorders of the glial limiting membrane.* Cereb Cortex, 2001. **11**(6): p. 498-505.
25. Hecht, J.H., et al., *Primary cellular meningeal defects cause neocortical dysplasia and dyslamination.* Ann Neurol, 2010. **68**(4): p. 454-64.
26. Li, S., et al., *GPR56 regulates pial basement membrane integrity and cortical lamination.* J Neurosci, 2008. **28**(22): p. 5817-26.
27. Gongidi, V., et al., *SPARC-like 1 regulates the terminal phase of radial glia-guided migration in the cerebral cortex.* Neuron, 2004. **41**(1): p. 57-69.
28. Franco, S.J., et al., *Reelin regulates cadherin function via Dab1/Rap1 to control neuronal migration and lamination in the neocortex.* Neuron, 2011. **69**(3): p. 482-97.
29. Greig, L.C., et al., *Molecular logic of neocortical projection neuron specification, development and diversity.* Nat Rev Neurosci, 2013. **14**(11): p. 755-69.
30. Zhao, S. and M. Frotscher, *Go or stop? Divergent roles of Reelin in radial neuronal migration.* Neuroscientist, 2010. **16**(4): p. 421-34.
31. Ayala, R., T. Shu, and L.H. Tsai, *Trekking across the brain: the journey of neuronal migration.* Cell, 2007. **128**(1): p. 29-43.
32. Schmidt, M.J. and K. Mirnics, *Neurodevelopment, GABA system dysfunction, and schizophrenia.* Neuropsychopharmacology, 2015. **40**(1): p. 190-206.
33. Furukawa, T., et al., *Roles of taurine-mediated tonic GABAA receptor activation in the radial migration of neurons in the fetal mouse cerebral cortex.* Front Cell Neurosci, 2014. **8**: p. 88.
34. Ginhoux, F., et al., *Fate mapping analysis reveals that adult microglia derive from primitive macrophages.* Science, 2010. **330**(6005): p. 841-5.
35. Kierdorf, K., et al., *Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways.* Nat Neurosci, 2013. **16**(3): p. 273-80.

36. Elmore, M.R., et al., *Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain.* Neuron, 2014. **82**(2): p. 380-97.
37. Nayak, D., T.L. Roth, and D.B. McGavern, *Microglia development and function.* Annu Rev Immunol, 2014. **32**: p. 367-402.
38. Swinnen, N., et al., *Complex invasion pattern of the cerebral cortex bymicroglial cells during development of the mouse embryo.* Glia, 2013. **61**(2): p. 150-63.
39. Hoeffel, G. and F. Ginhoux, *Ontogeny of Tissue-Resident Macrophages.* Front Immunol, 2015. **6**: p. 486.
40. Parkhurst, C.N., et al., *Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor.* Cell, 2013. **155**(7): p. 1596-609.
41. Arno, B., et al., *Neural progenitor cells orchestrate microglia migration and positioning into the developing cortex.* Nat Commun, 2014. **5**: p. 5611.
42. Nelson, L.H. and K.M. Lenz, *Microglia depletion in early life programs persistent changes in social, mood-related, and locomotor behavior in male and female rats.* Behav Brain Res, 2017. **316**: p. 279-293.
43. VanRyzin, J.W., et al., *Temporary Depletion of Microglia during the Early Postnatal Period Induces Lasting Sex-Dependent and Sex-Independent Effects on Behavior in Rats.* eNeuro, 2016. **3**(6).
44. Squarzoni, P., et al., *Microglia modulate wiring of the embryonic forebrain.* Cell Rep, 2014. **8**(5): p. 1271-9.
45. Buttgereit, A., et al., *Sall1 is a transcriptional regulator defining microglia identity and function.* Nat Immunol, 2016. **17**(12): p. 1397-1406.
46. Bennett, M.L., et al., *New tools for studying microglia in the mouse and human CNS.* Proc Natl Acad Sci U S A, 2016. **113**(12): p. E1738-46.
47. Frost, J.L. and D.P. Schafer, *Microglia: Architects of the Developing Nervous System.* Trends Cell Biol, 2016. **26**(8): p. 587-97.
48. Pierre, W.C., et al., *Neonatal microglia: The cornerstone of brain fate.* Brain Behav Immun, 2017. **59**: p. 333-345.

49. Ueno, M. and T. Yamashita, *Bidirectional tuning of microglia in the developing brain: from neurogenesis to neural circuit formation.* Curr Opin Neurobiol, 2014. **27**: p. 8-15.
50. Arnold, T. and C. Betsholtz, *The importance of microglia in the development of the vasculature in the central nervous system.* Vasc Cell, 2013. **5**(1): p. 4.
51. Casano, A.M. and F. Peri, *Microglia: multitasking specialists of the brain.* Dev Cell, 2015. **32**(4): p. 469-77.
52. Checchin, D., et al., *Potential role of microglia in retinal blood vessel formation.* Invest Ophthalmol Vis Sci, 2006. **47**(8): p. 3595-602.
53. Fantin, A., et al., *Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction.* Blood, 2010. **116**(5): p. 829-40.
54. Kubota, Y., et al., *M-CSF inhibition selectively targets pathological angiogenesis and lymphangiogenesis.* J Exp Med, 2009. **206**(5): p. 1089-102.
55. Rymo, S.F., et al., *A two-way communication between microglial cells and angiogenic sprouts regulates angiogenesis in aortic ring cultures.* PLoS One, 2011. **6**(1): p. e15846.
56. Stefater, J.A., 3rd, et al., *Regulation of angiogenesis by a non-canonical Wnt-Flt1 pathway in myeloid cells.* Nature, 2011. **474**(7352): p. 511-5.
57. Cunningham, C.L., V. Martinez-Cerdeno, and S.C. Noctor, *Microglia regulate the number of neural precursor cells in the developing cerebral cortex.* J Neurosci, 2013. **33**(10): p. 4216-33.
58. Aarum, J., et al., *Migration and differentiation of neural precursor cells can be directed by microglia.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15983-8.
59. Antony, J.M., et al., *Endogenous microglia regulate development of embryonic cortical precursor cells.* J Neurosci Res, 2011. **89**(3): p. 286-98.
60. Shigemoto-Mogami, Y., et al., *Microglia enhance neurogenesis and oligodendrogenesis in the early postnatal subventricular zone.* J Neurosci, 2014. **34**(6): p. 2231-43.

61. Dalmau, I., et al., *Development of microglia in the prenatal rat hippocampus.* J Comp Neurol, 1997. **377**(1): p. 70-84.
62. Dalmau, I., et al., *Development of microglia in the postnatal rat hippocampus.* Hippocampus, 1998. **8**(5): p. 458-74.
63. Santos, A.M., et al., *Embryonic and postnatal development of microglial cells in the mouse retina.* J Comp Neurol, 2008. **506**(2): p. 224-39.
64. Pont-Lezica, L., et al., *Microglia shape corpus callosum axon tract fasciculation: functional impact of prenatal inflammation.* Eur J Neurosci, 2014. **39**(10): p. 1551-7.
65. Stevens, B., et al., *The classical complement cascade mediates CNS synapse elimination.* Cell, 2007. **131**(6): p. 1164-78.
66. Tremblay, M.E., R.L. Lowery, and A.K. Majewska, *Microglial interactions with synapses are modulated by visual experience.* PLoS Biol, 2010. **8**(11): p. e1000527.
67. Paolicelli, R.C., et al., *Synaptic pruning by microglia is necessary for normal brain development.* Science, 2011. **333**(6048): p. 1456-8.
68. Schafer, D.P., et al., *Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner.* Neuron, 2012. **74**(4): p. 691-705.
69. Ji, K., et al., *Microglia actively regulate the number of functional synapses.* PLoS One, 2013. **8**(2): p. e56293.
70. Kim, H.J., et al., *Deficient autophagy in microglia impairs synaptic pruning and causes social behavioral defects.* Mol Psychiatry, 2016.
71. Lim, S.H., et al., *Neuronal synapse formation induced by microglia and interleukin 10.* PLoS One, 2013. **8**(11): p. e81218.
72. Wang, X., et al., *Requirement for Microglia for the Maintenance of Synaptic Function and Integrity in the Mature Retina.* J Neurosci, 2016. **36**(9): p. 2827-42.
73. Owen, M.J., A. Sawa, and P.B. Mortensen, *Schizophrenia.* Lancet, 2016. **388**(10039): p. 86-97.
74. McGrath, J.J., et al., *The neurodevelopmental hypothesis of schizophrenia: a review of recent developments.* Ann Med, 2003. **35**(2): p. 86-93.

75. Cardno, A.G. and Gottesman, II, *Twin studies of schizophrenia: from bow-and-arrow concordances to star wars Mx and functional genomics.* Am J Med Genet, 2000. **97**(1): p. 12-7.
76. Shepherd, A.M., et al., *Systematic meta-review and quality assessment of the structural brain alterations in schizophrenia.* Neurosci Biobehav Rev, 2012. **36**(4): p. 1342-56.
77. Bolkan, S.S., F. Carvalho Poyraz, and C. Kellendonk, *Using human brain imaging studies as a guide toward animal models of schizophrenia.* Neuroscience, 2016. **321**: p. 77-98.
78. Glantz, L.A. and D.A. Lewis, *Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia.* Arch Gen Psychiatry, 2000. **57**(1): p. 65-73.
79. Penzes, P., et al., *Dendritic spine pathology in neuropsychiatric disorders.* Nat Neurosci, 2011. **14**(3): p. 285-93.
80. Baumeister, A.A. and J.L. Francis, *Historical development of the dopamine hypothesis of schizophrenia.* J Hist Neurosci, 2002. **11**(3): p. 265-77.
81. Hess, E.J., et al., *Dopamine receptor subtype imbalance in schizophrenia.* Life Sci, 1987. **40**(15): p. 1487-97.
82. Mita, T., et al., *Decreased serotonin S2 and increased dopamine D2 receptors in chronic schizophrenics.* Biol Psychiatry, 1986. **21**(14): p. 1407-14.
83. Hu, W., et al., *The glutamate hypothesis of schizophrenia: evidence from human brain tissue studies.* Ann N Y Acad Sci, 2015. **1338**: p. 38-57.
84. Moghaddam, B. and D. Javitt, *From revolution to evolution: the glutamate hypothesis of schizophrenia and its implication for treatment.* Neuropsychopharmacology, 2012. **37**(1): p. 4-15.
85. Malhotra, D. and J. Sebat, *CNVs: harbingers of a rare variant revolution in psychiatric genetics.* Cell, 2012. **148**(6): p. 1223-41.
86. Rees, E., et al., *CNV analysis in a large schizophrenia sample implicates deletions at 16p12.1 and SLC1A1 and duplications at 1p36.33 and CGNL1.* Hum Mol Genet, 2014. **23**(6): p. 1669-76.

87. Schizophrenia Working Group of the Psychiatric Genomics, C., *Biological insights from 108 schizophrenia-associated genetic loci.* Nature, 2014. **511**(7510): p. 421-7.
88. Sullivan, P.F., M.J. Daly, and M. O'Donovan, *Genetic architectures of psychiatric disorders: the emerging picture and its implications.* Nat Rev Genet, 2012. **13**(8): p. 537-51.
89. Dean, K. and R.M. Murray, *Environmental risk factors for psychosis.* Dialogues Clin Neurosci, 2005. **7**(1): p. 69-80.
90. Buckley, P.F., et al., *Psychiatric comorbidities and schizophrenia.* Schizophr Bull, 2009. **35**(2): p. 383-402.
91. Mukherjee, S.B., *Autism Spectrum Disorders - Diagnosis and Management.* Indian J Pediatr, 2017.
92. Abrahams, B.S. and D.H. Geschwind, *Advances in autism genetics: on the threshold of a new neurobiology.* Nat Rev Genet, 2008. **9**(5): p. 341-55.
93. Dove, D., et al., *Medications for adolescents and young adults with autism spectrum disorders: a systematic review.* Pediatrics, 2012. **130**(4): p. 717-26.
94. Ghanizadeh, A., S. Tordjman, and N. Jaafari, *Aripiprazole for treating irritability in children & adolescents with autism: A systematic review.* Indian J Med Res, 2015. **142**(3): p. 269-75.
95. Jesner, O.S., M. Aref-Adib, and E. Coren, *Risperidone for autism spectrum disorder.* Cochrane Database Syst Rev, 2007(1): p. CD005040.
96. Jumah, F., et al., *Neuroanatomical variation in autism spectrum disorder: A comprehensive review.* Clin Anat, 2016. **29**(4): p. 454-65.
97. Yoo, H., *Genetics of Autism Spectrum Disorder: Current Status and Possible Clinical Applications.* Exp Neurobiol, 2015. **24**(4): p. 257-72.
98. Lyall, K., R.J. Schmidt, and I. Hertz-Picciotto, *Maternal lifestyle and environmental risk factors for autism spectrum disorders.* Int J Epidemiol, 2014. **43**(2): p. 443-64.
99. Bolton, P.F., et al., *Epilepsy in autism: features and correlates.* Br J Psychiatry, 2011. **198**(4): p. 289-94.

100. Frye, R.E. and D.A. Rossignol, *Identification and Treatment of Pathophysiological Comorbidities of Autism Spectrum Disorder to Achieve Optimal Outcomes.* Clin Med Insights Pediatr, 2016. **10**: p. 43-56.
101. Marin, O., *Interneuron dysfunction in psychiatric disorders.* Nat Rev Neurosci, 2012. **13**(2): p. 107-20.
102. Cho, K.K., et al., *Gamma rhythms link prefrontal interneuron dysfunction with cognitive inflexibility in Dlx5/6(+/-) mice.* Neuron, 2015. **85**(6): p. 1332-43.
103. Valente, M.F., et al., *Postnatal transplantation of interneuronal precursor cells decreases anxiety-like behavior in adult mice.* Cell Transplant, 2013. **22**(7): p. 1237-47.
104. Lemonnier, E., A. Lazartigues, and Y. Ben-Ari, *Treating Schizophrenia With the Diuretic Bumetanide: A Case Report.* Clin Neuropharmacol, 2016. **39**(2): p. 115-7.
105. Rahmanzadeh, R., et al., *Effect of bumetanide, a selective NKCC1 inhibitor, on hallucinations of schizophrenic patients; a double-blind randomized clinical trial.* Schizophr Res, 2017. **184**: p. 145-146.
106. Hadjikhani, N., et al., *Improving emotional face perception in autism with diuretic bumetanide: a proof-of-concept behavioral and functional brain imaging pilot study.* Autism, 2015. **19**(2): p. 149-57.
107. Lemonnier, E., et al., *A randomised controlled trial of bumetanide in the treatment of autism in children.* Transl Psychiatry, 2012. **2**: p. e202.
108. Frick, L.R., K. Williams, and C. Pittenger, *Microglial dysregulation in psychiatric disease.* Clin Dev Immunol, 2013. **2013**: p. 608654.
109. Radewicz, K., et al., *Increase in HLA-DR immunoreactive microglia in frontal and temporal cortex of chronic schizophrenics.* J Neuropathol Exp Neurol, 2000. **59**(2): p. 137-50.
110. Wierzba-Bobrowicz, T., et al., *Quantitative analysis of activated microglia, ramified and damage of processes in the frontal and temporal lobes of chronic schizophrenics.* Folia Neuropathol, 2005. **43**(2): p. 81-9.
111. Busse, S., et al., *Different distribution patterns of lymphocytes and microglia in the hippocampus of patients with residual versus paranoid*

schizophrenia: further evidence for disease course-related immune alterations? Brain Behav Immun, 2012. **26**(8): p. 1273-9.

