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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

The road from maternal immune activation to developmental disorders: rerouting via projection neurons

Promotor :
Prof. dr. Bert BRONE

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Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Content

Acknowledgements

Over the past 8 months, I have been part of the neurology group at the physiology department of the Biomedical Research institute of Hasselt University (BIOMED). Here, many opportunities were provided to me in order to expand my theoretical knowledge in the field of neurology as well as my methodological skills. I was stimulated to think in a problem solving manner and communicate my vision on various topics in a constructive way. Therefore, I would like to express a few word of grace towards all the people who made it possible for me to complete an internship in this research group.

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A word of appreciation goes to all members of the neurology group, and by extension all the staff at BIOMED, who helped me in successfully conducting my experimental work.

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Joris Winters, June 9th 2015

List of abbreviations

2D-DIGE	2D-fluorescence difference gel electrophoreses (2D-DIGE)
ANOVA	Analysis of variance
ApoER2	apolipoprotein E receptor type 2
ASD	Autism spectrum disorder
brdU	5-bromo-2-deoxyuridine
DAB1	Disabled1
DSHB	Developmental studies hybridoma bank
ECL	enhanced chemical luminescence
eGFP	enhanced green fluorescent protein
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	Horse raddish peroxidase
IPC	Intermediate progenitor cell
IUE	*In utero* electroporation
LGE	lateral ganglionec eminence
LPS	Lypopolysacharide
LV	Lateral ventricle
MGE	Medial ganglionec eminence
MGV	mean gray value
MIA	Maternal immune activation
MRI	magnetic resonance imaging
NMDA	N-methyl-D-aspartate
NPC	Neuronal precursor cell
pHH3	anti-phospho-histon H3
PI3K	phosphatidylinositol-3-kinase
Poly(I:C)	polyriboinosinic-polyribocytidilic acid
PVDF	Polyvinylidene fluoride
qPCR	Quantitative Real-time Polymerase chain reaction
RGP	Radial glial precursor
SDS	Sodium dodecyl phosphate
SVZ	Subventricular zone
TLR	Toll-like receptor
VLDR	Very low density receptor

Samenvatting

Een gebalanceerde neurotransmissie is het resultaat van een complexe interactie tussen inhiberende interneuronen en excitatoire projectieneuronen gedurende de ontwikkeling van het neurologische netwerk. De fundamenten van dit netwerk worden reeds gelegd tijdens de embryogenese. Een disbalans tussen excitatoire en inhibitoire stimuli die resulteert in neuro-ontwikkelingsstoornissen als schizofrenie en autisme is het resultaat van interactie tussen genetische elementen en omgevingsfactoren. Ook al zijn de processen die aan de basis liggen van deze stoornissen niet geheel gekend, toch is geweten dat blootstelling van de groeiende foetus aan een verstoorde cytokinebalans als gevolg van maternale infecties tijdens de zwangerschap één van de oorzaken is die aan de basis ligt van deze stoornissen. Met name voor projectieneuronen in het ontwikkelende brein zou wel eens een belangrijke rol weggelegd kunnen zijn.

Dit project vertrekt vanuit de hypothese dat een stimulatie van het maternale immuunsysteem tijdens de zwangerschap van invloed is op de proliferatie van neuronale voorloper cellen en hun migratie naar de cortex. Op die manier wordt de onstabiele basis gelegd voor verdere synaptogenese.

Eerst en vooral wordt het effect van maternale immuun stimulatie op de algemene vorming van de cortex in het nageslacht nagegaan. Hiervoor werd een immunohistochemische kleuring uitgevoerd voor enkele merkers die specifiek zijn voor één of meerdere lagen van de cortex: Tbr1, CTIP2 en CUX1. Stimulatie van het maternale immuunsysteem op embryonale leeftijd E11,5 had geen effect op de algemene vorming van de foetale cortex. Ten tweede werd de proliferatie van neuronale precursors gevisualiseerd aan de hand van een kleuring voor de mitotische merker Ki67. Een toename in het signaal afkomstig van mitotische cellen werd waargenomen. Ten derde werd de expressie van Reelin, een eiwit betrokken in de regulatie van neuronale migratie, gemeten met behulp van western blot analyse en qPCR. Immuun stimulatie in zwangere muizen verminderede de expressie van mRNA voor het RELN gen, maar liet de eiwitexpressie van Reelin onveranderd. Tenslotte werd de migratie van projectie neuronen in het zich ontwikkelende brein gemonitord na stimulatie van het maternale immuunsysteem. Door middel van *in utero* electroporatie werden voorlopercellen gelabeld met als doel hun migratie patroon te volgen tijdens de ontwikkeling. De verdeling van projectie neuronen over de verschillende lagen van de foetale cortex was niet beïnvloedt door maternale immuun activatie.

De data in deze studie laten niet toe om een eenzijdige conclusie te maken betreffende de impact van maternale immuun activatie op de ontwikkeling van projectie neuronen in de hersenen van het nageslacht.

Summary

Balanced neurotransmission is the result of complex interplay between inhibitory interneurons and excitatory projection neurons. The fundaments of this network are built during embryogenesis. Both genetic and environmental factors cause an imbalance between excitatory and inhibitory stimuli resulting in neurodevelopmental disorders, such as autism and schizophrenia. Although the underlying mechanisms are not fully understood, one factor known to distort the balance is exposure to maternal infection early during embryogenesis, with a disruptive effect on interneuron development. A hazardous impact of infection during pregnancy on the fate of excitatory projection neurons in the developing foetal brain may be equally important in neuropathology. In this project, it is hypothesized that stimulation of the maternal immune system during pregnancy will influence proliferation of neuronal progenitors and their migration into the neocortex.

First, general layering of the cortex was examined by staining the cortex for the layer(s)-specific markers Tbr1, CUX1 and CTIP2. Stimulation of the maternal immune system at E11,5 did not influence cortical layering. Second, proliferation of neuronal precursors was visualized by staining for the mitotic factor Ki67. The density of mitotic cells was shown to increase in the brains of offspring from immune-stimulated dams. Third, The expression of Reelin, a major regulator of neuronal migration, was measured using western blot analysis and qPCR. While immune-challenge in pregnant dams reduced the mRNA levels in the offspring, protein expression remained unaffected. Finally, migration of projection neurons in the offspring was monitored. By means of in *utero* electroporation, precursors were labeled, allowing to track their migration during development of the brain. Distribution of projection neurons along the cortex was not influenced.

Data collected in this study are insufficient and do not allow to make an unambiguous conclusion concerning the impact of maternal immune activation on the development of projection neurons in the offspring.

Introduction

1 Etiology of neurodevelopmental disorders and the importance of an appropriate animal model

More than ever, the sustainability of our health care system is topic for debate. In economically challenging times, medical costs have an increasingly large impact on political strategies towards health care and research. At the same time, the number of people requiring medical help remains immense. Therefore, fundamental research is needed. This work fits in a context of fundamental research regarding the causality of **neurodevelopmental disorders**. Despite intensive research, the etiology of most neurodevelopmental disorders is still elusive. In the interest of lowering the impact of neurological disorders on our health care system, fundamental research regarding the onset of these disorders is required. Particularly two disorders will be discussed: **schizophrenia** and **autism spectrum disorder (ASD)**.

The onset of schizophrenia usually situates around adolescence and is characterized by the appearance of positive and negative symptoms (1). Positive symptoms indicate the presence of atypical behaviour such as psychotic experiences (i.e. delusion, hallucination) and disorganized speech and behaviour. In contrast, negative symptoms indicate the absence of normal responses. Examples of negative symptoms are limited expression of emotion and goal-directed behaviour or speech and memory impairment (1). Furthermore, several abnormalities concerning brain volume and function have been correlated to schizophrenia (2). Enlarged lateral ventricles and the resulting shrinkage of surrounding brain tissue, the presence of abnormal neuronal clusters and synaptic anomalies with regard to the glutamate and dopamine system have been observed in schizophrenic subjects (3-5). Similar to schizophrenia, cognitive, social and communicative impairment has been reported in autistic subsets as well. Furthermore, several structural abnormalities including enlarged brain structures, abnormal grey and white matter distribution and structural irregularities have been linked to autism in post-mortem studies and through magnetic resonance imaging (MRI) (6).

Considering their complexity and the involvement of alterations in many human characteristics, comprehending every aspect of autism spectrum disorder and schizophrenia is challenging. Although the pathological processes underlying these disorders are unclear, a neurodevelopmental origin for both disorders has been suggested (7, 8). The notion that aberrant behaviour during adult life results from **early-onset insults** has been widely supported by various types of evidence. First and foremost, several epidemiological and animal studies have shown a link between prenatal infection and the incidence of both schizophrenia and autism (7). Second, the presence of brain abnormalities concerning brain volume and structure suggests the presence of insults already early during development (9, 10). Third, congenital anomalies and several ‘soft signs’ were observed in infants which later developed schizophrenia (11). Finally, expression of genes related to neuronal development and synaptogenesis was shown to be altered (12).

Over the last decade, it has become clear that an interplay between a multitude of parameters lies at the basis of autism and schizophrenia. An interaction between genetic components (13, 14) and several **environmental elements** influences the development of the brain (15). In this study, the focus will lie on the impact of exposure to an environmental element on early neurological

development. More specifically, the influence of **prenatal immune challenge** on the developing **embryonic** brain will be studied. Knowledge on the influence of prenatal immune challenge on early stage processes could contribute greatly in understanding the fundamental processes that form the basis for abnormal postnatal functioning.

The association between maternal disease and an increased risk for neurological disorders in the offspring was already made when a 'winter baby phenomenon' -in which pregnancy during winter months lead to a higher risk for ASD- was described. Already in 1988, Mednick et al. reported that viral exposure during the second trimester of foetal development predisposes for Schizophrenia (16). This hypothesis was eventually confirmed by Brown et al. (7). More recently, a case-control study (CHARGE-trial) showed an elevated risk for ASD in the offspring of mothers who had suffered from a fever during pregnancy as a result of an influenza infection (17). Using an animal model based on the intranasal administration of an adapted influenza strain, Fatemi et al. provided important insights with regard to the impact of environmental insults on neurodevelopment. In 2002, they showed that prenatal exposure to human influenza virus H1N1 influences neurodevelopment both short-term and long-term. Genes associated with schizophrenia or autism were altered in the offspring of immune challenged mice, pointing towards an interplay between environmental cues and genetic elements at the core of neurodevelopmental disorders (8). Soon after, infection with other micro-organisms (*Taxoplasma gondii*, *Herpes simplex* virus type 2 and urinary tract infections) were linked to increased occurrence of developmental brain disorders (18). Considering that respiratory infections are not expected to directly influence the growing foetus, it was concluded that the maternal immune challenge rather than foetal infection correlates to an increased prevalence of developmental brain disorders (19). It was hypothesized that exposure to maternal immune components present in the foetal environment influences neurodevelopment. This led to the development of rodent models mimicking exposure to viral and bacterial vectors in order to study neurodevelopmental disorders (20). These models do not require the exposure to live pathogens, as reviewed by Meyer (21). In addition to a model based on the administration of the bacterial endotoxin Lypopolysacharide (LPS), the most well-established model is based on the maternal exposure to polyriboinosinic-polyribocytidilic acid **[Poly(I:C)]**, which is a double stranded RNA molecule that activates Toll-like receptor 3 (TLR3) and causes a viral-like acute immune response (22, 23). Many insights regarding the influence of maternal infection on the developing foetal brain result from studies in which this technique, known as **maternal immune activation (MIA)** is used (24).

Using this model, a plethora of detrimental neurological effects have been linked to prenatal immune challenge, although the exact causal mechanisms have not yet been elucidated. Behavioural signs of psychotic inadequacy in the offspring of Poly(I:C)-stimulated dams have been described. More precisely, open-field exploratory behaviour, object recognition, social behaviour, selective attention and working memory were shown to be altered. Although impairments in cognitive flexibility are especially altered following late gestation exposure to poly (I:C), abnormalities such as altered prepulse and latent inhibition were the result of mid-gestational exposure to the pathogen (25). Latent inhibition describes the retardation in the learning about the significance of stimuli and is often observed in schizophrenic subjects (20). The term prepulse inhibition describes the attenuation of a startling response to a stimulus when the stimulus is preceded by another stimulus. This process is weakened in schizophrenic subjects (26). Moreover, data provided by Li et al. indicated a greater impact of early prenatal exposure to poly(I:C) on white matter tract integrity than exposure at a later

time during gestation (27). Combined, these findings strongly suggest the presence of an effect already **early during gestation**.