112. Morgan, J.T., et al., *Microglial activation and increased microglial density observed in the dorsolateral prefrontal cortex in autism.* Biol Psychiatry, 2010. **68**(4): p. 368-76.
113. Tetreault, N.A., et al., *Microglia in the cerebral cortex in autism.* J Autism Dev Disord, 2012. **42**(12): p. 2569-84.
114. Vargas, D.L., et al., *Neuroglial activation and neuroinflammation in the brain of patients with autism.* Ann Neurol, 2005. **57**(1): p. 67-81.
115. Kreisl, W.C., et al., *A genetic polymorphism for translocator protein 18 kDa affects both in vitro and in vivo radioligand binding in human brain to this putative biomarker of neuroinflammation.* J Cereb Blood Flow Metab, 2013. **33**(1): p. 53-8.
116. Rao, J.S., et al., *Increased neuroinflammatory and arachidonic acid cascade markers, and reduced synaptic proteins, in the postmortem frontal cortex from schizophrenia patients.* Schizophr Res, 2013. **147**(1): p. 24-31.
117. Banati, R. and I.B. Hickie, *Therapeutic signposts: using biomarkers to guide better treatment of schizophrenia and other psychotic disorders.* Med J Aust, 2009. **190**(4 Suppl): p. S26-32.
118. Doorduin, J., et al., *Neuroinflammation in schizophrenia-related psychosis: a PET study.* J Nucl Med, 2009. **50**(11): p. 1801-7.
119. Suzuki, K., et al., *Microglial activation in young adults with autism spectrum disorder.* JAMA Psychiatry, 2013. **70**(1): p. 49-58.
120. van Berckel, B.N., et al., *Microglia activation in recent-onset schizophrenia: a quantitative (R)-[11C]PK11195 positron emission tomography study.* Biol Psychiatry, 2008. **64**(9): p. 820-2.
121. Brown, A.S., *Epidemiologic studies of exposure to prenatal infection and risk of schizophrenia and autism.* Dev Neurobiol, 2012. **72**(10): p. 1272-6.
122. Brown, A.S., et al., *Serologic evidence of prenatal influenza in the etiology of schizophrenia.* Arch Gen Psychiatry, 2004. **61**(8): p. 774-80.

123. Brown, A.S., et al., *Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring.* Am J Psychiatry, 2005. **162**(4): p. 767-73.
124. Brown, A.S., et al., *Elevated maternal interleukin-8 levels and risk of schizophrenia in adult offspring.* Am J Psychiatry, 2004. **161**(5): p. 889-95.
125. Buka, S.L., et al., *Maternal infections and subsequent psychosis among offspring.* Arch Gen Psychiatry, 2001. **58**(11): p. 1032-7.
126. Brown, A.S., et al., *A.E. Bennett Research Award. Prenatal rubella, premorbid abnormalities, and adult schizophrenia.* Biol Psychiatry, 2001. **49**(6): p. 473-86.
127. Babulas, V., et al., *Prenatal exposure to maternal genital and reproductive infections and adult schizophrenia.* Am J Psychiatry, 2006. **163**(5): p. 927-9.
128. Brown, A.S., et al., *Maternal exposure to respiratory infections and adult schizophrenia spectrum disorders: a prospective birth cohort study.* Schizophr Bull, 2000. **26**(2): p. 287-95.
129. Sorensen, H.J., et al., *Association between prenatal exposure to bacterial infection and risk of schizophrenia.* Schizophr Bull, 2009. **35**(3): p. 631-7.
130. Atladottir, H.O., et al., *Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders.* J Autism Dev Disord, 2010. **40**(12): p. 1423-30.
131. Abdallah, M.W., et al., *Amniotic fluid inflammatory cytokines: Potential markers of immunologic dysfunction in autism spectrum disorders.* World J Biol Psychiatry, 2011.
132. Goines, P.E., et al., *Increased midgestational IFN-gamma, IL-4 and IL-5 in women bearing a child with autism: A case-control study.* Mol Autism, 2011. **2**: p. 13.
133. Fatemi, S.H., et al., *Differential expression of synaptosome-associated protein 25 kDa [SNAP-25] in hippocampi of neonatal mice following exposure to human influenza virus in utero.* Brain Res, 1998. **800**(1): p. 1-9.

134. Kneeland, R.E. and S.H. Fatemi, *Viral infection, inflammation and schizophrenia.* Prog Neuropsychopharmacol Biol Psychiatry, 2013. **42**: p. 35-48.
135. Boksa, P., *Effects of prenatal infection on brain development and behavior: a review of findings from animal models.* Brain Behav Immun, 2010. **24**(6): p. 881-97.
136. Meyer, U., J. Feldon, and S.H. Fatemi, *In-vivo rodent models for the experimental investigation of prenatal immune activation effects in neurodevelopmental brain disorders.* Neurosci Biobehav Rev, 2009. **33**(7): p. 1061-79.
137. Venkatasubramanian, G. and M. Debnath, *The TRIPS (Toll-like receptors in immuno-inflammatory pathogenesis) Hypothesis: a novel postulate to understand schizophrenia.* Prog Neuropsychopharmacol Biol Psychiatry, 2013. **44**: p. 301-11.
138. Hsiao, E.Y. and P.H. Patterson, *Placental regulation of maternal-fetal interactions and brain development.* Dev Neurobiol, 2012. **72**(10): p. 1317-26.
139. Meyer, U., *Prenatal Poly(I:C) Exposure and Other Developmental Immune Activation Models in Rodent Systems.* Biol Psychiatry, 2013.
140. Smith, S.E., et al., *Maternal immune activation alters fetal brain development through interleukin-6.* J Neurosci, 2007. **27**(40): p. 10695-702.
141. Bergeron, J., et al., *Activation of the IL-1beta/CXCL1/MMP-10 axis in chorioamnionitis induced by inactivated Group B Streptococcus.* Placenta, 2016. **47**: p. 116-123.
142. Cloarec, R., et al., *Cytomegalovirus Infection of the Rat Developing Brain In Utero Prominently Targets Immune Cells and Promotes Early Microglial Activation.* PLoS One, 2016. **11**(7): p. e0160176.
143. Kazlauskas, N., et al., *Postnatal behavioral and inflammatory alterations in female pups prenatally exposed to valproic acid.* Psychoneuroendocrinology, 2016. **72**: p. 11-21.
144. Maturana, C.J., A. Aguirre, and J.C. Saez, *High glucocorticoid levels during gestation activate the inflammasome in hippocampal oligodendrocytes of the offspring.* Dev Neurobiol, 2016.

145. Schwartzer, J.J., et al., *Behavioral impact of maternal allergic-asthma in two genetically distinct mouse strains.* Brain Behav Immun, 2016.
146. Terasaki, L.S. and J.M. Schwarz, *Effects of Moderate Prenatal Alcohol Exposure during Early Gestation in Rats on Inflammation across the Maternal-Fetal-Immune Interface and Later-Life Immune Function in the Offspring.* J Neuroimmune Pharmacol, 2016. **11**(4): p. 680-692.
147. Wong, A.H. and S.A. Josselyn, *Caution When Diagnosing Your Mouse With Schizophrenia: The Use and Misuse of Model Animals for Understanding Psychiatric Disorders.* Biol Psychiatry, 2016. **79**(1): p. 32-8.
148. Meyer, U., et al., *Adult brain and behavioral pathological markers of prenatal immune challenge during early/middle and late fetal development in mice.* Brain Behav Immun, 2008. **22**(4): p. 469-86.
149. Hadar, R., et al., *Using a maternal immune stimulation model of schizophrenia to study behavioral and neurobiological alterations over the developmental course.* Schizophr Res, 2015. **166**(1-3): p. 238-47.
150. Han, M., et al., *Intake of 7,8-Dihydroxyflavone During Juvenile and Adolescent Stages Prevents Onset of Psychosis in Adult Offspring After Maternal Immune Activation.* Sci Rep, 2016. **6**: p. 36087.
151. Batinic, B., et al., *Lipopolysaccharide exposure during late embryogenesis results in diminished locomotor activity and amphetamine response in females and spatial cognition impairment in males in adult, but not adolescent rat offspring.* Behav Brain Res, 2016. **299**: p. 72-80.
152. Borcoi, A.R., et al., *Effects of prenatal immune activation on amphetamine-induced addictive behaviors: Contributions from animal models.* Prog Neuropsychopharmacol Biol Psychiatry, 2015. **63**: p. 63-9.
153. Buschert, J., et al., *Prenatal immune activation in mice blocks the effects of environmental enrichment on exploratory behavior and microglia density.* Prog Neuropsychopharmacol Biol Psychiatry, 2016. **67**: p. 10-20.
154. Straley, M.E., et al., *Distinct alterations in motor & reward seeking behavior are dependent on the gestational age of exposure to LPS-induced maternal immune activation.* Brain Behav Immun, 2016.

155. Vorhees, C.V., et al., *Prenatal immune challenge in rats: altered responses to dopaminergic and glutamatergic agents, prepulse inhibition of acoustic startle, and reduced route-based learning as a function of maternal body weight gain after prenatal exposure to poly IC.* Synapse, 2012. **66**(8): p. 725-37.
156. Meehan, C., et al., *Effects of immune activation during early or late gestation on schizophrenia-related behaviour in adult rat offspring.* Brain Behav Immun, 2016.
157. Richetto, J., et al., *Prenatal immune activation induces maturation-dependent alterations in the prefrontal GABAergic transcriptome.* Schizophr Bull, 2014. **40**(2): p. 351-61.
158. Waterhouse, U., et al., *Nicotine ameliorates schizophrenia-like cognitive deficits induced by maternal LPS exposure: a study in rats.* Dis Model Mech, 2016. **9**(10): p. 1159-1167.
159. Fernandez de Cossio, L., et al., *Prenatal infection leads to ASD-like behavior and altered synaptic pruning in the mouse offspring.* Brain Behav Immun, 2016.
160. Wu, W.L., et al., *The placental interleukin-6 signaling controls fetal brain development and behavior.* Brain Behav Immun, 2016.
161. Zhu, F., et al., *Minocycline alleviates behavioral deficits and inhibits microglial activation in the offspring of pregnant mice after administration of polyriboinosinic-polyribocytidilic acid.* Psychiatry Res, 2014. **219**(3): p. 680-6.
162. Coiro, P., et al., *Impaired synaptic development in a maternal immune activation mouse model of neurodevelopmental disorders.* Brain Behav Immun, 2015. **50**: p. 249-58.
163. Onore, C.E., et al., *Maternal immune activation leads to activated inflammatory macrophages in offspring.* Brain Behav Immun, 2014. **38**: p. 220-6.
164. Fatemi, S.H., et al., *Prenatal viral infection leads to pyramidal cell atrophy and macrocephaly in adulthood: implications for genesis of autism and schizophrenia.* Cell Mol Neurobiol, 2002. **22**(1): p. 25-33.

165. Fatemi, S.H., et al., *Defective corticogenesis and reduction in Reelin immunoreactivity in cortex and hippocampus of prenatally infected neonatal mice.* Mol Psychiatry, 1999. **4**(2): p. 145-54.
166. Li, Q., et al., *Prenatal immune challenge is an environmental risk factor for brain and behavior change relevant to schizophrenia: evidence from MRI in a mouse model.* PLoS One, 2009. **4**(7): p. e6354.
167. Le Belle, J.E., et al., *Maternal inflammation contributes to brain overgrowth and autism-associated behaviors through altered redox signaling in stem and progenitor cells.* Stem Cell Reports, 2014. **3**(5): p. 725-34.
168. Fatemi, S.H., et al., *Maternal infection leads to abnormal gene regulation and brain atrophy in mouse offspring: implications for genesis of neurodevelopmental disorders.* Schizophr Res, 2008. **99**(1-3): p. 56-70.
169. Kumral, A., et al., *Erythropoietin attenuates lipopolysaccharide-induced white matter injury in the neonatal rat brain.* Neonatology, 2007. **92**(4): p. 269-78.
170. Paintlia, M.K., et al., *N-acetylcysteine prevents endotoxin-induced degeneration of oligodendrocyte progenitors and hypomyelination in developing rat brain.* J Neurosci Res, 2004. **78**(3): p. 347-61.
171. Paintlia, M.K., et al., *Modulation of peroxisome proliferator-activated receptor-alpha activity by N-acetyl cysteine attenuates inhibition of oligodendrocyte development in lipopolysaccharide stimulated mixed glial cultures.* J Neurochem, 2008. **105**(3): p. 956-70.
172. Rousset, C.I., et al., *Maternal exposure to LPS induces hypomyelination in the internal capsule and programmed cell death in the deep gray matter in newborn rats.* Pediatr Res, 2006. **59**(3): p. 428-33.
173. Yesilirmak, D.C., et al., *Activated protein C reduces endotoxin-induced white matter injury in the developing rat brain.* Brain Res, 2007. **1164**: p. 14-23.
174. Farrelly, L., et al., *Maternal immune activation induces changes in myelin and metabolic proteins, some of which can be prevented with risperidone in adolescence.* Dev Neurosci, 2015. **37**(1): p. 43-55.