In conclusion, in case of early, severe maternal immune challenge, the neuro-immune crosstalk in the embryonic brain is disturbed, resulting in aberrant brain development in the offspring.

2 Development of excitatory projection neurons

Despite the overwhelming evidence pointing towards an important role for prenatal immune challenge in aberrant brain development, little is known about the underlying etiology. It is assumed that a variety of neurodevelopmental disorders share common etiological mechanisms. A distorted excitation/inhibition balance in the cortex contributes to the cognitive and sensorimotor dysfunction observed in ASD and schizophrenia (28). In order to understand the causality of this imbalance, the composition of the cortex should be discussed in more detail. The mammalian cortex is known to consist of a well-defined structure of six layers, connected through a complex network of excitatory projection neurons and inhibitory interneurons (29). Cortical interneurons arise in the medial and lateral ganglionic eminences (MGE, LGE), from where they migrate tangentially into the cortex (30). An abnormal synaptic plasticity and cortical micro-circuitry has been attributed to an incorrect functioning of these inhibitory neurons. The involvement of GABAergic interneurons in the etiology of schizophrenia has been confirmed through post-mortem studies and *in vivo* models (31). However, in the light of recent publications, a potential role for excitatory projection neurons is also associated with neurodevelopmental disorders. Glutamatergic projection neurons arise from a neuronal precursor cell (NPC) pool localized in the ventricular zone. Development of projection neurons is typically characterized by three crucial steps: proliferation of NPCs, polarization of new-born neurons and migration of these neurons into the cortex where they will differentiate. Finally, communication with other neurons is established through a networking process known as synaptogenesis (32).

The peak of neuronal development in the neocortex occurs during a 6-day period (E11-E17) known as the neurogenetic interval. Both the length of the cell cycles and the number of NPCs that exit the cell cycle is dynamic during this interval. Projection neurons arise from radial glia progenitors (RGPs) through symmetrical and asymmetrical divisions (figure 1) (32). Early during embryogenesis, one RGP divides into two equal daughter cells (RGPs) through **symmetrical progenitor divisions**, expanding the progenitor pool. Subsequently, through **asymmetrical division**, one RGP divides into a daughter cell (self-renewal) and one slightly more differentiated cell. This can either be a single neuron (neurogenic division) or an intermediate progenitor cell (IPC, progenitor division). Following the asymmetrical division, the intermediate progenitor migrates into the subventricular zone (SVZ), where it generates either two post-mitotic neurons through **terminal neurogenic divisions**, or in some cases two equal intermediate progenitors. Finally, through **terminal gliogenic divisions**, one RGP gives rise to one neuron and a daughter cell, which differentiates into a glial morphology (29, 32).

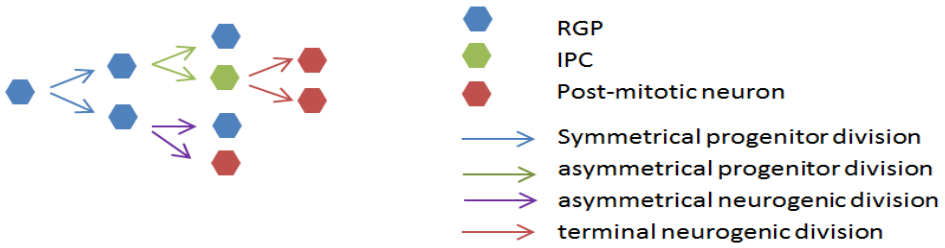


Figure 1:Different stages of neurogenesis. Projection neurons can either develop directly from radial glial cells (RGPs) through asymmetrical neurogenic divisions in the ventricular zone, or indirectly via intermediate progenitor cells (IPCs) in the subventricular zone. In that case, one RGP first divides into one RGP (self-renewal) and one IPC (asymmetrical progenitor division), which than later divides into two post-mitotic neurons (terminal neurogenic division).

Subsequently, new-born neurons **migrate** to their final location in the cortex using radial glia as scaffolds (figure 2, right panel), specifically termed **radial migration** (33). They can reach their final position in the cortex through **somal translocation**, where the neuronal cell body travels within an extended radial process, and through **glial-guided locomotion**, where the new-born neurons develop a leading and trailing process and migrate along radial pial fibers (34). Radial migration occurs through four distinct phases. In phase one, new-born neurons acquire a bipolar morphology as they migrate from the ventricular zone towards the sub-ventricular zone (figure 2, stage 2 to 3)). Next, in phase two, the new-born neurons cease migration for a 24-hour period as they obtain a multipolar morphology (figure 2, stage 3). However, during this period, they remain highly dynamic. Afterwards, a phase of retrograde movement towards the ventricular surface is observed (figure 2, stage 3 to 4). Finally, the new-born neurons experience a **multipolar-to-bipolar transition** (figure 2, stage 4), which is the starting signal for migration towards the pial surface (figure 2, stage 5) (29). This multipolar-to-bipolar transition is often referred to as **polarization**. The first migrating neurons compose a layer known as the primordial plexiform layer or pre-plate, which later splits into a marginal zone and a sub-plate, divided by the cortical plate (figure 2, left panel). Subsequently, six mantle layers of projection neurons are created in an 'inside-out' fashion where the newest progenitors migrate through previously formed layers (figure 2, left panel) (35-39).

Finally, after arrival at their definite position, precursors will further **differentiate** into specialized projection neurons. This differentiation includes the sprouting of neurites and synaptogenesis around birth (40). The final position of projection neurons after migration influences their cell fate and is therefore of importance for their functionality and for the connectivity between neurons (40). The ectopic positioning of projection neurons has been described in brains of autistic and schizophrenic humans (41). Furthermore, erroneous neuronal migration and aberrant neuronal layering was confirmed in rodent models (42).

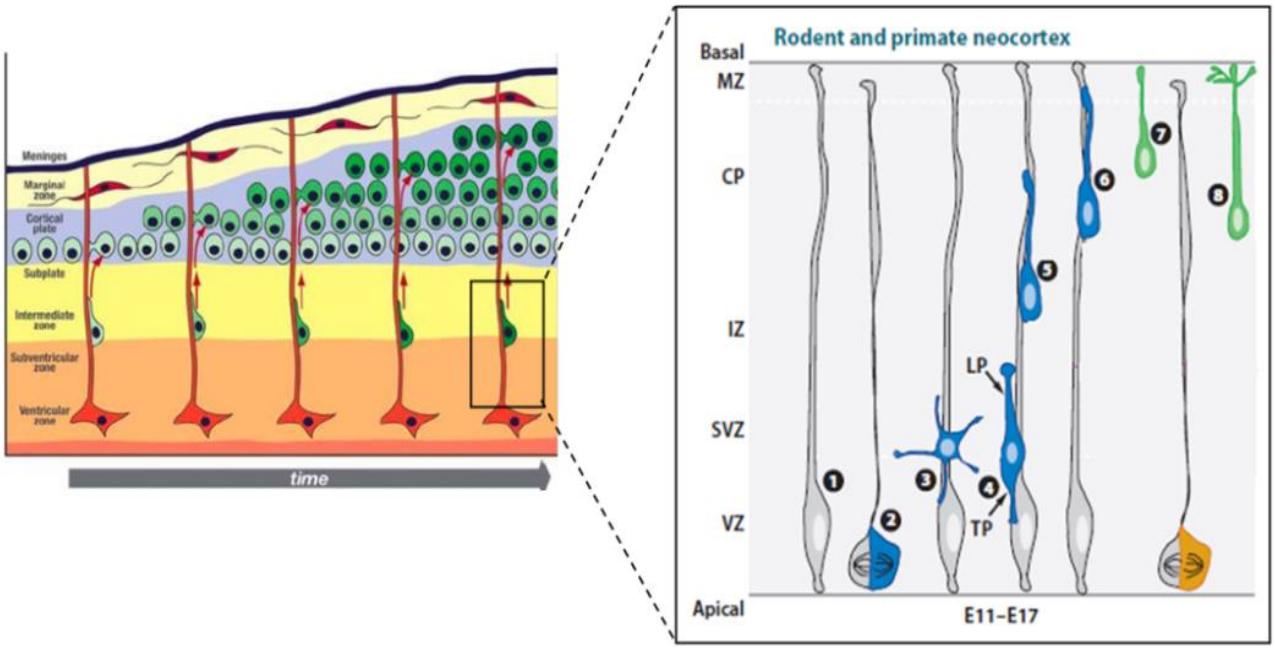


Figure 2: Projection neurons arise from NPCs in the ventricular zone. Left panel: Later-generated neurons migrate past earlier-generated neurons. This way, the cortical plate is created in an inside-out fashion. **Right panel**: Following division of radial glial cells (1), new-born neurons initially start migrating towards the cortical plate (2). Rather than migrating towards their final position continuously, new-born neurons transiently hold-up in the sub-ventricular zone (SVZ) and obtain a multipolar morphology (3). Following this interlude, a retrograde migration pattern occurs (3 to 4). Finally, neurons sprout two neurites, the leading (LP) and trailing (TP) process and obtain a bipolar morphology (4). This polarity shift marks the start of neuronal migration along radial glia towards the cortical plate (CP) (5). This figure was adapted from Bielas et al. (38) and Barnes and Polleux (39).

Evidence from animal studies points to an effect of MIA on projection neuron development (figure 3). In rats, LPS-induced MIA (on E15 and E16) influences the size of the neuronal progenitor pool indirectly through microglia by modulating NPC proliferation and apoptosis and by phagocytosis (43). It is not known whether polarization and migration of new-born neurons are affected in an immune challenged environment, although several observations are in favor of an effect on the **migration** of new-born neurons. In the case of MIA-induced deficits, a disrupted migration pattern for new-born neurons can be expected: offspring of H1N1 infected (E9) rodent dams exhibit a reduced expression of R**eelin**, a major regulator of radial migration of new-born neurons (44). Whether neuronal migration is effectively affected has yet to be determined.

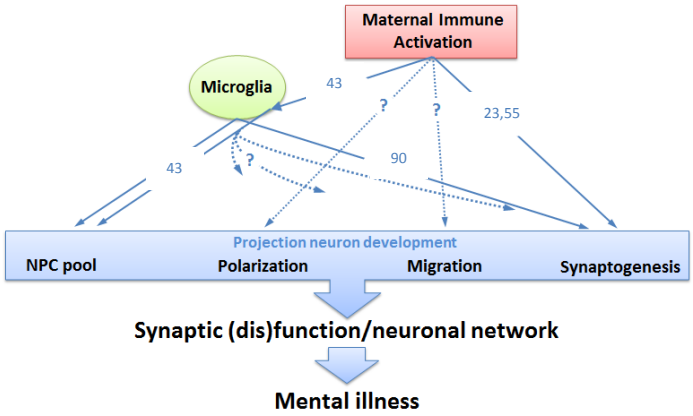


Figure 3: The impaired neuro-immune crosstalk induced by maternal immune activation (MIA) results in a direct impact on projection neuron development. Additionally, embryonic microglia integrate the inflammatory signals produced by the infected mother altering normal microglial function and interfering with early events in neurodevelopment. Both events increase the risk of developing behavioral defects later in life. (full lines with numbers indicate published connections and references respectively; dotted lines are putative connections that will be studied in the current project proposal).

3 Project outline

Based on recent publications the goal of this study is to determine the effects of Poly(I:C)-induced MIA on the developing projection neurons during embryogenesis. The immune system of pregnant mice is stimulated by injecting the double stranded RNA poly(I:C) (20 mg/kg) into the pregnant mice at E11,5. It is theorized that MIA may influence the proliferation, apoptosis, polarization and migration of progenitors, or that MIA may have a detrimental impact on the differentiation rate of these progenitors into excitatory projection neurons. This could eventually disturb the sprouting of neurites from these neurons and thus influence synaptogenesis. Particularly the influence of MIA on the polarization of new-born neurons and their migration into the cortex remains unexplained. In this study, it is **hypothesized** that MIA distorts the embryonic projection neuron development at the level of **proliferation** and **migration**.

In order to test this hypothesis, the following key **objectives** are set:

A. Observation of a Poly(I:C)-induced effect on the **general layering** of the foetal cortex.
B. characterization of the impact of MIA on the **proliferation** of progenitors.
C. Characterization of MIA-induced differences in **migration**-related processes and its effect on **migration** of new-born neurons into the cortical plate.

<u>Objective A: Observation of a Poly(I:C)-induced effect on the general layering of the foetal cortex</u>

As a primary objective, proper layering of the cortex will have to be assured. A total of three markers will be used to analyze the **general layering** and differentiation rate of the precursors in each section of the cortex: TBR1 (layer6), CTIP2 (layers 6,5) and CUX1 (layers 4 to 1). This way, major errors in the layering of the cortex can be discovered. The presence of every group of layers is the most important parameter for these stainings. For this objective, the effects of a single injection (E11,5) will be compared to the control. The potential influence of a secondary stimulation of the maternal immune system later during development (E15,5) on the effects observed after one injection will also be studied.

Objective B: characterization of the impact of MIA on the proliferation of progenitors

In the interest of studying the proliferation of progenitors in the foetal brain in a mouse model of MIA, immunohisotchemical stainings will be performed. After tissue collection (E17,5), a staining for Ki67 will be performed. Ki67 is a marker for mitosis and is present on all mitotic cells (45). Also for this objective, the potentially different effects of a single and double stimulation of the maternal immune system will be studied.

Objective C: Characterization of MIA-induced differences in migration-related processes and its effect on migration of new-born neurons into the cortical plate.

Considering earlier indications of a disturbance in the expression of Reelin upon maternal immune activation, the expression of Reelin in the foetal brain upon Poly(I:C)-induced MIA will be studied. A western blot analysis and quantitative PCR (qPCR) will be performed in order to examine respectively the protein and mRNA expression of Reelin in the offspring upon maternal immune challenge induced by administration of Poly(I:C).

Second, precursors for layer 4 pyramidal neurons will be labelled in order to ensure visualization. By applying in utero electroporation (IUE), a fluorescent marker will be introduced into progenitors for these projection neurons in the lateral ventricle. The technique is cell-specific as a result of a localized injection of the plasmid combined with developmental characteristics specific to the embryonic age of the developing brain when electroporated (46, 47). The usage of mild electrical stimuli will temporarily reorganize the phospholipids of which the cell membrane of the precursors is constructed, thereby temporarily deregulating the sturdy, hydrophobic character of the cell membrane. In order to achieve the correct spatiotemporal targeting of projection neurons in the lateral ventricle, the electroporation will be performed at embryonic age E14,5. The brain tissue will be analyzed after sacrificing the mice at E18, when most progenitors have differentiated into neurons and many settled in the cortical plate. Results will be quantified by measuring the distance from the projection neurons to the lateral ventricle (LV). The thickness of the cortex will be measured and divided into ten equal sections. The signal coming from the electroporated projection neurons in each section will be measured using fluorescence microscopy.

Materials and methods

Animal model

Female C57BL/6 mice were purchased from Harlan Laboratories (Gannat, France). All animal experiments were in compliance with guidelines provided by the institutional animal care and ethics committee of Hasselt University. Mice were fed ad libidum with an 18% protein rodent diet (Harlan laboratories, Horst, the Netherlands) and group-housed in the temperature and humidity-controlled animal facility of the BIOMED research institute.

Pregnant mice received an intraperitoneal injection of 20 mg/kg Poly (I:C) (LMW) (Invivogen, Toulouse, France) or physiological water. Unless otherwise mentioned, animals received a single injection of 100 µl at E11,5. After 5 hours, an immune response was confirmed by measurement of maternal serum interleukin-6 (IL-6) cytokine levels.

ELISA

Serum samples were obtained through tail tip removal and centrifugation of the sampled product for 10 minutes at 8000xg at 4°C. After collection of the serum samples, they were stored at -20°C. To measure the exression of IL-6 in the maternal serum, the Mouse IL-6 ELISA Ready-SET-Go kit from eBioscience (San Diego, United States of America) was used according to the suppliers instructions. In brief, a corning Costar 9018 96 well-plate was coated by incubation with anti-mouse IL-6 overnight at 4°C. Wells were blocked by addition of 1X assay diluent, provided with the kit. After preparation of a standard curve, 100 µl of the sample was added to each well. Following a 2-hour incubation period, anti-mouse IL-6 biotinylated detection antibody was added to the samples. An incubation period of 1 hour was respected. Subsequently, Avidin-HRP was added to the plate, followed by an incubation period of 30 minutes at room temperature. Next, substrate solution was added to each well. After 15 minutes of incubation, the stop solution was added. The plate was read at 450 nm using the BMG Labtech Fluostar optima (BMG Labtech, Offenburg, Germany).

Western blot analysis

Expression of Reelin was measured using western blot analysis. At E18, foetal brains were collected. Immediately following tissue isolation, foetal brains were freeze-dried in liquid nitrogen and stored at -80°C. Proteins were extracted by addition of RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1mM EDTA, 1% NP-40, 0,25% Na-deoxycholate and 1 EDTA-free ROCHE protease inhibitor for each 50ml) and centrifugation at 12500 g for 15 minutes at 4°C. The collected lysate was stored at -80°C.

Protein concentrations were determined using a BCA protein assay kit (Thermo-Fisher, Erembodegem, Belgium). For every sample, an amount of 20 µg was loaded onto a 12% Sodium Dodecyl Sulphate (SDS) polyacrylamide gel for 90 minutes at 200 V in electrophoresis buffer (25 mM Tris, 192 mM glycine, 0,1% SDS). Following activation of a polyvinylidene fluoride (PVDF) membrane for 15 minutes in 100% methanol, proteins were blotted onto the membrane for 90 minutes at 350 mA. Subsequently, aspecific binding spots were blocked with 2% non-fat milk in TBS-T (20 mM Tris, 150 mM NaCl, 0,1% Tween, pH 7,6) for 2 hours. Afterwards, the membrane was incubated overnight

at 4°C with mouse anti-reelin (R4B, Developmental Studies Hybridoma Bank, Iowa, USA) diluted in blocking solution at a final concentration of 0,5 µg/ml. Finally, the membrane was incubated for 2 hours at 20-24°C with horse raddish peroxidase (HRP)-conjugated rabbit anti mouse (Dako, Heverlee, Belgium) diluted in blocking solution to a final concentration of 0,65 µg/ml. Expression of Reelin was normalized for β-actin by overnight incubation of the membrane at 4°C with mouse anti-β-actin (Santa Cruz biotechnology, USA) at a concentration of 0,08 µg/ml, followed by a 1-hour incubation with rabbit anti-mouse HRP (DAKO, Heverlee, Belgium) at a concentration of 0,52 µg/ml. Both the primary and secondary antibody were diluted in 2% BSA-TBS-T. Expression of Reelin and B-actin was visualized with the enhanced chemiluminescence (ECL) technique by applying Pierce ECL Plus Western Blotting Substrate (Thermo Fisher, Erembodegem, Belgium) according to the instructions provided by the manufacturer. Membranes were incubated in the dark for 5 minutes before detection of the signal using the ImageQuant LAS 400 apparatus (GE Healthcare, Diegem, Belgium). For quantification of Reelin expression, ImageQuant TL software (GE Healthcare, Diegem,Belgium) was used.

Quantitative real-time PCR

Foetal brains were harvested at E18, followed by hemisection and immediate freeze-drying of the tissue in liquid nitrogen. Tissues were stored at -80°C until RNA was isolated. Total RNA was extracted from whole hemispheres using the RNeasy Plus Mini kit (Qiagen, Antwerp, Belgium) according to the guidelines provided by the manufacturer. RNA was converted into cDNA in a 20µl reaction by reverse transcriptase with random primers using the high capacity cDNA RT kit (Applied Biosystems, Erembodegem, Belgium). Starting with a 5 ng/µl cDNA concentration, RELN expression was measured in duplo in 3 samples per group by quantitative real time PCR (qPCR) and quantified using a SYBR green mix (Applied Biosystems, Erembodegem, Belgim). The reaction was run on a 7900 HT Fast-Real-Time PCR system (Applied Biosystems, Erembodegem, Belgium). Primer sequences for RELN (Integrated DNA Technologies, San Diego, USA) were as follows: (5' to 3'): GAAGGACTTCACACAAGCTC (Forward), TAAGCGACCTTCGTCTTCTG (Reverse). The reaction protocol contained an initial phase of 20s at 95°C, followed by 40 amplification cycles consisting of a denaturing step of 3s at 95°C and an extension step of 28s at 60°C. For analysis, the Lyvak method (ΔΔCT method) was used. Ct tresholds for every sample were normalized for the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Primers for the HPRT-gene were obtained from Qiagen. In order to calculate the fold change of RELN expression in every Poly(I:C)-treated sample compared to the control, the mean ΔCt of three control samples was calculated and substracted from the ΔCt of all three Poly(I:C) samples.

In utero electroporation

In order to visualize neuronal migration, a plasmid expressing dTomato under a pCAG promoter was purified using an EndoFree Plasmid kit (Qiagen, Antwerp, Belgium) and *in utero* electroporated into neuronal precursors targeting the lateral ventricle.

At E14.5, uterine horns were exposed after induction of general anesthesia with 3% Isoflurane ventilation (Abbot, Wavre, Belgium) and maintenance of anesthesia at 1.5% Isoflurane. The plasmid was mixed with 1% fast green and diluted to a final concentration of 2µg/µl in TE-buffer. In utero electroporation was performed as described earlier(47). Briefly, the plasmid mixture was injected

into the lateral ventricle using a glass micropipette (20 µm opening) derived from a microcapillary tube. Five electronic pulses of 37V each (50 ms on, 950 ms interval) were administered to the foetal head targeting the frontal-medial part of the cortex. Therefore, the positive electrode should be placed at a dorsal-medial position. Electroporation was achieved using the Electro Square Porator ECM 830 (BTX Harvard Apparatus, Massachussets, USA), provided with tweezers supplied with platinum coated electrodes. The uterine horns were re-positioned back into the abdominal cavity before closing the wound. Muscular layers were sutured using Vicryl 19mm sutures, while the skin is closed with wound clips. At E18, pregnant dams were sacrificed by means of cervical dislocation. Upon exposure of the uterine horns, foetuses were removed from the amniotic sac. After decapitation and removal of the skin and skull, the foetal brain was isolated and fixed in 4% Paraformaldehyde for 6 hours at 4°C.

To test the impact of the manipulations intrinsic to IUE on the brain and assure a lack of microglial activation upon in utero electroporation, a transgenic CX3CR1-eGFP mouse line was purchased from Harlan Laboratories (Harlan Laboratories, Gannat France). In these animals, a locus of the CX3CR1 receptor was replaced by a construction encoding for the enhanced green fluorescent protein (eGFP). CX3CR1-eGFP mice were sacrificed by cervical dislocation 24 hours post-IUE. Foetal brains were isolated and fixed overnight at 4°C.

Fluorescence imaging of neuronal migration in brain slices

To allow examination of the migration pattern of the electroporated projection neurons, foetal brains were submerged in PBS overnight at 4°C. Subsequently, coronal brain sections (100 µm) were obtained with the Microtome Prosan HM 650 Vibratome (Thermo Fisher, Erembodegem, Belgium). Brain slices were stained free floating with DAPI (4 v/v%) for 1 hour before mounting them with Vectashield Hard set mounting medium (Vector Laboratories, Burlingame, USA).