175. Richetto, J., et al., *Genome-Wide Transcriptional Profiling and Structural Magnetic Resonance Imaging in the Maternal Immune Activation Model of Neurodevelopmental Disorders.* Cereb Cortex, 2016.
176. Baharnoori, M., W.G. Brake, and L.K. Srivastava, *Prenatal immune challenge induces developmental changes in the morphology of pyramidal neurons of the prefrontal cortex and hippocampus in rats.* Schizophr Res, 2009. **107**(1): p. 99-109.
177. Li, W.Y., et al., *Prenatal infection affects the neuronal architecture and cognitive function in adult mice.* Dev Neurosci, 2014. **36**(5): p. 359-70.
178. Paylor, J.W., et al., *Developmental disruption of perineuronal nets in the medial prefrontal cortex after maternal immune activation.* Sci Rep, 2016. **6**: p. 37580.
179. Cui, K., et al., *Effects of prenatal immune activation on hippocampal neurogenesis in the rat.* Schizophr Res, 2009. **113**(2-3): p. 288-97.
180. Liu, Y.H., et al., *Effects of maternal immune activation on adult neurogenesis in the subventricular zone-olfactory bulb pathway and olfactory discrimination.* Schizophr Res, 2013. **151**(1-3): p. 1-11.
181. Meyer, U., et al., *The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology.* J Neurosci, 2006. **26**(18): p. 4752-62.
182. Mouihate, A., *Prenatal Activation of Toll-Like Receptor-4 Dampens Adult Hippocampal Neurogenesis in An IL-6 Dependent Manner.* Front Cell Neurosci, 2016. **10**: p. 173.
183. Giovanoli, S., et al., *Prenatal immune activation causes hippocampal synaptic deficits in the absence of overt microglia anomalies.* Brain Behav Immun, 2016. **55**: p. 25-38.
184. Gyorffy, B.A., et al., *Widespread alterations in the synaptic proteome of the adolescent cerebral cortex following prenatal immune activation in rats.* Brain Behav Immun, 2016. **56**: p. 289-309.
185. Ducharme, G., et al., *Early alterations in hippocampal circuitry and theta rhythm generation in a mouse model of prenatal infection: implications for schizophrenia.* PLoS One, 2012. **7**(1): p. e29754.
186. Savanthrapadian, S., et al., *Enhanced hippocampal neuronal excitability and LTP persistence associated with reduced behavioral flexibility in the*

maternal immune activation model of schizophrenia. Hippocampus, 2013. **23**(12): p. 1395-409.

187. Dickerson, D.D., A.R. Wolff, and D.K. Bilkey, *Abnormal long-range neural synchrony in a maternal immune activation animal model of schizophrenia.* J Neurosci, 2010. **30**(37): p. 12424-31.
188. Oh-Nishi, A., et al., *Maternal immune activation by polyriboinosinic-polyribocytidilic acid injection produces synaptic dysfunction but not neuronal loss in the hippocampus of juvenile rat offspring.* Brain Res, 2010. **1363**: p. 170-9.
189. Lau, C.I., et al., *Does the dopamine hypothesis explain schizophrenia?* Rev Neurosci, 2013. **24**(4): p. 389-400.
190. Bakos, J., et al., *Prenatal immune challenge affects growth, behavior, and brain dopamine in offspring.* Ann N Y Acad Sci, 2004. **1018**: p. 281-7.
191. Bitanihirwe, B.K., et al., *Late prenatal immune activation in mice leads to behavioral and neurochemical abnormalities relevant to the negative symptoms of schizophrenia.* Neuropsychopharmacology, 2010. **35**(12): p. 2462-78.
192. Landreau, F., et al., *Effects of two commonly found strains of influenza A virus on developing dopaminergic neurons, in relation to the pathophysiology of schizophrenia.* PLoS One, 2012. **7**(12): p. e51068.
193. Ling, Z., et al., *Rotenone potentiates dopamine neuron loss in animals exposed to lipopolysaccharide prenatally.* Exp Neurol, 2004. **190**(2): p. 373-83.
194. Ling, Z., et al., *In utero bacterial endotoxin exposure causes loss of tyrosine hydroxylase neurons in the postnatal rat midbrain.* Mov Disord, 2002. **17**(1): p. 116-24.
195. Ling, Z., et al., *Progressive dopamine neuron loss following supra-nigral lipopolysaccharide (LPS) infusion into rats exposed to LPS prenatally.* Exp Neurol, 2006. **199**(2): p. 499-512.
196. Ling, Z., et al., *Prenatal lipopolysaccharide does not accelerate progressive dopamine neuron loss in the rat as a result of normal aging.* Exp Neurol, 2009. **216**(2): p. 312-20.

197. Romero, E., et al., *Ontogeny of sensorimotor gating and immune impairment induced by prenatal immune challenge in rats: implications for the etiopathology of schizophrenia.* Mol Psychiatry, 2010. **15**(4): p. 372-83.
198. Snyder-Keller, A. and P.F. Stark, *Prenatal inflammatory effects on nigrostriatal development in organotypic cultures.* Brain Res, 2008. **1233**: p. 160-7.
199. Wang, S., et al., *Dopaminergic and serotoninergic deficiencies in young adult rats prenatally exposed to the bacterial lipopolysaccharide.* Brain Res, 2009. **1265**: p. 196-204.
200. Borrell, J., et al., *Prenatal immune challenge disrupts sensorimotor gating in adult rats. Implications for the etiopathogenesis of schizophrenia.* Neuropsychopharmacology, 2002. **26**(2): p. 204-15.
201. Luchicchi, A., et al., *Maternal Immune Activation Disrupts Dopamine System in the Offspring.* Int J Neuropsychopharmacol, 2016. **19**(7).
202. Romero, E., et al., *Neurobehavioral and immunological consequences of prenatal immune activation in rats. Influence of antipsychotics.* Neuropsychopharmacology, 2007. **32**(8): p. 1791-804.
203. Winter, C., et al., *Prenatal immune activation leads to multiple changes in basal neurotransmitter levels in the adult brain: implications for brain disorders of neurodevelopmental origin such as schizophrenia.* Int J Neuropsychopharmacol, 2009. **12**(4): p. 513-24.
204. Baharnoori, M., S.K. Bhardwaj, and L.K. Srivastava, *Effect of maternal lipopolysaccharide administration on the development of dopaminergic receptors and transporter in the rat offspring.* PLoS One, 2013. **8**(1): p. e54439.
205. Ozawa, K., et al., *Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia.* Biol Psychiatry, 2006. **59**(6): p. 546-54.
206. Canetta, S., et al., *Maternal immune activation leads to selective functional deficits in offspring parvalbumin interneurons.* Mol Psychiatry, 2016. **21**(7): p. 956-68.

207. Cassella, S.N., et al., *Maternal immune activation alters glutamic acid decarboxylase-67 expression in the brains of adult rat offspring.* Schizophr Res, 2016. **171**(1-3): p. 195-9.
208. Nouel, D., et al., *Prenatal exposure to bacterial endotoxin reduces the number of GAD67- and reelin-immunoreactive neurons in the hippocampus of rat offspring.* Eur Neuropsychopharmacol, 2012. **22**(4): p. 300-7.
209. Oskvig, D.B., et al., *Maternal immune activation by LPS selectively alters specific gene expression profiles of interneuron migration and oxidative stress in the fetus without triggering a fetal immune response.* Brain Behav Immun, 2012. **26**(4): p. 623-34.
210. Javitt, D.C., *Glutamatergic theories of schizophrenia.* Isr J Psychiatry Relat Sci, 2010. **47**(1): p. 4-16.
211. Pratt, L., et al., *Maternal inflammation promotes fetal microglial activation and increased cholinergic expression in the fetal basal forebrain: role of interleukin-6.* Pediatr Res, 2013. **74**(4): p. 393-401.
212. Depino, A.M., *Early prenatal exposure to LPS results in anxiety- and depression-related behaviors in adulthood.* Neuroscience, 2015. **299**: p. 56-65.
213. Winter, C., et al., *Dopamine and serotonin levels following prenatal viral infection in mouse--implications for psychiatric disorders such as schizophrenia and autism.* Eur Neuropsychopharmacol, 2008. **18**(10): p. 712-6.
214. Khan, D., et al., *Long-term effects of maternal immune activation on depression-like behavior in the mouse.* Transl Psychiatry, 2014. **4**: p. e363.
215. Wischhof, L., et al., *Maternal lipopolysaccharide treatment differentially affects 5-HT(2A) and mGlu2/3 receptor function in the adult male and female rat offspring.* Neuropharmacology, 2015. **97**: p. 275-88.
216. Lante, F., et al., *Neurodevelopmental damage after prenatal infection: role of oxidative stress in the fetal brain.* Free Radic Biol Med, 2007. **42**(8): p. 1231-45.

217. Roumier, A., et al., *Prenatal activation of microglia induces delayed impairment of glutamatergic synaptic function.* PLoS One, 2008. **3**(7): p. e2595.
218. Ashdown, H., et al., *The role of cytokines in mediating effects of prenatal infection on the fetus: implications for schizophrenia.* Mol Psychiatry, 2006. **11**(1): p. 47-55.
219. Dahlgren, J., et al., *Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation.* Pediatr Res, 2006. **60**(2): p. 147-51.
220. Kohmura, Y., et al., *Lipopolysaccharide (LPS)-induced intra-uterine fetal death (IUFD) in mice is principally due to maternal cause but not fetal sensitivity to LPS.* Microbiol Immunol, 2000. **44**(11): p. 897-904.
221. Hsiao, E.Y. and P.H. Patterson, *Activation of the maternal immune system induces endocrine changes in the placenta via IL-6.* Brain Behav Immun, 2011. **25**(4): p. 604-15.
222. Cai, Z., et al., *Cytokine induction in fetal rat brains and brain injury in neonatal rats after maternal lipopolysaccharide administration.* Pediatr Res, 2000. **47**(1): p. 64-72.
223. Liverman, C.S., et al., *Altered expression of pro-inflammatory and developmental genes in the fetal brain in a mouse model of maternal infection.* Neurosci Lett, 2006. **399**(3): p. 220-5.
224. Garay, P.A., et al., *Maternal immune activation causes age- and region-specific changes in brain cytokines in offspring throughout development.* Brain Behav Immun, 2013. **31**: p. 54-68.
225. Juckel, G., et al., *Microglial activation in a neuroinflammational animal model of schizophrenia--a pilot study.* Schizophr Res, 2011. **131**(1-3): p. 96-100.
226. Manitz, M.P., et al., *The role of microglia during life span in neuropsychiatric disease--an animal study.* Schizophr Res, 2013. **143**(1): p. 221-2.
227. Missault, S., et al., *The risk for behavioural deficits is determined by the maternal immune response to prenatal immune challenge in a neurodevelopmental model.* Brain Behav Immun, 2014. **42**: p. 138-46.

228. Basil, P., et al., *Prenatal maternal immune activation causes epigenetic differences in adolescent mouse brain.* Transl Psychiatry, 2014. **4**: p. e434.
229. Tang, B., et al., *Epigenetic changes at gene promoters in response to immune activation in utero.* Brain Behav Immun, 2013. **30**: p. 168-75.
230. Labouesse, M.A., et al., *Maternal immune activation induces GAD1 and GAD2 promoter remodeling in the offspring prefrontal cortex.* Epigenetics, 2015. **10**(12): p. 1143-55.
231. Reisinger, S.N., et al., *Maternal immune activation epigenetically regulates hippocampal serotonin transporter levels.* Neurobiol Stress, 2016. **4**: p. 34-43.
232. Richetto, J., et al., *Genome-wide DNA Methylation Changes in a Mouse Model of Infection-Mediated Neurodevelopmental Disorders.* Biol Psychiatry, 2016.
233. Bronson, S.L. and T.L. Bale, *The Placenta as a Mediator of Stress Effects on Neurodevelopmental Reprogramming.* Neuropsychopharmacology, 2016. **41**(1): p. 207-18.
234. Girard, S., et al., *IL-1 receptor antagonist protects against placental and neurodevelopmental defects induced by maternal inflammation.* J Immunol, 2010. **184**(7): p. 3997-4005.
235. Bronson, S.L. and T.L. Bale, *Prenatal stress-induced increases in placental inflammation and offspring hyperactivity are male-specific and ameliorated by maternal antiinflammatory treatment.* Endocrinology, 2014. **155**(7): p. 2635-46.
236. Tsukada, T., et al., *The suppression of maternal-fetal leukemia inhibitory factor signal relay pathway by maternal immune activation impairs brain development in mice.* PLoS One, 2015. **10**(6): p. e0129011.
237. Goeden, N., et al., *Maternal Inflammation Disrupts Fetal Neurodevelopment via Increased Placental Output of Serotonin to the Fetal Brain.* J Neurosci, 2016. **36**(22): p. 6041-9.
238. Semple, B.D., et al., *Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species.* Prog Neurobiol, 2013. **106-107**: p. 1-16.

239. Furukawa, S., Y. Kuroda, and A. Sugiyama, *A comparison of the histological structure of the placenta in experimental animals.* J Toxicol Pathol, 2014. **27**(1): p. 11-8.
240. Bauman, M.D., et al., *Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring.* Biol Psychiatry, 2014. **75**(4): p. 332-41.
241. Short, S.J., et al., *Maternal influenza infection during pregnancy impacts postnatal brain development in the rhesus monkey.* Biol Psychiatry, 2010. **67**(10): p. 965-73.
242. Willette, A.A., et al., *Brain enlargement and increased behavioral and cytokine reactivity in infant monkeys following acute prenatal endotoxemia.* Behav Brain Res, 2011. **219**(1): p. 108-15.
243. Machado, C.J., et al., *Maternal immune activation in nonhuman primates alters social attention in juvenile offspring.* Biol Psychiatry, 2015. **77**(9): p. 823-32.
244. Weir, R.K., et al., *Preliminary evidence of neuropathology in nonhuman primates prenatally exposed to maternal immune activation.* Brain Behav Immun, 2015. **48**: p. 139-46.
245. Rose, D.R., et al., *Long-term altered immune responses following fetal priming in a non-human primate model of maternal immune activation.* Brain Behav Immun, 2016.
246. Antonson, A.M., et al., *Maternal viral infection during pregnancy elicits anti-social behavior in neonatal piglet offspring independent of postnatal microglial cell activation.* Brain Behav Immun, 2017. **59**: p. 300-312.
247. Pineda, E., et al., *Maternal immune activation promotes hippocampal kindling epileptogenesis in mice.* Ann Neurol, 2013. **74**(1): p. 11-9.
248. Washington, J., 3rd, et al., *Cytokine-dependent bidirectional connection between impaired social behavior and susceptibility to seizures associated with maternal immune activation in mice.* Epilepsy Behav, 2015. **50**: p. 40-5.
249. Yin, P., et al., *Maternal immune activation increases seizure susceptibility in juvenile rat offspring.* Epilepsy Behav, 2015. **47**: p. 93-7.