For fluorescence microscopy, digital images were obtained using a C-HGFB Nikon IntensiLight fluorescence microscope equipped with a Nikon Eclipse 80i camera. Quantitative measurements were performed with Image J software. After marking the region of interest, the length of the cortex was divided in ten equal segments (bins). Mean gray values were measured for every segment using Image J and normalized for total mean gray value in the region of interest. Images were taken at a 10x magnification with an intensity of 250 ms and a gain of 1.70x.

Immunofluorescence

In order to study the general layering of the cortex, foetal heads were fixed in 4% PFA for 5 hours at 4°C and subsequently submerged overnight in a 30% (m/v%) sucrose solution at 4°C. Tissues were imbedded in OCT mounting medium prior to being snap-frozen in liquid nitrogen. Coronal brain sections of 10 µm were sliced using the Leica CM 3050S cryostat. Coupes were stored at 4°C.

Prior to immunehistochemical staining, coupes were re-hydrated in PBS and antigen retrieval was obtained by placing the coupes in Trisodium-citrate dehydrate solution (291.4M, pH6) at 90°C. After three PBS-washes, coupes were blocked in 10% NXS, 0.2% Triton X-100 for 1 hour at 20-24°C before incubation with a primary antibody overnight at 4°C. The following primary antibodies were applied: goat anti-Cux1 (1:25; Santa Cruz Biotechnologies), rat anti-CTIP2 (1:200; Abcam), rabbit anti-Tbr1

(1:200; Abcam) and rabbit anti-Ki67 (1:200, Abcam), as mentioned in table 1. Coupes were incubated with the proper secondary antibody (Invitrogen), dissolved in blocking solution (1:500), for 1 hour at 20-24°C (table 1). Finally, the coupes were washed in PBS three times and dipwashed in MilliQ prior to mounting with DAPI-containing Vectashield (Vector Laboratories, Burlingame, USA)

Table 1: List of primary and secondary antibodies used.

Marker	Primary antibody	Secondary antibody
Tbr1	Rabbit anti-Tbr1, 1:200 (Abcam)	Donkey anti-Rabbit Alexa fluor 555, 1:500 (Invitrogen)
CTIP2	Rat anti-CTIP2, 1:200 (Santa Cruz Biotechnologies)	Goat anti-Rat 555 Alexa fluor 555, 1:500 (Invitrogen)
CUX1	Goat anti-CUX1, 1:25 (Santa Cruz Biotechnologies)	Donkey anti-Goat Alexa fluor 555, 1:500 (Invitrogen)
Ki67	Rabbit anti-Ki67, 1:200 (Abcam)	Donkey anti-Rabbit Alexa fluor 555, 1:500 (Invitrogen)

Statistical analysis

Considering small sample sizes, absence of the assumption of homogeneity and normal distribution, non-parametric analyses were applied using Graphpad 5. Data are presented as the mean ± SEM and are considered significant when $p < 0,05$, as indicated with an asterix.

Results

Poly (I:C) injection results in an increased serum IL-6 expression.

In the interest of checking an immune response following a poly(I:C) injection, serum IL-6 expression was measured in immune-stimulated pregnant dams. Pregnant animals received an intraperitoneal injection of 20 mg/kg Poly(I:C) at E11,5. Expression of IL-6 was quantified in serum samples drawn 5 hours post-injection, shown in table 2. Average IL-6 concentration of samples from poly(I:C) injected dams were compared to those of pregnant mice injected with a physiological saline solution. Data were plotted in figure 4. Expression levels of IL-6 in the serum of pregnant mice significantly increased upon exposure to Poly(I:C) (3705,38 pg/ml ± 833,99) when compared to vehicle injected dams (2611,88 pg/ml ± 173,51) (p-value = 0,0283). An immune response was confirmed for all animals included in further experiments. However, IL-6 levels in one sample (indicated by an arrow) did not significantly differ from the mean of the control group (p-value = 0,4584). This animal was excluded from any further experiments.

Table 2: serum IL-6 concentration for every Poly(I:C)-injected dam

Poly(I:C) Sample	IL-6 (pg/ml)	Significantly different from the control group?, (p-value)	Included/excluded
1	4700,25	Yes (<0.0001)	Included
2	3767,50	Yes (<0.0001)	Included
3	3693,75	Yes (<0.0001)	Included
4	2660,00	No (0,4585)	Excluded

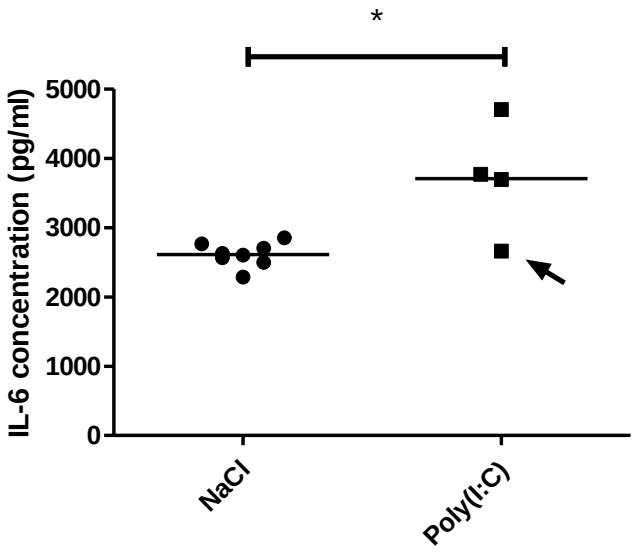
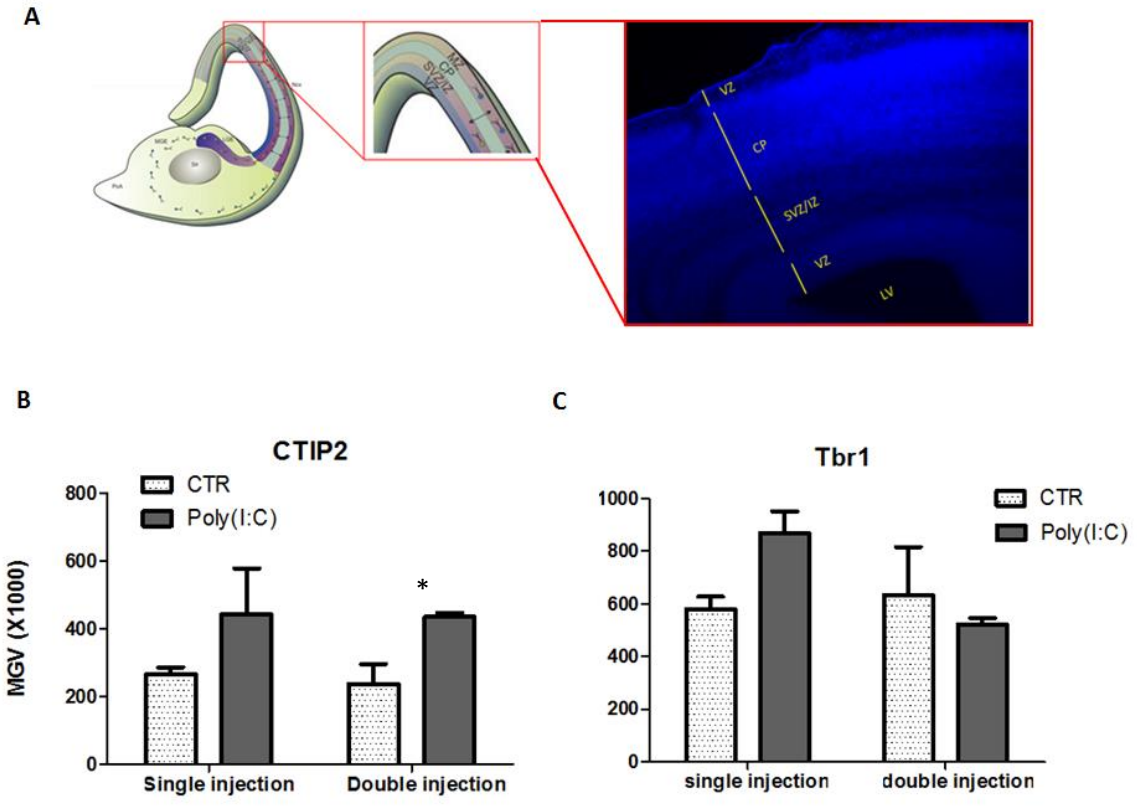


Figure 4: Serum levels of IL-6 increase upon stimulation of pregnant dams with Poly(I:C). In order to confirm an immune-stimulatory effect of Poly(I:C) in pregnant dams, serum concentration of IL-6 were measured. The concentration of IL-6 in serum samples in the Poly(I:C)-treated group (n=4) (3705,38 pg/ml ± 833,99) was significantly higher than in the control group (n=8), (2611,88 pg/ml ± 173,51), (p-value = 0.0283). All animals used for further experiments in this study showed a reaction to the injected Poly(I:C). IL-6 concentration of one sample (indicated by a black arrow) was not significantly different from the average IL-6 concentration in the control group (p-value = 0,4585) and was excluded from further experiments. *significant at α 0.05

MIA increases the number of CTIP2- expressing neurons in the foetal brain, but not the number of Tbr1- and CUX1-expressing neurons

Before any conclusions concerning neuronal migration can be made, the general cortical layering has to be investigated. Fluorescence imaging of E17,5 coronal sections, shown in figure 6A-C, was performed in order to study the effect of MIA on the neuronal distribution in the cortical plate. Lower layer neurons (L6, L5) in the cortical plate (CP) were stained with anti-CTIP2 (L6/5) and Tbr1 (L6), while upper layer neurons (L4 to L1) were visualized using anti-CUX1 (figure 5A). Mean gray values (MGV), which correlated to the amount of cells expressing respectively CTIP2, Tbr1 or CUX1, were measured in the layers of interest (respectively L6/5, L6 and L4 to L1). After subtraction of background signals, the presence of CTIP2- , Tbr1- and CUX1-expressing cells in the brains of offspring descending from infected mothers were compared to those descending from a saline injected mother. Furthermore, the potential presence of an altered response upon double injection of the pregnant dams with Poly(I:C) was also tested (figures 5B-D). To test for an effect of a single and double Poly(I:C) treatment, a two-way ANOVA followed by a Bonferoni post-hoc correction for multiple comparisons was performed. The density of CTIP2-expressing neurons in the lower cortical layers was not significantly higher following a single injection of the pregnant dams (n=3, p-value = 0,700). Double injection of Poly(I:C) in pregnant dams did significantly increase the density of CTIP2-expressing neurons in the offspring (n=3, p-value =0,0357). The density of Tbr1-expressing cells was not significantly influenced by a single (n=3, p-value = 0,4227) or double injection (n=3, p-value = 0,1900). The presence of CUX1-expressing, differentiated neurons in the upper cortical layers was not significantly influenced by a single injection of the pregnant mother (n=3, p-value = 0,7930), nor where the levels of CUX-1 expressing neurons after a double injection of the infected mother (n=3, p-value = 0,6579).