250. Hsiao, E.Y., et al., *Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders.* Cell, 2013. **155**(7): p. 1451-63.
251. Rao, M. and M.D. Gershon, *The bowel and beyond: the enteric nervous system in neurological disorders.* Nat Rev Gastroenterol Hepatol, 2016. **13**(9): p. 517-28.
252. Ibi, D., et al., *Combined effect of neonatal immune activation and mutant DISC1 on phenotypic changes in adulthood.* Behav Brain Res, 2010. **206**(1): p. 32-7.
253. Nagai, T., et al., *Effects of antipsychotics on the behavioral deficits in human dominant-negative DISC1 transgenic mice with neonatal polyI:C treatment.* Behav Brain Res, 2011. **225**(1): p. 305-10.
254. Abazyan, B., et al., *Prenatal interaction of mutant DISC1 and immune activation produces adult psychopathology.* Biol Psychiatry, 2010. **68**(12): p. 1172-81.
255. Lipina, T.V., et al., *Maternal immune activation during gestation interacts with Disc1 point mutation to exacerbate schizophrenia-related behaviors in mice.* J Neurosci, 2013. **33**(18): p. 7654-66.
256. Ehninger, D., et al., *Gestational immune activation and Tsc2 haploinsufficiency cooperate to disrupt fetal survival and may perturb social behavior in adult mice.* Mol Psychiatry, 2012. **17**(1): p. 62-70.
257. Vuillermot, S., et al., *Prenatal immune activation interacts with genetic Nurr1 deficiency in the development of attentional impairments.* J Neurosci, 2012. **32**(2): p. 436-51.
258. Wu, W.L., et al., *The interaction between maternal immune activation and alpha 7 nicotinic acetylcholine receptor in regulating behaviors in the offspring.* Brain Behav Immun, 2015. **46**: p. 192-202.
259. Giovanoli, S., et al., *Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice.* Science, 2013. **339**(6123): p. 1095-9.
260. Yee, N., et al., *Differential effects of maternal immune activation and juvenile stress on anxiety-like behaviour and physiology in adult rats: no evidence for the "double-hit hypothesis".* Behav Brain Res, 2011. **224**(1): p. 180-8.

261. Mattei, D., et al., *Minocycline rescues decrease in neurogenesis, increase in microglia cytokines and deficits in sensorimotor gating in an animal model of schizophrenia.* Brain Behav Immun, 2014. **38**: p. 175-84.
262. Giovanoli, S., et al., *Preventive effects of minocycline in a neurodevelopmental two-hit model with relevance to schizophrenia.* Transl Psychiatry, 2016. **6**: p. e772.
263. Li, Q., et al., *Dietary supplementation with n-3 fatty acids from weaning limits brain biochemistry and behavioural changes elicited by prenatal exposure to maternal inflammation in the mouse model.* Transl Psychiatry, 2015. **5**: p. e641.
264. Weiser, M.J., et al., *Dietary docosahexaenoic acid alleviates autistic-like behaviors resulting from maternal immune activation in mice.* Prostaglandins Leukot Essent Fatty Acids, 2016. **106**: p. 27-37.
265. Tronnes, A.A., et al., *Effects of Lipopolysaccharide and Progesterone Exposures on Embryonic Cerebral Cortex Development in Mice.* Reprod Sci, 2016. **23**(6): p. 771-8.
266. Fujita, Y., T. Ishima, and K. Hashimoto, *Supplementation with D-serine prevents the onset of cognitive deficits in adult offspring after maternal immune activation.* Sci Rep, 2016. **6**: p. 37261.
267. Naviaux, R.K., et al., *Antipurinergic therapy corrects the autism-like features in the poly(IC) mouse model.* PLoS One, 2013. **8**(3): p. e57380.
268. Soares, D.C., et al., *DISC1: Structure, Function, and Therapeutic Potential for Major Mental Illness.* ACS Chem Neurosci, 2011. **2**(11): p. 609-632.
269. Hikida, T., N.J. Gamo, and A. Sawa, *DISC1 as a therapeutic target for mental illnesses.* Expert Opin Ther Targets, 2012. **16**(12): p. 1151-60.
270. Brandon, N.J. and A. Sawa, *Linking neurodevelopmental and synaptic theories of mental illness through DISC1.* Nat Rev Neurosci, 2011. **12**(12): p. 707-22.
271. Lipina, T.V. and J.C. Roder, *Disrupted-In-Schizophrenia-1 (DISC1) interactome and mental disorders: impact of mouse models.* Neurosci Biobehav Rev, 2014. **45**: p. 271-94.

272. Wood, J.D., et al., *Disrupted-in-schizophrenia 1 and neuregulin 1 are required for the specification of oligodendrocytes and neurones in the zebrafish brain.* Hum Mol Genet, 2009. **18**(3): p. 391-404.
273. Seshadri, S., et al., *Disrupted-in-Schizophrenia-1 expression is regulated by beta-site amyloid precursor protein cleaving enzyme-1-neuregulin cascade.* Proc Natl Acad Sci U S A, 2010. **107**(12): p. 5622-7.
274. Katsel, P., et al., *Expression of mutant human DISC1 in mice supports abnormalities in differentiation of oligodendrocytes.* Schizophr Res, 2011. **130**(1-3): p. 238-49.
275. Hattori, T., et al., *DISC1 (disrupted-in-schizophrenia-1) regulates differentiation of oligodendrocytes.* PLoS One, 2014. **9**(2): p. e88506.
276. Ma, T.M., et al., *Pathogenic disruption of DISC1-serine racemase binding elicits schizophrenia-like behavior via D-serine depletion.* Mol Psychiatry, 2013. **18**(5): p. 557-67.
277. Abazyan, S., et al., *Mutant disrupted-in-schizophrenia 1 in astrocytes: focus on glutamate metabolism.* J Neurosci Res, 2014. **92**(12): p. 1659-68.
278. Xia, M., et al., *DISC1, astrocytes and neuronal maturation: a possible mechanistic link with implications for mental disorders.* J Neurochem, 2016. **138**(4): p. 518-24.
279. Krady, J.K., et al., *Ciliary neurotrophic factor and interleukin-6 differentially activate microglia.* J Neurosci Res, 2008. **86**(7): p. 1538-47.
280. Streit, W.J., et al., *Comparative evaluation of cytokine profiles and reactive gliosis supports a critical role for interleukin-6 in neuron-glia signaling during regeneration.* J Neurosci Res, 2000. **61**(1): p. 10-20.
281. Lacroix, S., et al., *Delivery of hyper-interleukin-6 to the injured spinal cord increases neutrophil and macrophage infiltration and inhibits axonal growth.* J Comp Neurol, 2002. **454**(3): p. 213-28.
282. Lee, K.M., S.M. Jeon, and H.J. Cho, *Interleukin-6 induces microglial CX3CR1 expression in the spinal cord after peripheral nerve injury through the activation of p38 MAPK.* Eur J Pain, 2010. **14**(7): p. 682 e1-12.

283. Jung, S., et al., *Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion.* Mol Cell Biol, 2000. **20**(11): p. 4106-14.
284. Shi, L., et al., *Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring.* J Neurosci, 2003. **23**(1): p. 297-302.
285. Rigato, C., et al., *Pattern of invasion of the embryonic mouse spinal cord by microglial cells at the time of the onset of functional neuronal networks.* Glia, 2011. **59**(4): p. 675-95.
286. © 2008 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas. E11.5, E12.5 and E17.5 coronal sections. Available from: http://developingmouse.brain-map.org/static/atlas.
287. Ratnayake, U., et al., *Behaviour and hippocampus-specific changes in spiny mouse neonates after treatment of the mother with the viral-mimetic Poly I:C at mid-pregnancy.* Brain Behav Immun, 2012. **26**(8): p. 1288-99.
288. Dumic, J., S. Dabelic, and M. Flogel, *Galectin-3: an open-ended story.* Biochim Biophys Acta, 2006. **1760**(4): p. 616-35.
289. Rotshenker, S., *The role of Galectin-3/MAC-2 in the activation of the innate-immune function of phagocytosis in microglia in injury and disease.* J Mol Neurosci, 2009. **39**(1-2): p. 99-103.
290. Hutton, L.C., et al., *Microglial activation, macrophage infiltration, and evidence of cell death in the fetal brain after uteroplacental administration of lipopolysaccharide in sheep in late gestation.* Am J Obstet Gynecol, 2008. **198**(1): p. 117 e1-11.
291. Kuypers, E., et al., *Effects of intra-amniotic lipopolysaccharide and maternal betamethasone on brain inflammation in fetal sheep.* PLoS One, 2013. **8**(12): p. e81644.
292. Mallard, C., et al., *White matter injury following systemic endotoxemia or asphyxia in the fetal sheep.* Neurochem Res, 2003. **28**(2): p. 215-23.
293. Monji, A., et al., *Neuroinflammation in schizophrenia especially focused on the role of microglia.* Prog Neuropsychopharmacol Biol Psychiatry, 2013. **42**: p. 115-21.

294. Patterson, P.H., *Immune involvement in schizophrenia and autism: etiology, pathology and animal models.* Behav Brain Res, 2009. **204**(2): p. 313-21.
295. Jonakait, G.M., *The effects of maternal inflammation on neuronal development: possible mechanisms.* Int J Dev Neurosci, 2007. **25**(7): p. 415-25.
296. Gayle, D.A., et al., *Maternal LPS induces cytokines in the amniotic fluid and corticotropin releasing hormone in the fetal rat brain.* Am J Physiol Regul Integr Comp Physiol, 2004. **286**(6): p. R1024-9.
297. Bauer, S., B.J. Kerr, and P.H. Patterson, *The neuropoietic cytokine family in development, plasticity, disease and injury.* Nat Rev Neurosci, 2007. **8**(3): p. 221-32.
298. Deverman, B.E. and P.H. Patterson, *Cytokines and CNS development.* Neuron, 2009. **64**(1): p. 61-78.
299. Nakanishi, M., et al., *Microglia-derived interleukin-6 and leukaemia inhibitory factor promote astrocytic differentiation of neural stem/progenitor cells.* Eur J Neurosci, 2007. **25**(3): p. 649-58.
300. Holloway, T., et al., *Prenatal stress induces schizophrenia-like alterations of serotonin 2A and metabotropic glutamate 2 receptors in the adult offspring: role of maternal immune system.* J Neurosci, 2013. **33**(3): p. 1088-98.
301. Milior, G., et al., *Fractalkine receptor deficiency impairs microglial and neuronal responsiveness to chronic stress.* Brain Behav Immun, 2016. **55**: p. 114-25.
302. Rimmerman, N., et al., *The hippocampal transcriptomic signature of stress resilience in mice with microglial fractalkine receptor (CX3CR1) deficiency.* Brain Behav Immun, 2017. **61**: p. 184-196.
303. Reshef, R., et al., *Microglia and their CX3CR1 signaling are involved in hippocampal- but not olfactory bulb-related memory and neurogenesis.* Brain Behav Immun, 2014. **41**: p. 239-50.
304. Zabel, M.K., et al., *Microglial phagocytosis and activation underlying photoreceptor degeneration is regulated by CX3CL1-CX3CR1 signaling in a mouse model of retinitis pigmentosa.* Glia, 2016. **64**(9): p. 1479-91.

305. Lee, S., et al., *CX3CR1 deficiency alters microglial activation and reduces beta-amyloid deposition in two Alzheimer's disease mouse models.* Am J Pathol, 2010. **177**(5): p. 2549-62.
306. Wieghofer, P., K.P. Knobeloch, and M. Prinz, *Genetic targeting of microglia.* Glia, 2015. **63**(1): p. 1-22.
307. Heppner, F.L., et al., *Experimental autoimmune encephalomyelitis repressed by microglial paralysis.* Nat Med, 2005. **11**(2): p. 146-52.
308. Grathwohl, S.A., et al., *Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia.* Nat Neurosci, 2009. **12**(11): p. 1361-3.
309. Yona, S., et al., *Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.* Immunity, 2013. **38**(1): p. 79-91.
310. Feil, S., N. Valtcheva, and R. Feil, *Inducible Cre mice.* Methods Mol Biol, 2009. **530**: p. 343-63.
311. Bruttger, J., et al., *Genetic Cell Ablation Reveals Clusters of Local Self-Renewing Microglia in the Mammalian Central Nervous System.* Immunity, 2015. **43**(1): p. 92-106.
312. Smith, A.M., et al., *The transcription factor PU.1 is critical for viability and function of human brain microglia.* Glia, 2013. **61**(6): p. 929-42.
313. Dakic, A., et al., *PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis.* J Exp Med, 2005. **201**(9): p. 1487-502.
314. Butovsky, O., et al., *Identification of a unique TGF-beta-dependent molecular and functional signature in microglia.* Nat Neurosci, 2014. **17**(1): p. 131-43.
315. Olson, M.C., et al., *PU. 1 is not essential for early myeloid gene expression but is required for terminal myeloid differentiation.* Immunity, 1995. **3**(6): p. 703-14.
316. McKercher, S.R., et al., *Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities.* EMBO J, 1996. **15**(20): p. 5647-58.

317. Beers, D.R., et al., *Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis.* Proc Natl Acad Sci U S A, 2006. **103**(43): p. 16021-6.
318. Schulz, C., et al., *A lineage of myeloid cells independent of Myb and hematopoietic stem cells.* Science, 2012. **336**(6077): p. 86-90.
319. Jego, G., et al., *Dual regulation of SPI1/PU.1 transcription factor by heat shock factor 1 (HSF1) during macrophage differentiation of monocytes.* Leukemia, 2014. **28**(8): p. 1676-86.
320. Kueh, H.Y., et al., *Positive feedback between PU.1 and the cell cycle controls myeloid differentiation.* Science, 2013. **341**(6146): p. 670-3.
321. Tay, T.L., N. Hagemeyer, and M. Prinz, *The force awakens: insights into the origin and formation of microglia.* Curr Opin Neurobiol, 2016. **39**: p. 30-7.
322. Bergon, A., et al., *CX3CR1 is dysregulated in blood and brain from schizophrenia patients.* Schizophr Res, 2015. **168**(1-2): p. 434-43.
323. Rogers, J.T., et al., *CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity.* J Neurosci, 2011. **31**(45): p. 16241-50.
324. Zhan, Y., et al., *Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior.* Nat Neurosci, 2014. **17**(3): p. 400-6.
325. Maggi, L., et al., *CX(3)CR1 deficiency alters hippocampal-dependent plasticity phenomena blunting the effects of enriched environment.* Front Cell Neurosci, 2011. **5**: p. 22.
326. Corona, A.W., et al., *Fractalkine receptor (CX3CR1) deficiency sensitizes mice to the behavioral changes induced by lipopolysaccharide.* J Neuroinflammation, 2010. **7**: p. 93.
327. Cunningham, C., et al., *The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I:C).* Brain Behav Immun, 2007. **21**(4): p. 490-502.
328. Gonzalez-Liencres, C., et al., *Emotional Contagion is not Altered in Mice Prenatally Exposed to Poly (I:C) on Gestational Day 9.* Front Behav Neurosci, 2016. **10**: p. 134.