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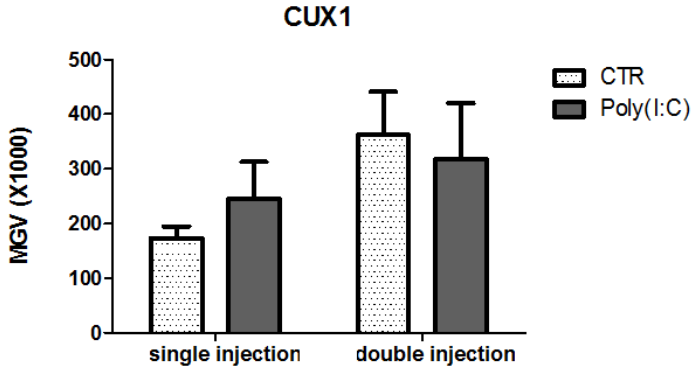
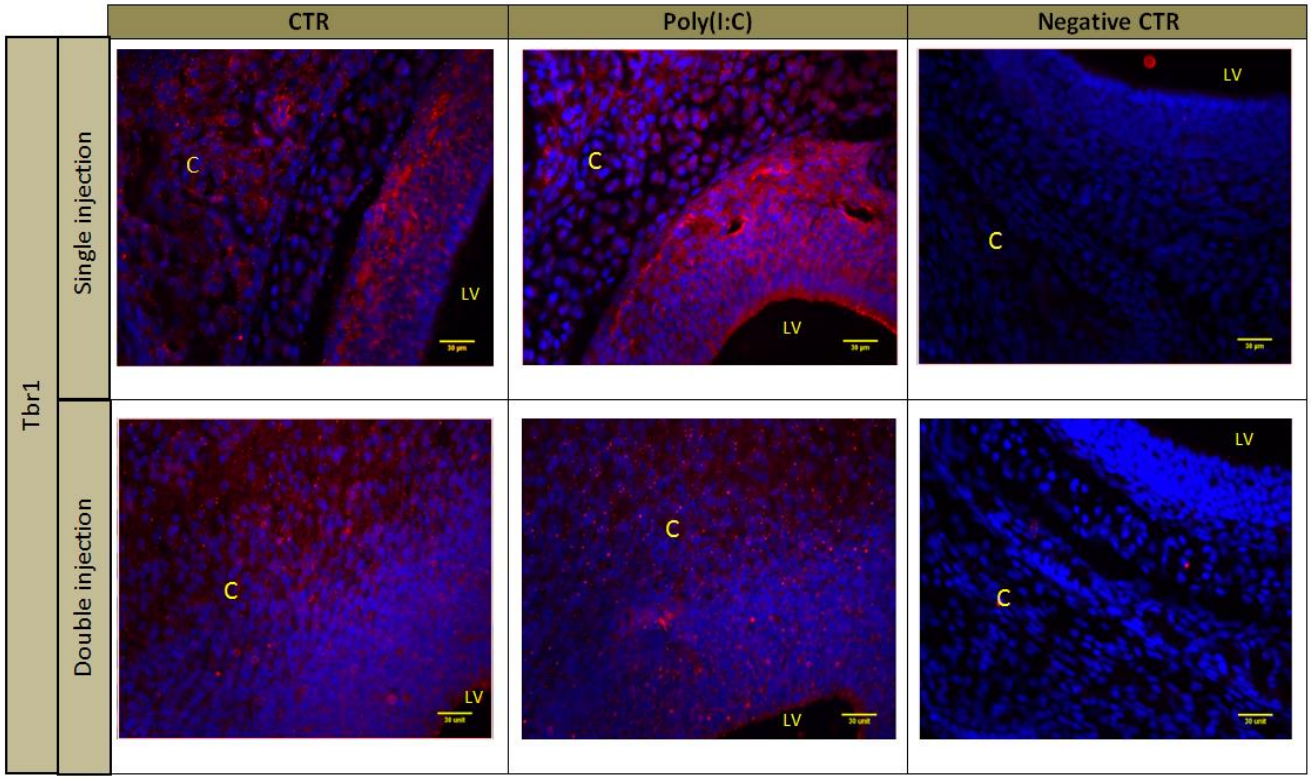


Figure 5:Expression of Tbr1, CTIP2 and CUX1 in the offspring of immune-challenged dams. A) Schematic representation of one half of a coronal brain section, figure adjusted from Tabata et al. (47) The targeted cortical plate (CP) and the location of each of its layers is shown in the red box. **B-D**) Mean gray value (MGV) measured in the targeted layer of the cortical plate after staining for respectively Tbr1 (L6), CTIP2 (L6,L5) and CUX1 (L4 to L1). A single injection of the pregnant dams with Poly(I:C) did not significantly increase the expression of CTIP2, Tbr1 and CUX1 in the foetal brain (n=3). A double injection however did increase the density of CTIP2-positive cells, but not the expression of Tbr1 and CUX1. * significant at α 0.05.

A



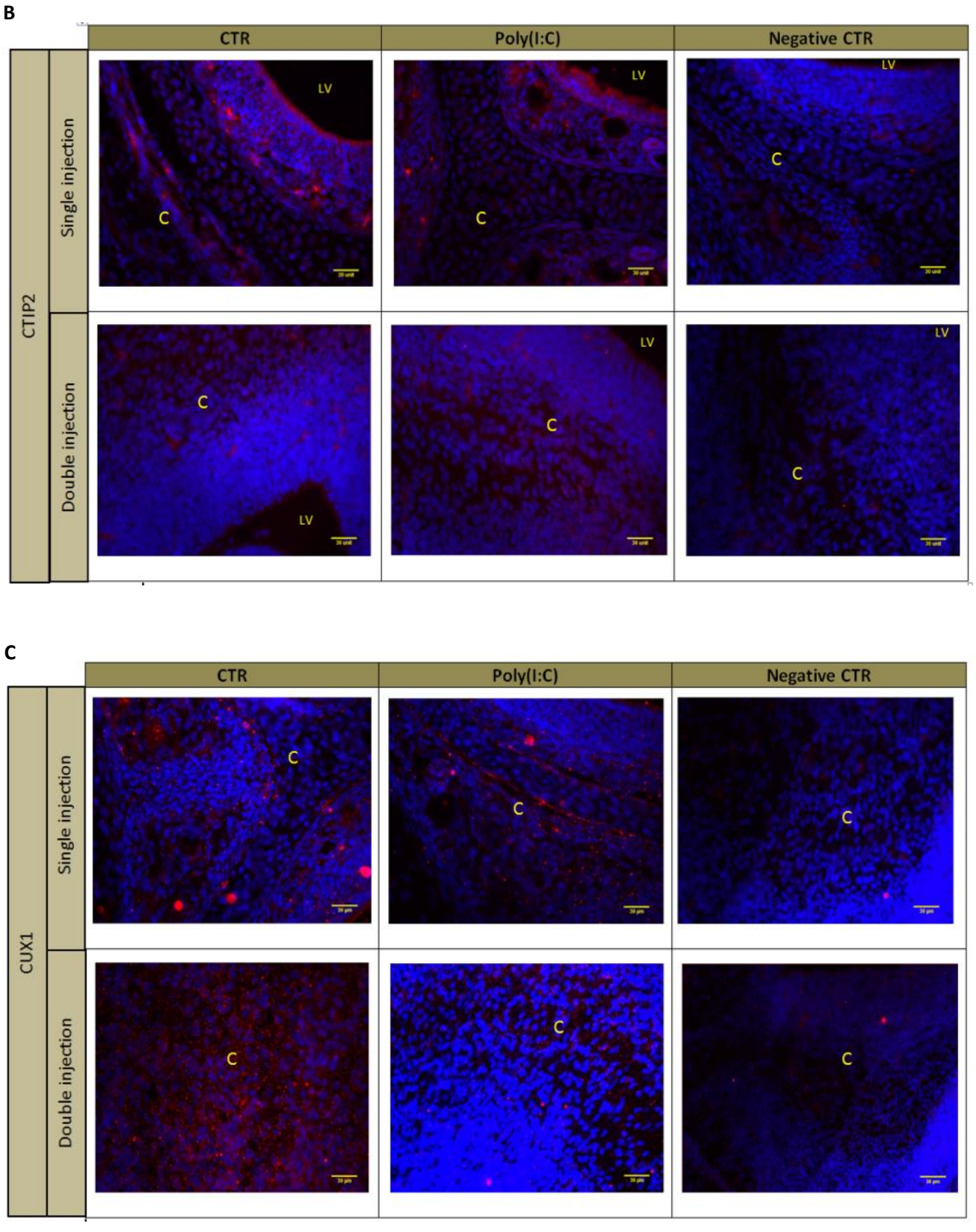


Figure 6: Fluorescence imaging (40x) of the cortex on E17,5 coronal brain slices after single and double injection with Poly(I:C) compared to a control. **A**) Images represent a staining for Tbr1 targeting cortical layer 6. Tbr1-positive cells are shown in red, Nuclei stained with DAPI are represented in blue. **B**) Staining for CTIP2 where cortical layers 6 and 5 are targeted. CTIP2-positive cells are shown in red, DAPI-positive nuclei in blue. **C**) Cortical layers 4 to 1 visualized with a staining for CUX1. Red cells represent CUX-1 positive cells, DAPI-positive nuclei are shown in blue. C indicates the cortex, LV the lateral ventricle. Scale bars represent 30 µm.

MIA influences the number of mitotic neuronal cells in the embryonic brain (E17,5) of the offspring.

Subsequently, the effect of Poly(I:C) on the proliferation of neuronal precursors was investigated. E17,5 coronal brain slices prepared from the brains of the offspring of immune-challenged dams were stained with anti- Ki67, as is shown in figure 7A. Mitotic cells express Ki67. Following immunohistochemical staining of the coronal sections, MGVs were measured and background noise was subtracted using ImageJ. A Two-way ANOVA and subsequent Bonferroni post-hoc analysis revealed a significant influence of both the Poly(I:C) treatment and the number of injections into the pregnant mice (figure 7B). The level of Ki67-expressing mitotic cells in the foetal cortex was significantly higher after stimulation of the maternal immune system with Poly(I:C) at E11,5 (n=3, p-value < 0.0001). An additional injection of Poly(I:C) at E15,5 further increased the presence of Ki67-expressing cells (n=3, p-value < 0.0001).

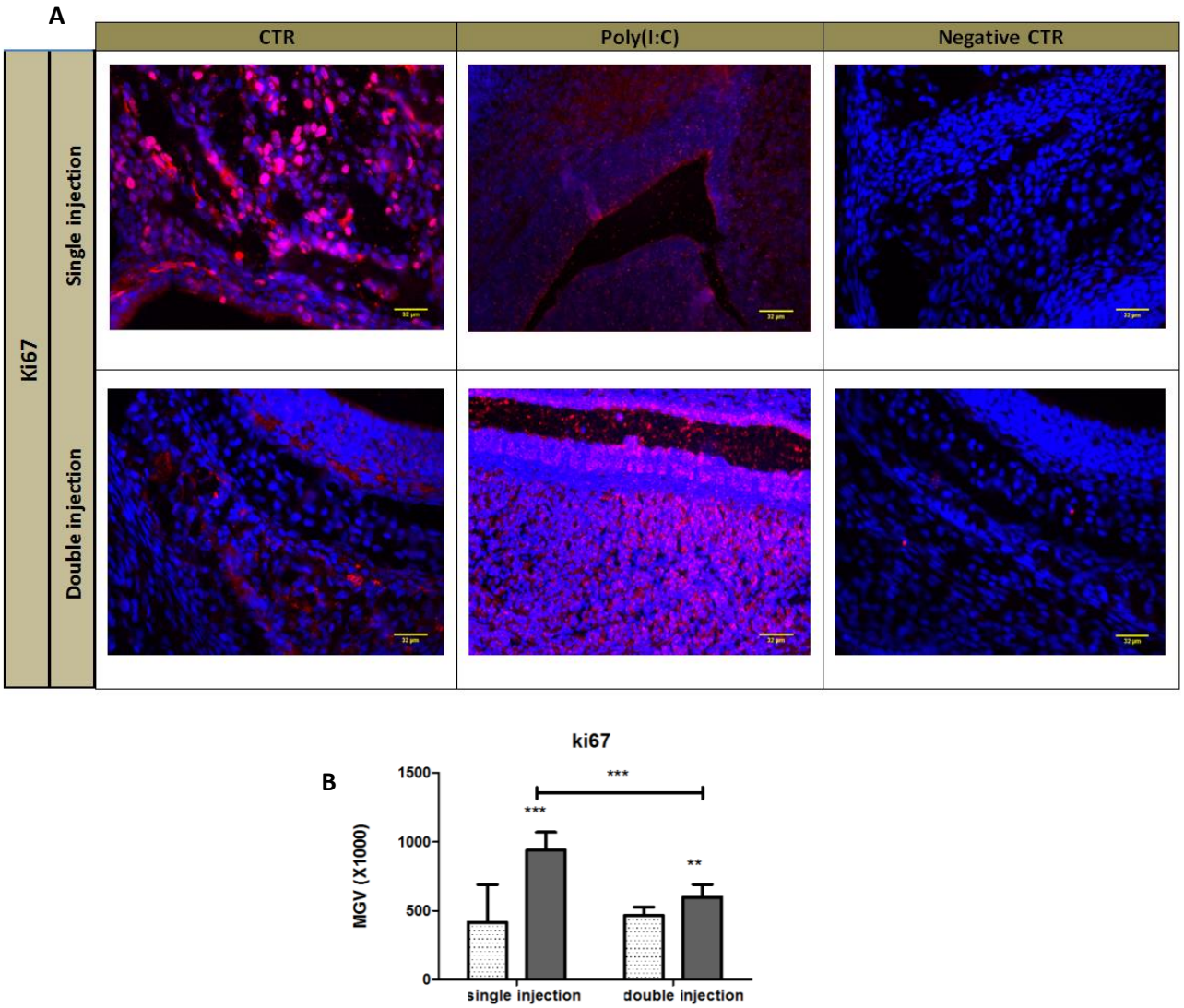


Figure 7: Mitoses of foetal brain cells is stimulated following stimulation of the maternal immune system with Poly(I:C). **A**) Fluorescence images of E17,5 coronal sections stained for Ki67 after stimulation of the maternal immune system during pregnancy with both a single and double injection of Poly(I:C). Ki67-positive cells are depicted in red, while nuclei stained with DAPI are shown in blue. **B**) Quantification of the expression of Ki67 by measurement of Mean Gray Value (MGV), corrected for background signal. Both injection with Poly(I:C) of pregnant dams and the number of injections correlates to the number of mitotic cells in the foetal brain. ** significant at α 0.01, ***significant at α 0.0001.