329. Mouihate, A. and H. Mehdawi, *Toll-like receptor 4-mediated immune stress in pregnant rats activates STAT3 in the fetal brain: role of interleukin-6.* Pediatr Res, 2016. **79**(5): p. 781-7.
330. Stumm, R.K., et al., *CXCR4 regulates interneuron migration in the developing neocortex.* J Neurosci, 2003. **23**(12): p. 5123-30.
331. Tiveron, M.C., et al., *Molecular interaction between projection neuron precursors and invading interneurons via stromal-derived factor 1 (CXCL12)/CXCR4 signaling in the cortical subventricular zone/intermediate zone.* J Neurosci, 2006. **26**(51): p. 13273-8.
332. Islam, O., et al., *Interleukin-6 and neural stem cells: more than gliogenesis.* Mol Biol Cell, 2009. **20**(1): p. 188-99.
333. Valerio, A., et al., *Soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro differentiation of purified rat oligodendroglial lineage cells.* Mol Cell Neurosci, 2002. **21**(4): p. 602-15.
334. La Fata, G., et al., *FMRP regulates multipolar to bipolar transition affecting neuronal migration and cortical circuitry.* Nat Neurosci, 2014. **17**(12): p. 1693-700.
335. Kamiya, A., et al., *A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development.* Nat Cell Biol, 2005. **7**(12): p. 1167-78.
336. Steinecke, A., et al., *Disrupted-in-Schizophrenia 1 (DISC1) is necessary for the correct migration of cortical interneurons.* J Neurosci, 2012. **32**(2): p. 738-45.
337. Stolp, H.B., et al., *Reduced ventricular proliferation in the foetal cortex following maternal inflammation in the mouse.* Brain, 2011. **134**(Pt 11): p. 3236-48.
338. Charil, A., et al., *Prenatal stress and brain development.* Brain Res Rev, 2010. **65**(1): p. 56-79.
339. Fujioka, A., et al., *Differential effects of prenatal stress on the morphological maturation of hippocampal neurons.* Neuroscience, 2006. **141**(2): p. 907-15.
340. Tamura, M., et al., *Prenatal stress inhibits neuronal maturation through downregulation of mineralocorticoid receptors.* J Neurosci, 2011. **31**(32): p. 11505-14.

341. Palacios-Garcia, I., et al., *Prenatal stress down-regulates Reelin expression by methylation of its promoter and induces adult behavioral impairments in rats.* PLoS One, 2015. **10**(2): p. e0117680.
342. Huang, Y., et al., *Pre-gestational stress reduces the ratio of 5-HIAA to 5-HT and the expression of 5-HT1A receptor and serotonin transporter in the brain of foetal rat.* BMC Neurosci, 2012. **13**: p. 22.
343. Meyer, U., et al., *Towards an immuno-precipitated neurodevelopmental animal model of schizophrenia.* Neurosci Biobehav Rev, 2005. **29**(6): p. 913-47.
344. Cain, D.W. and J.A. Cidlowski, *Immune regulation by glucocorticoids.* Nat Rev Immunol, 2017.
345. Rugerio-Vargas, C., et al., *Prenatal corticosterone influences the trajectory of neuronal development, delaying or accelerating aspects of the Purkinje cell differentiation.* Histol Histopathol, 2007. **22**(9): p. 963-9.
346. Yu, I.T., et al., *Differential effects of corticosterone and dexamethasone on hippocampal neurogenesis in vitro.* Biochem Biophys Res Commun, 2004. **317**(2): p. 484-90.
347. Wang, S., et al., *Anesthesia-induced neurodegeneration in fetal rat brains.* Pediatr Res, 2009. **66**(4): p. 435-40.
348. Wei, H., et al., *Isoflurane and sevoflurane affect cell survival and BCL-2/BAX ratio differently.* Brain Res, 2005. **1037**(1-2): p. 139-47.
349. Garcia, P.S., S.E. Kolesky, and A. Jenkins, *General anesthetic actions on GABA(A) receptors.* Curr Neuropharmacol, 2010. **8**(1): p. 2-9.
350. Luhmann, H.J., A. Fukuda, and W. Kilb, *Control of cortical neuronal migration by glutamate and GABA.* Front Cell Neurosci, 2015. **9**: p. 4.
351. Cuffe, J.S., et al., *Maternal corticosterone exposure in the mouse has sex-specific effects on placental growth and mRNA expression.* Endocrinology, 2012. **153**(11): p. 5500-11.
352. Vaughan, O.R., A.N. Sferruzzi-Perri, and A.L. Fowden, *Maternal corticosterone regulates nutrient allocation to fetal growth in mice.* J Physiol, 2012. **590**(21): p. 5529-40.

353. Sachs, N.A., et al., *A frameshift mutation in Disrupted in Schizophrenia 1 in an American family with schizophrenia and schizoaffective disorder.* Mol Psychiatry, 2005. **10**(8): p. 758-64.
354. Crepel, A., et al., *DISC1 duplication in two brothers with autism and mild mental retardation.* Clin Genet, 2010. **77**(4): p. 389-94.
355. Williams, J.M., et al., *A 1q42 deletion involving DISC1, DISC2, and TSNAX in an autism spectrum disorder.* Am J Med Genet A, 2009. **149A**(8): p. 1758-62.
356. Song, W., et al., *Identification of high risk DISC1 structural variants with a 2% attributable risk for schizophrenia.* Biochem Biophys Res Commun, 2008. **367**(3): p. 700-6.
357. Song, W., et al., *Identification of high risk DISC1 protein structural variants in patients with bipolar spectrum disorder.* Neurosci Lett, 2010. **486**(3): p. 136-40.
358. Camargo, L.M., et al., *Disrupted in Schizophrenia 1 Interactome: evidence for the close connectivity of risk genes and a potential synaptic basis for schizophrenia.* Mol Psychiatry, 2007. **12**(1): p. 74-86.
359. Steinecke, A., et al., *DISC1 knockdown impairs the tangential migration of cortical interneurons by affecting the actin cytoskeleton.* Front Cell Neurosci, 2014. **8**: p. 190.
360. Uhlemann, R., et al., *Actin dynamics shape microglia effector functions.* Brain Struct Funct, 2016. **221**(5): p. 2717-34.
361. St Clair, D., et al., *Association within a family of a balanced autosomal translocation with major mental illness.* Lancet, 1990. **336**(8706): p. 13-6.
362. Tsuboi, D., et al., *Disrupted-in-schizophrenia 1 regulates transport of ITPR1 mRNA for synaptic plasticity.* Nat Neurosci, 2015. **18**(5): p. 698-707.
363. Zhang, X., J.P. Edwards, and D.M. Mosser, *The expression of exogenous genes in macrophages: obstacles and opportunities.* Methods Mol Biol, 2009. **531**: p. 123-43.
364. Nimmerjahn, A., F. Kirchhoff, and F. Helmchen, *Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo.* Science, 2005. **308**(5726): p. 1314-8.

365. Duca, L.M., et al., *Zika Virus Disease and Associated Neurologic Complications.* Curr Infect Dis Rep, 2017. **19**(1): p. 4.
366. Walter, L.T., et al., *Evaluation of Possible Consequences of Zika Virus Infection in the Developing Nervous System.* Mol Neurobiol, 2017.
367. Harvey, L. and P. Boksa, *A stereological comparison of GAD67 and reelin expression in the hippocampal stratum oriens of offspring from two mouse models of maternal inflammation during pregnancy.* Neuropharmacology, 2012. **62**(4): p. 1767-76.
368. Fortier, M.E., G.N. Luheshi, and P. Boksa, *Effects of prenatal infection on prepulse inhibition in the rat depend on the nature of the infectious agent and the stage of pregnancy.* Behav Brain Res, 2007. **181**(2): p. 270-7.
369. Arsenault, D., et al., *The different effects of LPS and poly I:C prenatal immune challenges on the behavior, development and inflammatory responses in pregnant mice and their offspring.* Brain Behav Immun, 2014. **38**: p. 77-90.
370. Xuan, I.C. and D.R. Hampson, *Gender-dependent effects of maternal immune activation on the behavior of mouse offspring.* PLoS One, 2014. **9**(8): p. e104433.
371. Meyer, U., *Prenatal poly(i:C) exposure and other developmental immune activation models in rodent systems.* Biol Psychiatry, 2014. **75**(4): p. 307-15.
372. Arrode-Bruses, G. and J.L. Bruses, *Maternal immune activation by poly I:C induces expression of cytokines IL-1beta and IL-13, chemokine MCP-1 and colony stimulating factor VEGF in fetal mouse brain.* J Neuroinflammation, 2012. **9**: p. 83.
373. Bergeron, J.D., et al., *White matter injury and autistic-like behavior predominantly affecting male rat offspring exposed to group B streptococcal maternal inflammation.* Dev Neurosci, 2013. **35**(6): p. 504-15.
374. Allard, M.J., et al., *A sexually dichotomous, autistic-like phenotype is induced by Group B Streptococcus maternofetal immune activation.* Autism Res, 2017. **10**(2): p. 233-245.

375. Akarsu, E.S. and S. Mamuk, *Escherichia coli lipopolysaccharides produce serotype-specific hypothermic response in biotelemetered rats.* Am J Physiol Regul Integr Comp Physiol, 2007. **292**(5): p. R1846-50.
376. Connor, C.M., et al., *Maternal immune activation alters behavior in adult offspring, with subtle changes in the cortical transcriptome and epigenome.* Schizophr Res, 2012. **140**(1-3): p. 175-84.
377. Rana, S.A., T. Aavani, and Q.J. Pittman, *Sex effects on neurodevelopmental outcomes of innate immune activation during prenatal and neonatal life.* Horm Behav, 2012. **62**(3): p. 228-36.
378. Ibi, D., et al., *Neonatal polyI:C treatment in mice results in schizophrenia-like behavioral and neurochemical abnormalities in adulthood.* Neurosci Res, 2009. **64**(3): p. 297-305.
379. Wolff, A.R. and D.K. Bilkey, *Immune activation during mid-gestation disrupts sensorimotor gating in rat offspring.* Behav Brain Res, 2008. **190**(1): p. 156-9.
380. Zuckerman, L., et al., *Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia.* Neuropsychopharmacology, 2003. **28**(10): p. 1778-89.
381. Richetto, J., et al., *Prenatal versus postnatal maternal factors in the development of infection-induced working memory impairments in mice.* Brain Behav Immun, 2013. **33**: p. 190-200.
382. Giovanoli, S., et al., *Late prenatal immune activation causes hippocampal deficits in the absence of persistent inflammation across aging.* J Neuroinflammation, 2015. **12**: p. 221.
383. Babri, S., M.H. Doosti, and A.A. Salari, *Strain-dependent effects of prenatal maternal immune activation on anxiety- and depression-like behaviors in offspring.* Brain Behav Immun, 2014. **37**: p. 164-76.
384. Schwartzer, J.J., et al., *Maternal immune activation and strain specific interactions in the development of autism-like behaviors in mice.* Transl Psychiatry, 2013. **3**: p. e240.

385. Brenna, J.T., *Animal studies of the functional consequences of suboptimal polyunsaturated fatty acid status during pregnancy, lactation and early post-natal life.* Matern Child Nutr, 2011. **7 Suppl 2**: p. 59-79.
386. Fedorova, I., et al., *Deficit in prepulse inhibition in mice caused by dietary n-3 fatty acid deficiency.* Behav Neurosci, 2009. **123**(6): p. 1218-25.
387. Weiser, M.J., et al., *Dietary DHA during development affects depression-like behaviors and biomarkers that emerge after puberty in adolescent rats.* J Lipid Res, 2015. **56**(1): p. 151-66.
388. Jones, K.L., et al., *Maternal diet rich in omega-6 polyunsaturated fatty acids during gestation and lactation produces autistic-like sociability deficits in adult offspring.* Behav Brain Res, 2013. **238**: p. 193-9.
389. Deslauriers, J., et al., *Combination of prenatal immune challenge and restraint stress affects prepulse inhibition and dopaminergic/GABAergic markers.* Prog Neuropsychopharmacol Biol Psychiatry, 2013. **45**: p. 156-64.
390. Ambree, O., et al., *Reduction of amyloid angiopathy and Abeta plaque burden after enriched housing in TgCRND8 mice: involvement of multiple pathways.* Am J Pathol, 2006. **169**(2): p. 544-52.
391. Chourbaji, S., C. Brandwein, and P. Gass, *Altering BDNF expression by genetics and/or environment: impact for emotional and depression-like behaviour in laboratory mice.* Neurosci Biobehav Rev, 2011. **35**(3): p. 599-611.
392. van Dellen, A., et al., *Delaying the onset of Huntington's in mice.* Nature, 2000. **404**(6779): p. 721-2.
393. van Praag, H., G. Kempermann, and F.H. Gage, *Neural consequences of environmental enrichment.* Nat Rev Neurosci, 2000. **1**(3): p. 191-8.
394. Taylor, P.V., et al., *Sexually dimorphic effects of a prenatal immune challenge on social play and vasopressin expression in juvenile rats.* Biol Sex Differ, 2012. **3**(1): p. 15.
395. Grayton, H.M., et al., *Altered social behaviours in neurexin 1alpha knockout mice resemble core symptoms in neurodevelopmental disorders.* PLoS One, 2013. **8**(6): p. e67114.

396. Laarakker, M.C., et al., *Sex-dependent novelty response in neurexin-1alpha mutant mice.* PLoS One, 2012. **7**(2): p. e31503.
397. Rosenfeld, C.S., *Sex-Specific Placental Responses in Fetal Development.* Endocrinology, 2015. **156**(10): p. 3422-34.
398. Gabory, A., et al., *Maternal diets trigger sex-specific divergent trajectories of gene expression and epigenetic systems in mouse placenta.* PLoS One, 2012. **7**(11): p. e47986.
399. Gallou-Kabani, C., et al., *Sex- and diet-specific changes of imprinted gene expression and DNA methylation in mouse placenta under a high-fat diet.* PLoS One, 2010. **5**(12): p. e14398.
400. Reynolds, C.M., et al., *Maternal high fat and/or salt consumption induces sex-specific inflammatory and nutrient transport in the rat placenta.* Physiol Rep, 2015. **3**(5).
401. Kim, D.W., et al., *Obesity during pregnancy disrupts placental morphology, cell proliferation, and inflammation in a sex-specific manner across gestation in the mouse.* Biol Reprod, 2014. **90**(6): p. 130.
402. Cox, L.A., et al., *Expression of the placental transcriptome in maternal nutrient reduction in baboons is dependent on fetal sex.* J Nutr, 2013. **143**(11): p. 1698-708.
403. McCarthy, M.M., *The two faces of estradiol: effects on the developing brain.* Neuroscientist, 2009. **15**(6): p. 599-610.
404. McCarthy, M.M., et al., *Mechanisms mediating oestradiol modulation of the developing brain.* J Neuroendocrinol, 2008. **20**(6): p. 777-83.
405. Schwarz, J.M., P.W. Sholar, and S.D. Bilbo, *Sex differences in microglial colonization of the developing rat brain.* J Neurochem, 2012. **120**(6): p. 948-63.
406. Matcovitch-Natan, O., et al., *Microglia development follows a stepwise program to regulate brain homeostasis.* Science, 2016. **353**(6301): p. aad8670.
407. Loram, L.C., et al., *Sex and estradiol influence glial pro-inflammatory responses to lipopolysaccharide in rats.* Psychoneuroendocrinology, 2012. **37**(10): p. 1688-99.

408. Santos-Galindo, M., et al., *Sex differences in the inflammatory response of primary astrocytes to lipopolysaccharide.* Biol Sex Differ, 2011. **2**: p. 7.
409. Manitz, M.P., et al., *Flow cytometric characterization of microglia in the offspring of PolyI:C treated mice.* Brain Res, 2016. **1636**: p. 172-82.
410. Van den Eynde, K., et al., *Hypolocomotive behaviour associated with increased microglia in a prenatal immune activation model with relevance to schizophrenia.* Behav Brain Res, 2014. **258**: p. 179-86.
411. de Souza, D.F., et al., *Changes in Astroglial Markers in a Maternal Immune Activation Model of Schizophrenia in Wistar Rats are Dependent on Sex.* Front Cell Neurosci, 2015. **9**: p. 489.
412. Choi, G.B., et al., *The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring.* Science, 2016. **351**(6276): p. 933-9.
413. Hao, L.Y., et al., *Prenatal exposure to lipopolysaccharide results in cognitive deficits in age-increasing offspring rats.* Neuroscience, 2010. **166**(3): p. 763-70.
414. Krstic, D., et al., *Systemic immune challenges trigger and drive Alzheimer-like neuropathology in mice.* J Neuroinflammation, 2012. **9**: p. 151.
415. Doehner, J., et al., *Extrusion of misfolded and aggregated proteins--a protective strategy of aging neurons?* Eur J Neurosci, 2012. **35**(12): p. 1938-50.
416. Knuesel, I., et al., *Age-related accumulation of Reelin in amyloid-like deposits.* Neurobiol Aging, 2009. **30**(5): p. 697-716.
417. Dean, D.C., 3rd, et al., *Brain differences in infants at differential genetic risk for late-onset Alzheimer disease: a cross-sectional imaging study.* JAMA Neurol, 2014. **71**(1): p. 11-22.
418. Knickmeyer, R.C., et al., *Common variants in psychiatric risk genes predict brain structure at birth.* Cereb Cortex, 2014. **24**(5): p. 1230-46.
419. Carvey, P.M., et al., *Prenatal exposure to the bacteriotoxin lipopolysaccharide leads to long-term losses of dopamine neurons in offspring: a potential, new model of Parkinson's disease.* Front Biosci, 2003. **8**: p. s826-37.

420. Akbarian, S., et al., *Altered distribution of nicotinamide-adenine dinucleotide phosphate-diaphorase cells in frontal lobe of schizophrenics implies disturbances of cortical development.* Arch Gen Psychiatry, 1993. **50**(3): p. 169-77.
421. Akbarian, S., et al., *Maldistribution of interstitial neurons in prefrontal white matter of the brains of schizophrenic patients.* Arch Gen Psychiatry, 1996. **53**(5): p. 425-36.
422. Arnold, S.E., et al., *Some cytoarchitectural abnormalities of the entorhinal cortex in schizophrenia.* Arch Gen Psychiatry, 1991. **48**(7): p. 625-32.
423. Arnold, S.E., D.D. Ruscheinsky, and L.Y. Han, *Further evidence of abnormal cytoarchitecture of the entorhinal cortex in schizophrenia using spatial point pattern analyses.* Biol Psychiatry, 1997. **42**(8): p. 639-47.
424. Falkai, P., T. Schneider-Axmann, and W.G. Honer, *Entorhinal cortex pre-alpha cell clusters in schizophrenia: quantitative evidence of a developmental abnormality.* Biol Psychiatry, 2000. **47**(11): p. 937-43.
425. Jakob, H. and H. Beckmann, *Prenatal developmental disturbances in the limbic allocortex in schizophrenics.* J Neural Transm, 1986. **65**(3-4): p. 303-26.
426. Fry, A.E., et al., *Neuropsychiatric disease in patients with periventricular heterotopia.* J Neuropsychiatry Clin Neurosci, 2013. **25**(1): p. 26-31.
427. Wegiel, J., et al., *Differences between the pattern of developmental abnormalities in autism associated with duplications 15q11.2-q13 and idiopathic autism.* J Neuropathol Exp Neurol, 2012. **71**(5): p. 382-97.
428. Iossifov, I., et al., *De novo gene disruptions in children on the autistic spectrum.* Neuron, 2012. **74**(2): p. 285-99.
429. Orosco, L.A., et al., *Loss of Wdfy3 in mice alters cerebral cortical neurogenesis reflecting aspects of the autism pathology.* Nat Commun, 2014. **5**: p. 4692.
430. Stoner, R., et al., *Patches of disorganization in the neocortex of children with autism.* N Engl J Med, 2014. **370**(13): p. 1209-19.
431. Wegiel, J., et al., *The neuropathology of autism: defects of neurogenesis and neuronal migration, and dysplastic changes.* Acta Neuropathol, 2010. **119**(6): p. 755-70.

432. Penagarikano, O., et al., *Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits.* Cell, 2011. **147**(1): p. 235-46.
433. Lang, B., et al., *Control of cortex development by ULK4, a rare risk gene for mental disorders including schizophrenia.* Sci Rep, 2016. **6**: p. 31126.
434. Ma, L., et al., *Interleukin-1 beta guides the migration of cortical neurons.* J Neuroinflammation, 2014. **11**: p. 114.
435. Crampton, S.J., et al., *Exposure of foetal neural progenitor cells to IL-1beta impairs their proliferation and alters their differentiation - a role for maternal inflammation?* J Neurochem, 2012. **120**(6): p. 964-73.
436. Simo, S., Y. Jossin, and J.A. Cooper, *Cullin 5 regulates cortical layering by modulating the speed and duration of Dab1-dependent neuronal migration.* J Neurosci, 2010. **30**(16): p. 5668-76.
437. Solmi, M., et al., *Systematic review and meta-analysis of the efficacy and safety of minocycline in schizophrenia.* CNS Spectr, 2017: p. 1-12.
438. Xiang, Y.Q., et al., *Adjunctive minocycline for schizophrenia: A meta-analysis of randomized controlled trials.* Eur Neuropsychopharmacol, 2017. **27**(1): p. 8-18.
439. Willi, R., et al., *Altered GSK3beta signaling in an infection-based mouse model of developmental neuropsychiatric disease.* Neuropharmacology, 2013. **73**: p. 56-65.
440. Schmidt, A.F., et al., *Intra-amniotic LPS causes acute neuroinflammation in preterm rhesus macaques.* J Neuroinflammation, 2016. **13**(1): p. 238.
441. Cao, M., et al., *Fetal microglial phenotype in vitro carries memory of prior in vivo exposure to inflammation.* Front Cell Neurosci, 2015. **9**: p. 294.
442. Xing, J., A.R. Titus, and M.B. Humphrey, *The TREM2-DAP12 signaling pathway in Nasu-Hakola disease: a molecular genetics perspective.* Res Rep Biochem, 2015. **5**: p. 89-100.
443. Cronk, J.C., et al., *Methyl-CpG Binding Protein 2 Regulates Microglia and Macrophage Gene Expression in Response to Inflammatory Stimuli.* Immunity, 2015. **42**(4): p. 679-91.

444. Gholizadeh, S., S.K. Halder, and D.R. Hampson, *Expression of fragile X mental retardation protein in neurons and glia of the developing and adult mouse brain.* Brain Res, 2015. **1596**: p. 22-30.

Curriculum Vitae

Silke Smolders was born at the 15th of December in 1989 in Hasselt. In 2007, she graduated from high school in Science - Modern Languages at Virga-Jesse College Hasselt and started her Bachelor in Biomedical Sciences at Hasselt University. In 2010, she did her Master in Biomedical Sciences at the Catholic University of Leuven, where she graduated in 2012. In 2013, she started her joint PhD at Hasselt University and Catholic University of Leuven.

Publications

Silke Smolders*, Sophie MT Smolders*, Nina Swinnen, Annette Gärtner, Jean-Michel Rigo°, Pascal Legendre°, Bert Brône°. Maternal immune activation evoked by polyinosinic:polycytidylic acid does not evoke microglial cell activation in the embryo. Frontiers in Cellular Neuroscience. 2015 Aug 5;9:301 *° equally contributing. IF = 4.609

Sophie MT Smolders, Nina Swinnen, Sofie Kessels, Kaline Arnauts, **Silke Smolders**, Barbara Le Bras, Jean-Michel Rigo , Pascal Legendre°, Bert Brone °. Age-specific function of α5β1 integrin in microglial migration during early colonization of the developing mouse cortex. GLIA. Accepted. IF = 5.997

Selected poster presentations

EMBL Symposium 2014: Microglia - Guardians of the brain
Silke Smolders, Nina Swinnen, Sophie Smolders, Bert Brône, Pascal Legendre, Jean-Michel Rigo - Can embryonic microglia bridge the gap between maternal immune activation and neurodevelopmental disorders?

FENS/SFN Summer school 2014: Neurodevelopmental Psychiatric Disorders
Silke Smolders, Nina Swinnen, Sophie Smolders, Bert Brône, Pascal Legendre, Jean-Michel Rigo - Can embryonic microglia bridge the gap between maternal immune activation and neurodevelopmental disorders?

GLIA Symposium 2015: European meeting on glial cells in health and disease
Silke Smolders, Nina Swinnen, Sophie Smolders, Bert Brône, Pascal Legendre, Jean-Michel Rigo - Can embryonic microglia bridge the gap between maternal immune activation and neurodevelopmental disorders?

Marguerite-Marie Delacroix Steunfonds 2016: Autism research workshop
Silke Smolders, Sophie MT Smolders, Nina Swinnen, Annette Gärtner, Jean-Michel Rigo, Pascal Legendre, Bert Brône - Does maternal immune activation lead to neuropsychiatric disorders through altered brain development?

Bursaries

Rotary Hope in Head grant to support my research.

FWO travel grant, BSCDB travel grant and Howard Hughes Medical Institute grant to attend the CSHL workshop**.**

Acknowledgements

Een vershoudfolietje over mijn toetsenbord, zakdoekjes in de aanslag, comfortfood a volonté en een lichtelijk melancholisch muziekje, stuk voor stuk een absolute must bij het schrijven van dit onderdeel van mijn thesis want toegegeven, ik heb toch wel een traantje (of waren het er meerdere? ☺) gelaten. Ik heb de afgelopen 4 jaar heel wat hoogtepunten en dieptepunten meegemaakt (wat meer van de een dan van de ander ☺) en daarbij heb ik gelukkig heel wat mensen aan mijn zijde gehad. Bij deze wil ik dan ook elk van hun bedanken, want zonder hun zou het er helemaal anders hebben uitgezien tijdens mijn doctoraat. Voor alle duidelijkheid, de volgorde waarin ik iedereen bedank staat niet in relatie tot mijn dankbaarheid! ☺

First of all, I would like to thank my promotor and co-promotors, Bert Brône, Carlos Dotti and Annette Gärtner, for giving me the opportunity to work on this project and to guide me all the way through it. Many thanks also to my jury members, Niels Hellings, Jean-Michel Rigo, Eve Seuntjens, Evgenia Salta and Urs Meyer for all the valuable input in my PhD Thesis and to our collaborators in Leuven Rudi D'Hooge, Paul Van Veldhoven, Leen Van Aerschot and Zsuzsanna Vegh for all the help and guidance in the complex world of mouse behavioral testing and high-performance liquid chromatography.

Annette, we already go back a long time, when I was still doing my master's thesis you were the one guiding me in the *in utero electroporation* technique, teaching me everything there was to know. You were also my motivation to come to Hasselt University. It was a bit scary for me to make this transition, but I knew that as long you would be there, everything would be fine. I could always come to you when experiments didn't work or when I wanted to discuss the results. Thank you for all the input in and help with my PhD and for all the non-work related talks we had. You were a pleasure to work with and I hope you are enjoying now the new challenges of your position in Hamburg, thank you so much!