Maternal immune challenge reduces mRNA levels of RELN, but not expression of the Reelin protein, in the offspring.

Prior to studying the effect of Poly(I:C)-induced MIA in gestating dams on the cortical distribution of projection neurons in the offspring (E18), the influence of MIA on the expression of the Reelin protein, a migration-guiding protein, was studied. First, mRNA expression of the RELN gene was assessed in 3 individual samples per group by means of qPCR (figure 8A). Poly(I:C) treatement of gestating dams at E11,5 significantly reduced the expression levels of the RELN gene compared to those in cortical samples (n=3, p-value = 0,0500). Second, protein expression of Reelin was measured using western blot analysis (figure 8B). After quantification, the amount of Reelin protein in the foetal brains of offspring from Poly(I:C) stimulated mothers did not significantly differ from to the control (n=6, p-value = 0.4686).

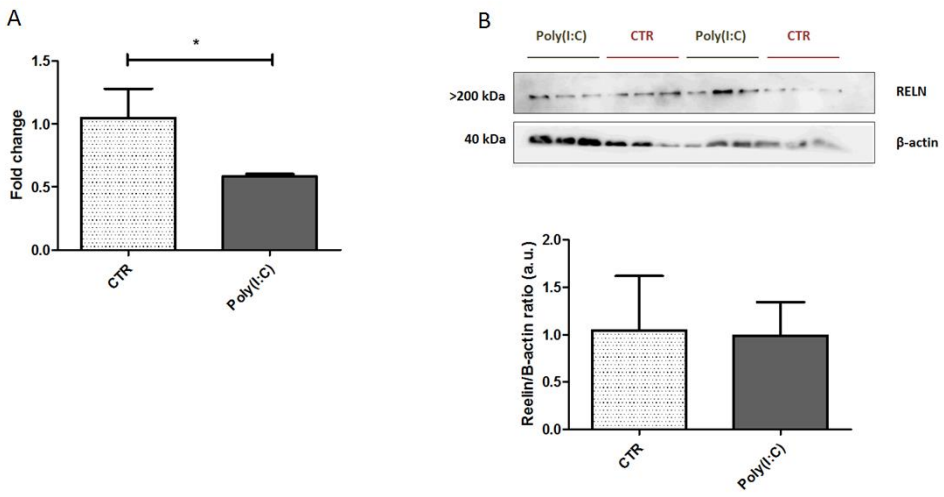


Figure 8: MIA reduces the mRNA expression of RELN, but not the expression of the Reelin protein. A) qPCR analysis demonstrate that Poly(I:C)-induced MIA significantly reduces RELN mRNA expression in the foetal brains of offspring (n=3, p-value = 0,0500). **B)** Western blot analysis demonstrate that Poly(I:C)-induced MIA in gestating dams does not influence the expression of Reelin protein in the foetal brains of the offspring (n=6, p-value = 0,4686). For quantification, Reelin expression was normalized to β-actin. * significant at $\alpha = 0,05$.

In utero electroporation does not result in the infiltration of microglia into the electroporated area.

Prior to electroporation of embryos of immune challenged mothers, the potency of manipulations intrinsic to the technique to stimulate a glial response was tested. For this experiment, CX3CR1-eGFP mice, with enhanced Green fluorescent proteins (eGFP) expressing monocytes, were used. Animals were electroporated with a plasmid expressing dTomato under a pCAG promoter at E14,5 and sacrificed 24 hours later. After selection of the targeted area, or region of interest (figure 9A, yellow selection), mean gray values within this region were measured. The intensity of the signal corresponds to the presence of microglia, as they are the sole eGFP expressing cells present. Data were normalized for mean gray value of the entire cortex (figure 9B). A Wilcoxon signed rank test revealed that the density of eGFP-expressing microglia did not significantly increase upon IUE (n=5, p-value = 0,1563).

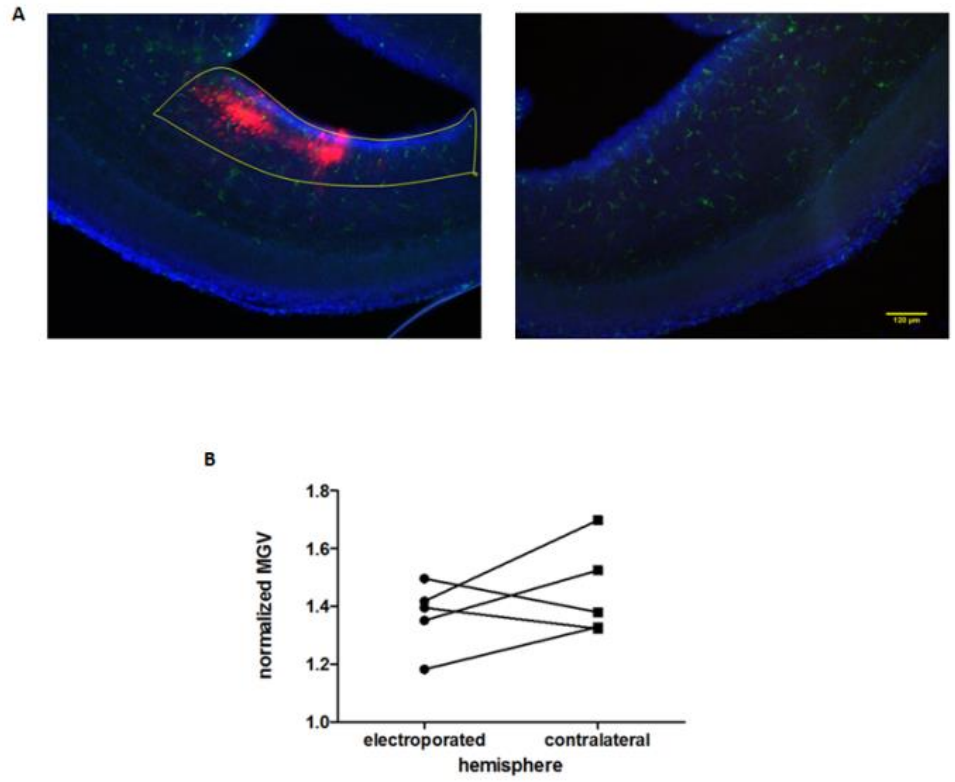


Figure 9: In utero electroporation (IUE) does not result in a glial response in the electroporated region. A) Fluorescence image of an electroporated (left) and contralateral (right) hemisphere of a CX3CR1-eGFP heterozygous foetus. The presence of microglia (green) in the electroporated region of interest (yellow) did not change as a result of the IUE. After IUE, dTomato (red) is expressed in projection neurons under a pCAG promoter. **B)** For every electroporated brain and every contralateral hemisphere, microglial infiltration was assessed by measuring the mean gray value in the region of interest and corrected for total mean gray value in the cortex. IUE did not significantly increase the density of eGFP expressing microglia (n=5, p-value = 0,1563). MGV = mean gray value.

Poly(I:C)-induced MIA does not influence the distribution of projection neurons over the E18 foetal cortex.

To study the fate of layer 4 projection neurons, a plasmid expressing dTomato under a pCAG promoter region was in utero electroporated into progenitor cells in the lateral ventricle at E14,5. Analysis was performed on fluorescent images of E18 coronal brain sections (fig 10A). The electroporated area overlapping the region of interest (geen box) was used for analysis. After measuring the cortical thickness, the cortex was divided into 10 equal bins. The density of projection neurons in every bin was quantified by measuring the MGV for every bin and normalizing it to the total mean gray value in the region of interest (figure 10B). The distribution of projection neurons across the cortex did not change as a result of Poly(I:C)-induced MIA, as the density of projection neurons in the Poly(I:C) group (n=3) was not significantly different from the control (n=5) in any of the bins.

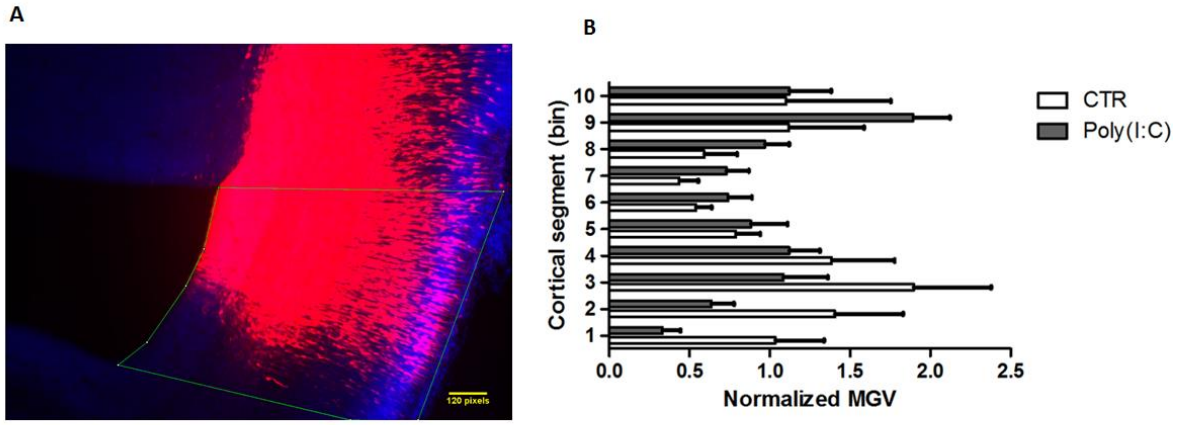


Figure 10: Neuronal positioning across the cortex is not altered in the offspring of Poly(I:C)-stimulated dams. A) Fluorescence image of a foetal brain (E18) following in utero electroporation (E14,5) of progenitors for layer 4 projection neurons with a plasmid containing dTomato expressed under a pCAG promoter. Cells expressing dTomato are recognized by a red color. The region of interest was marked with a green box**. B)** After division of the cortex into ten equal bins, the density of dTomato-expressing cells within the region of interest was measured. The density of projection neurons in the Poly(I:C)-group (n=3) did not significantly differ from that in the control group (n=5) in any of the bins analyzed. MGV = mean gray value.

Discussion

Immunohistochemical stainings for CTIP2, Tbr1 and CUX1 did not reveal an aberrant effect of Poly(I:C)-induced MIA on cortical layering in the foetus. However, a second stimulus of the maternal immune system did increase the density of CTIP2-expressing neurons in the deeper cortical layers. Mitosis of projection neurons was increased as a result of Poly(I:C)-induced MIA. Furthermore, mRNA levels of the RELN gene were significantly decreased in the offspring of immune challenged mothers. However, the Reelin protein expression was not influenced. Distribution of layer 4 projection neurons across the foetal cortex was not influenced by MIA, indicating immune challenge does not influence neuronal migration during development.