Bert, ... Ik herinner me nog goed toen ik de eerste keer een sarcastische opmerking maakte via e-mail, na op verzenden te klikken heb ik in vreselijke spanning (lees: schrik) afgewacht of je hier überhaupt wel mee zou kunnen lachen. Was ik opgelucht toen je een nog beter grapje terugmailde! En was ik blij dat er bij jou op tijd en stond wel een grapje vanaf kon, dat heb ik kunnen

gebruiken op de momenten dat ik weer in een dipje zat (en dat zijn er wel genoeg geweest ...). Dankjewel om keer op keer in het project te blijven geloven wanneer ik het niet meer zag zitten, jouw enthousiasme heeft me er op die momenten mee doorheen geholpen. Dankjewel ook voor je begrip voor mijn close-to-deadline werkmethode. Je bent natuurlijk zelf ook wel een heel klein beetje zo (☺) maar het zal toch af en toe wel de nodige frustratie met zich meegebracht hebben. Bedankt voor je flexibiliteit om mij nieuwe dingen te laten proberen, ook al draaide dat niet altijd even positief uit en waren ze niet altijd van de goedkoopste. Het was een ongelooflijk plezier om jou als supervisor te hebben de afgelopen 4 jaar, dankjewel daarvoor!

Lieve, lieve **Sophie**… hoe moet ik aan dit paragraafje beginnen… Ik kan gemakkelijk een heel boekje schrijven over 'het PhD leven van Sophie en Silke' en dan zou ik nog een heel deel moeten schrappen (mede omdat sommige passages niet voor publicatie vatbaar zijn waarschijnlijk :-D). 'Zouden dat 2 zusjes zijn?' hoorden we vaak op de gang als er weer een rondleiding op BIOMED was. Onze papa's vertoonden dan ook nog eens schrikwekkend veel gelijkenissen… je zou voor minder beginnen twijfelen ☺ Maar ook al zijn we dan officieel geen familie, we hebben de afgelopen jaren zoveel samen meegemaakt dat het toch niet meer heel veel zal schelen! Je was mijn bureaugenootje voor de afgelopen 3.5 jaar, mijn steun en toeverlaat bij de moeilijke momenten, mijn klaagmuur en motivator, mijn sportbuddy, mijn persoonlijke nagelstyliste, mijn microgliamaatje en zoveel meer. Wat hebben we toch samen afgezien met het tellen van al die microglia (of waren het nu soms toch eerder menigeale macrofagen of monocyten? ☺)! Goddank dat we dit samen konden doen want met het nodige geklaag en gezaag hebben we ons hier toch maar prima doorheen gewerkt! ☺ Ook als je in Parijs zat kon ik altijd bij jou terecht, ellenlange e-mails gingen bijna daags op en af met vragen, frustraties en blijde gebeurtenissen van de voorbije dag(en). Dankjewel voor al je hulp met de experimenten, voor al het nalezen van mijn schrijfsels, voor al je 'What's New NCBI' updates en voor al je input tijdens de meetings (al dan niet officieel ☺). Oneindig bedankt voor de vele filmavondjes, veggie-dinner nights, nagellak en make-up sessies (hier heb je je roeping toch wel een beetje gemist hoor :-D), BH-fittings (opgepast met dit voor een raam te doen!), je goede zorgen na het feestje op de microglia meeting in Heidelberg (lang leve The Voice Kids) en nog

zo veel meer, je was een absolute droomcollega! Heel veel succes met je eigen verdediging binnenkort, maar dat ga je uiteraard helemaal prima doen, en met je nieuwe uitdaging erna ☺. You are the best!

Dorien en **Ilse**, mijn medeoprichters van de toffe peren club, waarom we tijdens de bachelor al geen vrienden waren blijft voor mij een mysterie maar shame on me! Ik weet niet hoe het voor jullie zit, maar wat mij betreft was het duidelijk tijdens mijn eerste BIOMED-kerstfeestje aan de chocolade fontein: samen zouden we de komende jaren nog veel plezier maken! We deelden namelijk een grote passie: eten en drinken! Martini bellini, aperol spritz, kiwitini, frituurhapjes, fried ice cream, recepties, barbecue, moelleux, … als er eten en/of drinken bij te pas kwam waren wij altijd wel van de partij. Voor een lach en een traan konden we ook altijd bij elkaar terecht, de bureau van Dorien werd dan van tijd tot tijd wel eens omgevormd tot een psychologen praktijk ☺. Hopelijk hebben we BIOMED niet TE veel overlast bezorgd, want ons gegiechel was naar het schijnt toch wel regelmatig te horen tot aan de andere kant van de gang (vreemd, toch? ☺). Liefste toffe peertjes, dankjulliewel voor al die jaren die we hebben mogen delen op BIOMED en hopelijk toch ook nog wat meer in de toekomst! Heel veel succes ook met de eindfase van jullie doctoraat, maar georganiseerd als jullie zijn zal dat vast geen al te groot probleem vormen ☺. Toitoitoi!

Ook onmisbaar in mijn dankwoord is **Patrick**, de GRASP van BIOMED. En nee, dan bedoel ik niet die techniek die wel heel leuk klonk in theorie maar verdomd veel frustraties met zich meebracht in praktijk en waarvan we dus ook hadden gezworen er de naam niet meer van uit te spreken. Nee hoor, in deze context staat GRASP voor Geweldig Rappe Alleswetende Super Patrick, iemand die ze in elk lab zouden moeten hebben (jammer genoeg niet commercieel beschikbaar). Jij was altijd van alle projecten op de hoogte en zat er ook helemaal niks mee in om daarin mee te brainstormen en leuke nieuwe experimenten te bedenken. Je kon ook zowat alles snel laten gaan: denken, praten, pipetteren, …, jij ging altijd als een wervelwind doorheen BIOMED. Maar wat mij keer op keer het meest deed verbazen was hoe snel je de tijd kon laten gaan. ‘Even’ bijpraten met jou kon gemakkelijk een uur duren zonder het door te hebben. Als ik daardoor dan later thuis was dan voorzien moest ik gewoon zeggen ‘Patrick was nog even

binnengesprongen' en dan was het wel duidelijk. Patrick, dankjewel voor al je input in mijn project, al je hulp met het kloneren en al je leuke babbels, al dan niet vergezeld van een kopje (in jouw geval kommetje) koffie. Ik heb verschrikkelijk veel van je mogen bijleren en zal je daar dan ook eeuwig dankbaar voor zijn, dankjewel!

Minstens even onmisbaar op BIOMED was, en is nog steeds, **Petra**. Ik heb regelmatig gezegd 'Petra, zonder jou zou BIOMED op zijn gat liggen' en dat heb ik altijd gemeend! Petra, op jou kon ik altijd rekenen, voor hulp met mijn experimenten, de laatste nieuwtjes van BIOMED, een goeie oppepper wanneer alles weer eens tegenzat en natuurlijk ook voor je onvergetelijke lach. Als jij verlof had heerste er een onverdraagbare (hoewel, tijdens het schrijven van mijn thesis soms welgekomen ☺) stilte op de gang. Ook tijdens de BBB hebben we heel wat afgelachen, dat kwam onze oefeningen niet altijd helemaal ten goede, ma bon, we hebben er ons toch maar goed geamuseerd ☺! Een heel dikke merci voor de uitmuntende taxiservice toen ik in het gips lag, zonder jou had ik noodgedwongen ziekenverlof moeten nemen ☺! Ik had niet beter in de watten gelegd kunnen worden, ook tijdens de lab retreat ☺. Dankjewel ook voor al je hulp tijdens de bachelorstages en al het magnetofectiewerk, je bent ondertussen wel echt uitgeroeid tot DE transfectiekoningin! We komen ons vast en zeker nog wel tegen in Kermt/Tuilt (waar jij dan de helft van het volk kent, een bekende Tuiltenaar ☺), dus tot snel!

En dan ons Kessie, **Sofie**, mijn DISC1 partner in crime. Tijdens je junior stage had je al laten zien wat je allemaal in huis had. Je leerde snel, had geen schrik van nieuwe dingen en was niet vies van wat later door te werken. Ook al liep echt elke stap van de western blot mis waardoor we er een late-nighter van moesten maken, jij gaf niet op. Dat je dan ook je senior stage bij ons kwam doen konden wij alleen maar toejuichen. Daar heb je dan het DISC1 project in volle vaart verdergezet en je helemaal eigen gemaakt. Je was toen al helemaal geen student meer maar een volwaardig lid van fysiologie. Je senior stage hebben we dan geslaagd afgesloten met een tripje naar New York en dan kon je beginnen aan je doctoraat bij fysiologie. Al snel werd duidelijk dat je de afgelopen maanden veel tijd met mij had doorgebracht: ik voelde een zekere bezorgdheid maar ook gekke vreugde toen ik die beschimmelde agar platen van

jou overlaatst tegenkwam in de frigo ☺. Sofie, bedankt voor al je hulp en enthousiasme tijdens je stages bij ons, voor alle hulp met de magnetofectiepaper en alle gezellige babbels en ongelooflijk veel succes met je doctoraat, you're gonna kick some ass!

Ruth, my personal Spanish lab book and clean-desk police. You joined our lab for 'just a few weeks' to learn the phagocytosis assay from Sofie, but those few weeks quickly turned into a few months and due to your kick-ass defense at FWO you can now stay here for another 4 years ☺. On the work floor, the difference between us could not be any bigger! During your relatively short time in our lab, you already managed to fill 2 lab books (coffee stain free) while I still had to catch up for the last few months. And how you could nod your head when you saw the mess on my desk ☺. We often wondered how it was possible that we became friends ☺. Shop(lift)ing in IKEA, cheering for Gunther's football team on Sunday, eating kebab and drinking martini bellini and having sleep-overs (either planned or accidental), we spent quite some time together outside of the lab. Thank you so much for everything and good luck with the PhD! (PS: Please, keep me updated when I can come over to make that coffee stain on your lab book ☺!)

"Hé, jij kent Sanne ook?! Willen we anders samen iets voor haar verjaardag doen?" En zo leerde ik **Joke** kennen. Ritjes naar Elen werden de laatste tijd meer regel dan uitzondering, om nog eens gezellig bij te babbelen, een filmpje te kijken in jullie home cinema of ergens lekker te gaan eten. Ook op BIOMED werden de koffie- en lunchpauzes regelmatig gecombineerd met een goed babbeltje. Als ik met weer wat muizenissen (☺) zat hielp jij altijd met het zoeken naar een oplossing. Het kweken van Cre-Put muizen, samen sperma cryopreserveren, fostering experimenten helpen opzetten, …, niets was voor jou te veel. Ongelooflijk bedankt voor al die jaren van praktische en mentale ondersteuning, je was onmisbaar voor mij tijdens mijn PhD!

Quirine, de moederkloek van BIOMED, dat kunnen we toch wel stellen denk ik he?! Jij was er al van toen ik begon aan mijn PhD tot dat ik weg ging altijd bij geweest, ik zou me BIOMED niet zonder jou kunnen voorstellen. Toen jij van bureau verhuisde werd het ineens een stuk stiller aan onze côté... Gelukkig kwam je op tijd en stond nog eens van onze spiegel gebruik maken ☺. En

tijdens de lunchpauzes was het altijd genieten van jou verhalen over Anna en Gita, 2 schatten van kinderen, maar ja, dat kan ook moeilijk anders met zo een mama ☺. Dankjewel Quirine voor al die jaren van lachjes en traantjes, voor alle steun wanneer ik in een dipje zat en voor al je gekkigheid, je bent een toppertje!

Lize, jouw komst op BIOMED herinner ik me nog goed. Jij zou Nina zowat vervangen als postdoc van neurofysiologie, maar wat toch gedaan als jij helemaal niet zo leuk zou zijn? Allemaal gepieker op niets natuurlijk, want ineens stond daar een supertoffe madam bij ons op de gang! Superveel ervaring met muizen gedragsexperimenten en een krak in statistiek, precies wat ik nodig had dus! Tijdens mijn leuke, maar soms best frustrerende, tijd in Leuven stond je mij altijd met raad en daad bij, ik had het niet beter kunnen treffen! Ook de middagpauzes werden er een heel stuk leuker op wanneer jij ook daar was! En achteraf kon ik ook weer altijd bij jou terecht voor hulp met analyses en statistische vraagstukken, god weet hoe ik dat zonder jou had klaargespeeld... . Samen met jou en Petra de eerste fysiologie lab retreat organiseren was ook een uitermate groot plezier, hopelijk een traditie die nog een tijdje verder gezet kan worden ☺. Lize, een ongelooflijke merci voor alles, je bent er eentje uit de duizend!

McFlurry **Maxim**, mijn vaste partner voor regelmatige bezoekjes aan de McDonalds! Ze zullen ons daar weer graag hebben zien komen als we zo een grote bestelling doorgaven of als we daar afkwamen met onze doos ijs (maar dat hield wel echt goed koud!)! We konden natuurlijk niet anders, want die poging tot eigen creatie was niet bepaald een groot succes (om het zacht uit te drukken!) ☺. Bij jou kon ik ook altijd wel terecht om mijn hoofd even leeg te maken: ons getetter was altijd overladen met sarcastische opmerkingen en slechtgezindheid kende jij amper (behalve als je ratten niet goed gelopen hadden natuurlijk ☺). Uitgezonderd al de keren dat je mij de stuipen op het lijf joeg, dankjewel voor de leuke tijd op BIOMED, je was een topcollega ☺. (PS: zorg goed voor Dorien ☺)

Jens, als mijn allereerste bachelorstudent legde jij de lat al hoog voor wie nog moest volgen! Gelukkig voor ons patchte je ook zo graag want daarom kwam je (denk ik toch ☺) naar ons terug voor je doctoraat. Daar werd je al snel de man

van de onvoorstelbaar droge humor! Wat heb ik toch vaak krom gelegen van het lachen met weer een van je hilarische opmerkingen! 'Petra, wat een ferm fietstassen!' steekt er dan toch wel met kop en schouders bovenuit! Als er iets te beleven viel was jij altijd van de partij, en maar goed ook want jij blies leven in elk feestje. Dankjewel Jens, voor alle leuke momenten op BIOMED en superveel succes met je doctoraat, je gaat dat nog goed doen!

Giovanni and **Joris**, the original Glyrα2-guys and probably among the hardest working PhD students at BIOMED. Thank you so much for all the help in the lab and for the great years at BIOMED, it wouldn't have been the same without you guys! Good luck with the remainder of your PhD (Joris) and with your new postdoc (Giovanni) ☺.

Jirka, het perfecte voorbeeld van 'stille waters, diepe gronden', op het eerste zicht een stil en ingetogen meisje, maar eens losgekomen een spraakwaterval ☺. Jij hebt ondertussen ook al de nodige tegenslag meegemaakt in je doctoraat, maar je komt zeker en vast wel op je pootjes terecht! Dankjewel voor de afgelopen jaren op BIOMED en heel veel succes met de rest van je doctoraat!

Virginie, waarschijnlijk een van de hipste PI's die ik ken en dan ook nog eens een overheerlijke macaronnekes bakker ☺, ik ben echt blij dat ik jou heb mogen leren kennen op BIOMED. Heel erg bedankt voor al je wetenschappelijke input en voor alle minder wetenschappelijke maar des te meer leuke babbeltjes. Heel veel succes met het in bedwang houden van je huidige doctoraatsstudenten (☺) en hopelijk tot nog eens!

Ook buiten onze supertastische groep van fysiologie heb ik de nodige labohulp, PhD ondersteuning en gezellige babbeltjes gehad (wie had dat ooit mogelijk geacht ☺)! **Stefanie**, **Jessica** en **Hannelore**, samen hebben we de oh zo gezellige periode van het thesis-schrijven, achteraf gezien, toch best goed doorstaan! Frustraties waren onvermijdelijk, maar het spreekwoord 'gedeeld leed is halve smart' kon niet beter toegepast worden dan bij ons. Wetende dat ik niet de enige was die deze periode moest doormaken heeft me er heel vaak doorheen geholpen, dankjulliewel daarvoor! Ook bedankt natuurlijk voor alle verre van werk gerelateerde praatjes, feestjes en filmavondjes, jullie waren echt topcollega's! **Selien**, mijn eerste junior student en mede-oprichtster van het

DISC1 project, ik ben blij dat je na die stage toch nog de moed hebt om aan western blots te beginnen tijdens je doctoraat (hoewel het nu met de imager al wat leuker is dan in de donkere kamer natuurlijk ☺)! Dankjewel voor de leuke tijd op BIOMED en heel veel succes met de rest van je doctoraat, je gaat dat nog goed doen ☺. **Lieselotte**, **Elien**, **Petra H**, **Daniela, Yorg** en **Jo**, bedankt voor de leuke babbels op de gangen van BIOMED! Het waren keer op keer welgekomen afleidingen ☺.

En oh nog zo veel mensen van BIOMED die ik moet bedanken voor alle dagelijkse dingen die toch absoluut niet zo evident zijn. **Rosette**, **Jo**, **Igna**, **Regine**, **Katrien** en **Yennick**, dankjulliewel voor alle hulp met het dagelijks reilen en zeilen in het lab en in het animalium! **Rani** en **Veronique**, bedankt voor alle administratieve ondersteuning die ik echt wel broodnodig heb gehad! **Paul**, heel erg bedankt voor alle klusjes op BIOMED, het bijvullen van de koffiemachines en voor alle gezellige babbeltjes en **Zina**, dankjewel om mijn bureau toch nog in de mate van het mogelijke wat netjes te helpen houden!

Ook niet te vergeten in mijn dankwoord zijn mijn ex-BIOMED collega's waar ik toch een hele tijd lief en leed mee heb gedeeld. **Nina**, dankjewel om mij op te leiden (of lijden? ☺) in de wereld van de maternale immuun activatie en mij op gang te helpen in de eerste maanden van mijn doctoraat. Heel erg bedankt ook voor wat je allemaal voor mij gedaan hebt bij de MIA paper, ik zal je hier oprecht eeuwig dankbaar voor zijn! **Kathleen**, ook al was je officieel lid van biofysica, je hoorde minstens evenveel bij fysiologie. Bedankt voor de leuke tijden op en buiten BIOMED en hopelijk blijven we af en toe nog eens de tijd vinden om nog eens af te spreken ☺.

Jasmine, we hebben ons eigenlijk pas echt leren kennen op de GLIA meeting in Bilbao, maar wat ben ik daar blij van. We hebben er daar toen samen een leuk congres van gemaakt, met het nodige shoppen tussendoor en op regelmatige basis een lekker Tinto de Verano (toen we dan toch uiteindelijk wisten wat dat was ☺). Op BIOMED hebben we uren en uren getetterd, over de leuke dingen die we meemaakten, maar ook al onze muizenfrustraties moesten eraan geloven. Een showke van Jeff Dunham kon ons gelukkig altijd wel weer opbeuren ☺. Heel erg bedankt voor de leuke jaren op BIOMED (en er buiten) en

ongelooflijk veel succes nog met je nieuwe job! We blijven ongetwijfeld af en toe nog eens een gaatje vinden om wat bij te babbelen ☺!

Ann, mijn ex- maar ondertussen weer huidig bureaugenootje! Toen ik op BIOMED begon mocht ik mij bij jou en Marjolein op het bureau komen installeren, jullie hebben mij daar supergoed opgevangen de eerste paar dagen/weken/maanden! Ook toen ik daarna naar het bureau erlangs verhuisde was een bijna dagelijks bezoekje bij jullie niet te vermijden, we hadden dan ook altijd heel wat te vertellen! Zelfs buiten het werk stond onze tetter zelden stil, onze goede voornemens om lekker gezond te gaan lopen kwamen dan ook vaak in het gedrang. Elke uitvlucht was goed genoeg om misschien toch maar beter een ijsje te eten of iets te gaan drinken in de square (dat babbelde dan ook makkelijker met meer adem ☺). Heel erg bedankt Ann voor alle hulp en steun tijdens mijn doctoraat en nu ook tijdens mijn postdoc ☺!

Uiteraard hebben mijn fantastische (ex-)collega's/vrienden van BIOMED een ongelooflijk groot deel van mijn leven uitgemaakt de afgelopen 4 jaren, maar zonder alle vrienden en familie zou ik nooit zo ver geraakt zijn. Bij deze wil ik elk van hun dan ook heel erg bedanken voor alle steun de afgelopen jaren!

Katrien L, beste vriendinnen sinds onze kleutertijd, altijd samengezeten in de klas en dan uiteindelijk zelfs allebei op dezelfde campus werken, wat leuk dat ik je tijdens mijn doctoraat zo dicht bij mij heb gehad! Ons fantastisch plan om 2-wekelijks lunch voor elkaar te maken heeft niet heel erg lang standgehouden (het werden al snel gewoon boterhammekes ☺) maar ons Basic Fit avontuur was des te meer een groot succes! We hebben verschrikkelijk afgezien die eerste bodypump lessen maar beetje bij beetje boekten we toch vooruitgang ☺. Met kleine grote Ivan doe je nu aan een heel ander soort fitness, maar wat doe je dat goed! Hij mag zich maar gelukkig prijzen met zo een TOPmama! Lieve Katrien, dankjewel voor al die jaren dat wel al lief en leed hebben gedeeld en dat er zo nog vele jaren mogen volgen ☺!

Valerie en **Katrien S**, mijn beste turnmaatjes! Ookal zijn we tijdens mijn doctoraat nog amper aan turnen toegeraakt (oud en versleten ☺), we vonden altijd wel andere activiteiten om ons mee bezig te houden. Samen een festivalleke meepikken, naar de sauna, klimmen in landgraaf, een filmpje zien,

heel veel en lekker eten (met als grote favorieten natuurlijk Thais, Mexicaans en Indisch), het maakte eigenlijk niet uit, zolang we maar samen weg waren. De 3 maanden dat jullie weg waren konden dan ook niet snel genoeg vooruitgaan! Vallie, dankjewel voor de vele uren in de Basic Fit (Bilzen, Diepenbeek of Hasselt, allemaal om het even) en om mij telkens weer te motiveren om toch dat kilootje extra op te leggen tijdens de bodypump! Ladies, jullie zijn een fantastische steun en toeverlaat geweest doorheen mijn hele doctoraat en daarom bij deze echt een welgemeende heel erg dankjewel!

Sanne, ik ben zelfs de tel al kwijt hoelang we mekaar eigenlijk al kennen! Ook al woonde je niet bepaald naast de deur, we namen desnoods wel een uur de bus om nog eens af te kunnen spreken. Dat werd wel een stuk makkelijker toen jij ook in Leuven op kot kwam en tijdens mijn doctoraat door de gezamenlijke dates met Joke, 2 vliegen in 1 klap ☺! Sanne, heel erg bedankt voor de steun en ontspanning tijdens mijn PhD, onze afspraakjes waren keer op keer welgekomen!

Nathalie L, mijn steun en toeverlaat tijdens mijn master thesis en tijdens mijn overgang van Leuven naar Diepenbeek! Ondanks onze drukke agenda's door mijn doctoraat en de komst van jullie kleine Emilia zijn we er gelukkig toch op regelmatige basis in geslaagd nog eens af te spreken ☺. Dankjewel voor al de ontspannende afspraakjes en de gezellige babbeltjes de afgelopen jaren!

Annelies, **Marlies**, **Katrien M** en **Sharona**, mijn Leuvense studiegenootjes! Nu ja, eigenlijk ook al in Diepenbeek, maar we zijn mekaar eigenlijk pas echt tegengekomen op Gasthuisberg ☺. Dankjulliewel voor de leuke jaren op kot in Leuven en alle leuke afspraakjes die daarop volgden. Katrien M, we moesten altijd hennig zoeken om een datumpje te kunnen prikken, maar als we er dan een vonden maakten we er ook gewoon een supertoffe namiddag/avond van! Merci voor alle plezier en steun de afgelopen jaren en superveel succes in Aken, laat ze daar maar eens een dermatologisch poepie ruiken ☺. Sharona, a.k.a. Vonckie, bedankt voor alle supergezellige lunch dates in Diepenbeek of Genk. Ik ga ze vreselijk moeten missen vanaf nu, maar hopelijk kunnen we ze gewoon omzetten in paar-jaarlijkse diner dates in Hasselt of Overijse ☺. Doe dat nog goed de laatste maanden van je doctoraat!

Julie, **Justine**, **Tahnee** en **Karolien**, onafscheidelijk tijdens onze bachelorjaren in Diepenbeek maar dan toch elk onze eigen weg gegaan in onze master (van Leuven over Brussel tot Antwerpen). We doen nu ook allemaal iets anders in ons dagelijks leven, en toch blijft het een groot plezier om op tijd en stond nog eens allemaal samen te komen. De doodles om nog eens met ons vijven af te kunnen spreken gaan noodgedwongen best ver in de toekomst, maar uiteindelijk geraken we er altijd wel! Ladies, dankjulliewel voor de leuke jaren in Diepenbeek, de fijne tijd op kot in Leuven en de gezellige ontbijtjes, lunches en diners tijdens mijn doctoraat!

Nathalie VD, beste vriendin en mama van mijn crème van een metekindje, ongelooflijk bedankt voor alles de afgelopen jaren! Of het nu onder ons tweetjes was of samen met **Kiara** en **Ilona**, bij jullie kon ik altijd even mijn gedachten verzetten en de problemen van mijn doctoraat relativeren ☺. **Ben**, ook een superdikke merci voor al je hulp tijdens onze verbouwingen, jouw raad en (vooral) daad hebben voor een goede afloop gezorgd!

Nonke **Arnaut**, beste peter van de wereld, en tante **Joske**, jullie kunnen natuurlijk ook niet ontbreken in mijn dankwoord! Heel erg bedankt voor jullie steun tijdens mijn studies en doctoraat en voor alle etentjes.

Schoonfamilie kan je niet kiezen, maar ik heb toch wel geluk met die van mij ☺. **Nathalie H**, **Dieter**, **Bart H**, **Luc** en **Linda**, en **Rita** dankjulliewel om mijn toch af en toen wel stresserende (☺) PhD periode te verlichten door al jullie hulp bij de verhuis, de verbouwingen, het huishouden, de was en de plas en het avondeten op dinsdagen en donderdagen. We hoefden maar een kik te geven en jullie stonden al klaar om ons uit de nood te helpen. Ik zou niet weten waar we zonder jullie waren uitgekomen, dus bij deze, oprecht bedankt!

En dan natuurlijk zij die mij dag in dag uit, jaar in jaar uit hebben gesteund bij alles wat ik deed/doe en mij in mijn allerbeste maar zeker ook in mijn allerslechtste momenten hebben meegemaakt. Allerliefste **zusje**, topschoonbroer **Bart VR** en mijn liefste kleine spruit, mijn oogappeltje **Janne**, het was even slikken toen jullie een aantal jaar geleden besloten naar Breda te verhuizen, mijn lieve zus zo ver weg van huis! Gelukkig bestond er een telefoon en skype, zo konden we die oh zo grote afstand toch wat overbruggen! En toen

ik mijn eigen autootje had aan het begin van mijn doctoraat kon ik gewoon lekker zelf tot bij jullie bollen als ik eens een dagje wou komen shoppen of naar de sauna gaan! De bezoekfrequentie ligt nu natuurlijk nog een stukje hoger sinds Janne geboren is ☺. Zusje, ondanks je toch niet zo kortbij zijnde woonplaats ben je er altijd voor mij geweest en heb je mij altijd met raad en daad bijgestaan, dankjewel daarvoor!

Mam en **pap** (alias moeke en vake), dankjulliewel voor jullie onvoorwaardelijke steun tijdens mijn doctoraat en om altijd in mij te blijven geloven! Ookal was het vaak chinees waar ik het over had, bij jullie kon ik altijd mijn frustraties en irritaties kwijt en ongegeneerd gewoon een potje brommen als dat nodig was. Ook natuurlijk uitermate bedankt voor de uitmuntende was- en strijkdienst, grasmaaiservices en restaurantvoorzieningen waar we de afgelopen jaren altijd op hebben kunnen rekenen. Jullie hebben hiermee altijd een enorme huishoudelijke last van onze schouders genomen, dat is die 4 jaren onbetaalbaar geweest voor mij, dus bij deze nog eens een welgemeende dankjewel!

Lieve schat, **Lievie**, al 10 jaar en een klets heb je het bij mij uitgehouden, en dat moet niet altijd even gemakkelijk geweest zijn, zeker niet de afgelopen 4 jaar! Als er weer iets was misgelopen in het lab (en dat was natuurlijk niet zeldzaam) was ik zeker niet altijd de leukste thuis en vele weekends en feestdagen zijn opgeofferd geweest aan die muisjes van mij. Daarbij dan nog de aankoop en verbouwingen van ons eigen stekje, tja, we hebben zeker en vast de nodige frustraties gekend. Toch kon ik altijd bij jou terecht om wat te ventileren of te mokken, maar ook om mij op te beuren, te motiveren en te ontspannen of om mij gewoon eens een goei sjot onder mjin gat te geven. Dankjewel voor al die keren dat het eten al op tafel stond als ik thuis kwam, voor mij en verschrikkelijk huishoudelijk taakje en daarom eens zo fijn als ik mij hier weer eens niets van moest aantrekken. Lieve schat, vanuit de grond van mijn hart, dankjewel voor alles!