Relevance of the poly(I:C) model in neurodevelopmental studies

Ever since the development of animal models simulating an infection during pregnancy, these models contributed greatly in the search for answers to some burning questions concerning the etiology of neurodevelopmental disorders. Several methods of mimicking an immune response in the gestating dam are common practice. Experimental design of studies employing animal models to study the relevance of MIA on specific neurodevelopmental aspects often rely on the administration of either a live virus or a synthetic agent mimicking a bacterial or viral immune response in the infected host (48). Optimal foetal development relies on a balanced interaction between the maternal and foetal environment, tightly controlled under physiological conditions (49). However, when strong maternal immune stimulation occurs, maternally derived cytokines may intrude the foetal environment and interfere with optimal development. The resulting disturbance of the cytokine balance in the developing brain may result in neurodevelopmental abnormalities founding the basis for psychotic illness later in life (48). Older studies, based on the administration of live pathogens such as the influenza virus H1N1 have proven to be effective and undeniably resemble human viral infections the closest. However, the use of live pathogens in a laboratory setting requires specific safety measures that cannot be implemented in all laboratories. Furthermore, some ethical concerns arise when administering these live pathogens to experimental animals, especially considering the availability of alternative strategies. Therefore, the use of synthetic molecules to stimulate an immune response by activation of the TLR gated pathways is a most welcomed alternative. In addition to the poly(I:C) model discussed in this study, the injection of the bacterial endotoxin LPS, which is part of the gram negative bacterial cell wall, is another well-established model, focusing on the stimulation of TLR4. For this study, however, MIA was induced by injection of poly(I:C), an analog for a double-stranded RNA molecule used to stimulate TLR3 on macrophages, dendritic cells and B-cells (20). In response, an activation of the immune system occurs, with specific interest to the activation of pathways resulting in an imbalance of the maternal cytokine balance. Particularly the expression of IL-1β, IFN-γ, IL-6, TNF-α and MCP-1 has been described (48). One of the most important advantages of the poly(I:C) model over alternative models such as the LPS-model relates to the timing of an immune response. With poly(I:C), the induction of an immune response is restricted to a 24 to 48 hour window, allowing for a very specific timing of the immune insult (50). As a result, a more standardized response, correlated to a more precise stage of embryonic development is possible. This in contrast to a rather uncontrollable timing and severity of an immune reaction introduced by LPS or live pathogens. The susceptibility of the growing foetus towards the

introduced cytokine imbalance at a specific time during its development may be crucial in determining the response and later consequences of MIA (19, 51). Furthermore, the intensity of the maternal immune response itself, and thus the exposure of the developing fetus to immune-stimulatory mediators depends on the gestational age (48). As described by Carpentier et al., the placenta is most sensitive to a cytokine imbalance at early-embryogenesis and switches towards a less-sensitive barrier around E13 to eventually become resistant to immune challenge around E14,5 (52). In addition, specific types of documented schizophrenic behavior in the offspring of immune-challenged mothers have been traced back to induction of MIA at specific gestational ages (19).

Nevertheless, like any animal model, the poly(I:C) model poses several limitations. Traditionally, one of the key arguments against the use of the poly(I:C) model concerns the use of rodent models to study complex neuropsychiatric disorders. For this study, as the objectives are limited to the observation of neuronal characteristics and not involve the observation of animal behavior, the validity of this argument is limited. A more crucial note however, as mentioned, is that no actual exposure to a live pathogen occurs, resulting in a much more restricted immune response. With the well-characterized activation of the TLR3-pathway, only one aspect of the innate immune response is stimulated. In real life, in addition to an early innate immune response, an acquired immune response is activated, strongly influencing the reaction towards the pathogenic agent. A fact that should be bared in mind when interpreting findings based on the poly(I:C) model (21, 50).

Poly(I:C) injection results in an increased expression of IL-6

Before any conclusions can be drawn from subsequent experiments, a Poly(I:C)-induced immune response in pregnant dams should be confirmed. Therefore, serum expression of IL-6 was measured and compared to samples from vehicle injected dams. The expression of IL-6 in the serum during late pregnancy was correlated to an increased incidence of schizophrenia in a cohort study (53). After applying the MIA-model, an enhanced expression of IL-6 in maternal serum as well as amniotic fluid, placenta and foetal brain was shown, suggesting an important role for the cytokine balance during pregnancy (54). Furthermore, IL-6 is known to be a key mediator of the effect observed upon Poly(I:C)-induced MIA. It has been indicated that the maternal injection of IL-6 alone already causes behavioral abnormalities in the offspring (54, 55). A significant difference between the Poly(I:C) and vehicle-treated group was observed (figure 4). For one animal, the measured concentration of IL-6 in the serum did not differ from those observed in the control group. This can be explained by a number of possibilities ranging from technical errors to physiological differences. First, it is possible a loading error was made when preparing the injection. Second, an error may have occurred during the injection of the animal. Third, it is possible the injected animal did not react to the injection as strongly as expected. This may have influenced the progression of the IL-6 concentration in the serum over time, causing the peak concentration to differ from the observed concentration. Finally, technical errors may have occurred during the processing of the serum sample and the preparation of the ELISA plate. This animal was excluded from further experiments.

MIA induces an increased cortical expression of CTIP2, but not Tbr1 and CUX1

Neuronal progenitors of the SVZ and VZ in the dorsal telencephalon give rise to neurons of six different cortical layers. These layers can be divided into upper layers (L4 tot L1) and lower layers (L6/5). Early during embryogenesis, projection neurons that inhabit the deeper cortical layers (L6/5) arise from progenitors in the VZ. Later, the upper-layer cortico-cortical projecting neurons arise from progenitors in the SVZ (56, 57). Deep-layer neurons (L6/5) arise much earlier than upper-layer neurons (L4 to L1) and are less limited in their developmental potential. Upper-layer neurons however have a much more restricted cell fate (58, 59). Neurons located in one cortical layer are generated at similar times and possess common qualities and characteristics. Nonetheless, multiple phenotypes co-exist within a single layer. Layer 5 of the cerebral cortex contains both pyramidal neurons projecting axons to the contralateral hemisphere, callosal neurons, and neurons projecting axons towards subcortical targets in the spinal cord, pons, thalamus and tectum, known as long-range subcortical neurons (58, 60). Both types of neurons have unique electrophysiological and morphological identities. Subcortical projection neurons project long apical dendrites into upper layers of the cerebral cortex, whereas the callosal pyramidal neurons project much shorter neurites(59).

Despite the fact that both types of neurons arise from the same progenitors at the same developmental stage, molecular mechanisms involved in their development differ greatly. The processes determining cellular identity and cell fate rely on the expression of specific molecular pathways. A chromatin remodeling protein, Satb2, lies at the basis of a callosal subtype, whereas subcortical projection neurons rely on the expression of the transcription factors Fezf2 and CTIP2 (also known as Bcl11b) (56). The latter is most pronounced in corticospinal motor neurons projecting axons into the spinal cord. Besides subcortical projection neurons in cortical layer 5, CTIP2 is also expressed in corticothalamic projection neurons of layer 6. Neuronal progenitors as well as post-mitotic progeny in layers 5 and 6 can therefore be marked with a staining for CTIP2 (58, 59). Tbr1 on the other hand is a putatively expressed transcription factor expressed mainly in early-born deep layer neurons that inhabit the preplate and cortical layer 6 and has a crucial role in cortical development (37). In addition, Tbr1 is responsible for the corticothalamic identity of layer 6 neurons, while downregulating the expression of CTIP2 and thus repressing subcerebral cell fates (61). In short, a staining for Tbr1 allows for a staining of layer 6 cortical neurons, while CTIP2 allows the visualization of specific subsets of deep-layer neurons in both layers 6 and 5. These early-born projection neurons are highly relevant in obtaining an organized neuronal network in the cerebral cortex. In addition, upper layer neurons can be stained with a staining for CUX1, expressed in all upper layer pyramidal neurons (L4 to L1), but not in interneurons. This transcription factor encoded by the CUX1 gene is classified within the homeodomain family of DNA binding proteins and has known influences in the repression of gene expression throughout various developmental stages, ranging from early-embryogenesis to adulthood. CUX1 is also involved in differentiation and cell cycle progression (62, 63).

The results of this study indicate that a second stimulation of the maternal immune system at E15,5 does not cause the density ofTbr1- or CUX1-positive neurons to differ from values observed after a single Poly(I:C)-induced stimulation of the maternal immune system. It can therefore be concluded that a second stimulation of the maternal immune system does not influence the placental cytokine

balance sufficiently in order to affect foetal cortical layering. This was expected based on the observations of Carpentier et al., who indicated that the placenta experiences a shift from very sensitive to immune stimulation towards insensitive to immune stimulation around E14,5 (52).

An increase in the expression of CTIP2 upon a double injection of Poly(I:C) as compared to the control was however observed. As CTIP2 is required for the correct formation of the corticospinal tract, an upregulation of CTIP2 as a result of MIA may indicate overpopulation of the cortex with corticospinally projecting layer 5 neurons. However, no significant change in CTIP2-, Tbr1 or CUX1-expressing cells was observed in the foetal cortex upon a single injection with Poly(I:C) in pregnant dams. These findings were in controversy with reports by Carperntier et al., who showed an altered laminar distribution of early-born and late-born projection neurons in the adult murine brain as a result of an LPS injection at E12,5. This resulted in an abundance of Tbr1 and CTIP2 expressing cells in superficial cortical layers, and a depletion of early-born neurons form deeper cortical layers. This effect was not observed for the later-born CUX1 positive projection neurons (64). However, before any rash conclusions can be drawn, it should be noted that the measuring of MGVs on photos derived with fluorescent microscopy is suboptimal. It would be much more accurate to obtain confocal images of the precise targeted layers and perform a more accurate analysis on the laminar positioning of projection neurons, normalizing for slice thickness as well. In addition, it would be more reliable to study the general cortical layering by performing a CTIP2/CUX1 double staining. For practical reasons, confocal imaging was beyond the scope of this project. In addition, as described in literature, the peak of CTIP2 expression has passed at E17.5 (56, 58, 65). It would therefore be advised to confirm a potential effect of MIA at an earlier gestational age. Later during development, another transcription factor known to be expressed in upper layer neurons, Satb2, directly binds to CTIP2 and initiates the recruitment of histone deacetylases in order to convert CTIP2 into a less active product (60, 65).

As a follow-up, the expression of CTIP2 and CUX1 should be quantified using alternative set-ups such as qPCR and western blot analysis. Furthermore, layering of the murine cortex should be controlled using other immunohistochemical markers. Whereas CTIP2 only stains subcortically projecting pyramidal layer 6 and 5 neurons, layer 5 pyramidal neurons could also be targeted with a staining for ER81. This transcription factor is found in all layer 5 neurons projecting to the spinal cord, and in 1/3th of the callosal projection neurons (66).

MIA increases the density of mitotic cells in the foetal brain

In addition to the layering of the cortex and the abundance in which deeper-layer and upper-layer neurons are present upon stimulation of the gestating mother with Poly(I:C), it is also highly interesting to obtain information on the mitotic ratio of cortical neurons in the offspring of immune-stimulated dams. In this regard, the expression of Ki67, a mitotic marker expressed by all mitotic cells, was studied. Data revealed a significant increase in the expression of Ki67 in foetal brains after a single stimulation of pregnant dams with Poly(I:C) at E11.5, indicating that the density of proliferating progenitors at E17,5 was influenced by the induction of MIA in the pregnant mothers at E11,5. However, this effect attenuated significantly when pregnant animals were stimulated again at E15,5. Little is known about the effect of a Poly(I:C) injection at E11,5 on the expression of Ki67. However, An increased density of pyramidal cells has been observed upon exposure of pregnant dams to the H1N1 virus at E9 (67). In addition, Stolp at al. showed reduced ventricular proliferation in

the foetal cortex after stimulation of gestating dams with LPS at E13,5 (68). The effect was already observed 8 hours later by staining against anti-phospho-histon H3 (pHH3). Therefore, the effects of a single Poly(I:c-C) injection early during development were in line with the expectations based on findings by other groups. However, opposite effects have been described resulting from Poly(I:C)-induced MIA during late-gestation. Poly(I:C) has been reported to induce a cell cycle arrest in radial glial precursors stunting their progression towards intermediate precursors. In this case, cortical neurogenesis was shown to be inhibited as a result of a Poly(I:C) injection, resulting in the absence of normal population of superficial layers, resulting in behavioral disturbances (69). These findings were based on the injection of Poly(I:C) at E16. This conclusion was confirmed by Soumiya et al., who stated that specific abnormalities in the cortical progenitors precede deficits in neuronal phenotypes (70), but also in this case, Poly(I:C) was injected during late-gestation. In this study, findings based on a second injection of Poly(I:C) at E 15,5 also showed an attenuation of the effects observed after one Poly(I:C) injection. Combined, this could indicate a different outcome between Poly(I:C)-induced MIA during early-gestation and late-gestation.

It is possible a disproportionate effect was observed as a consequence of the low number of animals in the study or the absence of multiple, sensitive markers. For future experiments, it is therefore useful to include measurements for additional markers expressed on radial glial precursors and intermediate precursor cells, as well as post-mitotic neurons. Radial progenitors sequentially express Sox2 and PAX6, Tbr2 and Tbr1. Therefore, while expression of PAX6 is typical for radial progenitors, intermediate progenitors are known to express Tbr2 and decrease the expression of PAX6. Postmitotic neurons however tend to express Tbr1 while downregulating the level at which Tbr2 is expressed (71). Furthermore, in addition to a Ki67-stain, the injection of 5-bromo-2-deoxyuridine (brdU) - which is a thymidine analog and therefore intercalates into the DNA of the dividing precursors- at E16,5 has a high deterministic value as well. All precursors dividing within a 24-hour window will be labeled with brdU. After selection of all dividing cells with confocal microscopy or flow cytometry, the rate at which the precursors leave the cell cycle can be determined. This way, a much more accurate conclusion concerning the effect of MIA on the proliferative rate of progenitors in the offspring can be made. Finally, it has been noted that Poly(I:C) exerts an effect on the rate at which projection neurons become apoptotic (69). The influence of Poly(I:C) on the apoptotic rate could also influence the size of the cell pool and should be studied.

Upon Poly(I:C)-induced MIA, mRNA expression of the RELN gene is reduced in the offspring, but Reelin protein expression remains unaffected.

As previously described, first born cortical neurons populate the preplate. Subsequent neuronal precursors migrate into the preplate and form the cortical plate. Next, later generated progenitors populate the cortical plate in an inside-out fashion and form the six cortical layers. The preplate is split into Cajal-Retzius cells and suplate cells by the formation of the cortical plate (37). Reelin, an extracellular matrix protein secreted by Cajal-Retzius cells in the marginal zone, is crucial in guiding neuronal migration, cortical lamination and radial glia morphology (72). Already in 1995, insufficient formation of the cortical plate was shown in reeler mice, lacking functional Reelin (73). A reduced expression of the protein has been described in several neurodevelopmental disorders amongst which autism and schizophrenia (72). An explanation as to why Reelin expression is altered in these

disorders is currently absent from the literature, although a role for early developmental insults has been suggested (72). Other possible explanations for a reduced expression of Reelin are hypermethylation of the promoter region for the RELN gene, mutation of the RELN gene, silencing of RELN by miRNA and posttranslational modulation of the Reelin protein.

Functional Reelin binds to two lipoprotein receptors: very low density lipoprotein receptor (VLDR) and apolipoprotein E receptor type 2 (ApoER2)(74), causing the clustering of these receptors and the activation of Src-tyrosine kinase. As a result, an adaptor protein downstream of these receptors, disabled1 (DAB1) is phosphorylated. Through downstream signaling cascades including the activation of the phosphatidylinositol-3-kinase (PI3K) pathway, glycogen synthase kinase is eventually inhibited. Under standard conditions, this kinase is responsible for the phosphorylation of the microtubule-stabilizing protein Tau in migrating cortical cells, preventing it from fulfilling its task. Therefore, Reelin is crucial to the cytoskeleton of migrating pyramidal neurons (72). In addition, phosphorylation of DAB1 also results in the mobilization of Lissencephaly 1, which is involved in neuronal migration and cortical lamination as well (72). Eventually, when the targeted neuron has reached its final cortical destination, a subpopulation of DAB1 is responsible for the ubiquination of DAB1 in order to put the effects of Reelin on the migration of pyramidal neurons to an end (72). It has also been suggested that Reelin itself functions as a stop signal to neuronal migration, although this has not yet been confirmed (75). Besides its crucial roles in the regulation of neuronal migration, Reelin is involved in the maintenance of synaptic plasticity as well. Later during development, the secretion of Reelin by Cajal-Ratzius cells decreases as its expression by GABAergic interneurons increases. Regulating the entry of Ca^{2+} through N-methyl-d-aspartate (NMDA) receptors and the phosphorylation of two of its subunits, Reelin is an important contributor to synaptic plasticity (72).

The Reelin protein consists of an N-terminal region, followed by 8 nucleotide repeat regions and a C-terminus. Multiple splicing variants of the Reelin protein have been described (74). Full length Reelin shows as a 410 kDa band on an SDS-Page gel. In addition, 5 splicing variants have been reported: NR6 (N-terminus to end of nucleotide repeat region 6), R3-8 (nucleotide repeat regions 3 to 8), R3-6 (nucleotide repeat regions 3 to 6), NR-2 (N-terminus to end of nucleotide repeat 2) and R7-8 (nucleotide repeat regions 7 to 8). Molecular weights of these splicing variants are respectively: 370 kDa, 270 kDa, 190 kDa, 180 kDa and 80 kDa (74). In this project, a western blot analysis was performed using the R4B antibody obtained from the Developmental Studies Hybridoma Bank (DSHB). This antibody recognizes the fourth nucleotide repeat region of the Reelin protein. Therefore, theoretically, all splice variants but R7-8 and N-R2 could be recognized with SDS-Page.

Three protein bands were present on the blot. The first protein band was larger than 200 kDa, which indicates it is either the full length Reelin protein, the NR6-isoform or the R3-8-isoform. In order to be certain which isoform is presented on the blot, it is recommended to repeat the western blot using a gel with a lower percentage of acrylamide and methylenebisacrylamide in order to increase the pore size. This way, proteins with a higher molecular weight (such as full length Reelin) have more time to be separated on the gel. In combination with a molecular weight indicator which is more accurate for bigger proteins, this will increase the accuracy of the blot. Considering the wide range of molecular weights between the R3-6-isoform and full-length Reelin, the use of a gradient gel would also be recommended. Another solution would be to run a 2D-fluorescence difference gel electrophoreses (2D-DIGE). In addition to the vertical separation based on molecular size, the different proteins are separated in another (horizontal) dimension based on their isoelectric point.

Nevertheless, the expression of Reelin protein in the foetal brain of offspring from Poly(I:C) treated dams did not differ from that in control mice. In addition to the >200kDa band, two more protein bands were present (data not shown). Considering they are both located between 50 and 75 kDa, they cannot be intact isoforms of the Reelin protein. Likely, these bands result from posttranslational modifications such as enzymatic cleavage or proteolytic cleavage by metalloproteases. Although it should be pointed out that the lysis buffer used did contain 1mM EDTA, a substance known to block metalloproteases. It has been hypothesized that the cleavage of full length Reelin by metalloproteases secreted by migrating neurons is essential in order to release central Reelin (splice variant R3-6), which than binds to the target cells and influences its migration (76). Despite loading a sufficiently high amount of protein (20 µg/well), it should be noted that protein signals on the blot appeared rather mild. Adjusting the concentration of primary and secondary antibody did not provide the desired improvement. In order to further improve the protein signal, blocking time may be reduced from 2 hours to 1 hour and excessive washing may be limited.

In contrast to the lack of significant changes in the protein expression of Reelin, a significant change in the mRNA expression of RELN was observed in the offspring of Poly(I:C) treated dams. The discrepancy between a reduced mRNA expression and a stable protein expression observed can be explained by post-transcriptional modification of the mRNA, translation of the mRNA into protein, posttranslational modifications of the protein or protein degradation. It is ultimately the protein expression which relates to migration as it is the Reelin protein which is involved in the regulation of migration.

The findings described above were only partially in line with expectations based on several publications. Reduced RELN mRNA expression in this study fits with earlier publications from Impagnatiello et al. who reported a 50% reduction in the mRNA expression of RELN in patients with schizophrenia (77), a finding which was confirmed by some (78, 79) yet contrasted by others (80). However, Impagnatiello et al. also showed a 50% reduction in Reelin protein expression, In addition, a reduction in Reelin levels as a result of prenatal (E9) infection with the H1N1 influenza virus has been shown (44). Furthermore, RELN was positively associated with schizophrenia in woman (81), suggesting a gender-specific effect, and in combination with other genes (82). However, it should be mentioned that a lack of significant correlation was observed in several other gene association studies (83). In autistic subjects, a reduced expression of both full length Reelin and two of its splice variants (NR6 and R3-6) was shown in several brain regions including the superior frontal cortex, parietal cortex and cerebellum (84). In the same regions, mRNA levels of both RELN and DAB1 have been shown to be decreased. Gene association studies concerning the RELN gene were inconsistent, which is not surprising considering the complex, heterogeneous nature of autism. A positive association was found between autism and the increased length of a GGC repeat just 5' of the RELN start codon, also termed 'long triplet repeat alleles' (>11 GGC repeats rather than the usual 8) (85). The same GGC repeat also causes reduced expression of the Reelin protein in autistic individuals (86). Furthermore, single nucleotide repeats (SNPs) in the RELN gene have been contributed to autism (87). A conclusion which was later dismissed by others (88).

The lack of significant changes in the expression of neither Reelin nor RELN was unexpected considering the multitude of evidence linking the expression of both the RELN gene and its protein product to schizophrenia as well as autism and other neurodevelopmental disorders. However, most evidence results from genome wide association studies and retrospective population studies.

Furthermore, changes in the expression of Reelin are often correlated to specific brain regions. Since samples in this study were obtained from entire hemispheres, it is possible the read-outs used in this study were simply not accurate enough to pick up small changes in particular regions, as they may have been masked by the abundance of total Reelin levels in the hemisphere. For future experiments, it may therefore be preferred to study the expression of Reelin in specific regions of interest. Furthermore, not a lot is known about the expression of other players in the Reelin-activated pathways, which is also something that may be taken into consideration for future studies.

Poly(I:C)-induced MIA does not influence the distribution of projection neurons over the E18 foetal cortex.

Despite several indications, including publications about a reduced expression of Reelin in the offspring of mothers suffering from a respiratory infection during pregnancy, whether or not migration of projection neurons is actually influenced in the offspring of immune-challenged mothers had yet to be determined. In the interest of visualizing the migration of precursors for projection neurons at a desired developmental time point, in a specific region of the brain, IUE was performed. By performing an IUE at E14,5 targeting the lateral ventricle, progenitors for layer 4 projection neurons are targeted (46, 89). Earlier electroporation targets deeper cortical layers, while electroporation at a later embryonic age reaches more superficial layers of the cortex. The set-up of this study is based on direct labeling of these precursors, as was previously described by Tabata et al. (47) and Matsui et al. (46). Studying various processes by means of IUE on foetal brains is a well-established technique that has proven to be effective previously (89). This set-up is particularly beneficial in neurodevelopmental settings as it allows for a direct, relatively non-invasive in utero approach. The technique allows for a direct gene-transfer into the targeted progenitors at a desired developmental age and location. In addition, it has practical advances that cannot be denied in regard of safety considerations. In contrast to other set-ups, the use of live viruses is not required. A plasmid carrying genetic material required for the fluorescent labeling of the target cell cell is expressed under a specific promotor. For this study, DNA for the fluorescent marker dTomato was expressed under a pCAG promotor region. After injection of the construct carrying the genetic material required to obtain optimal labeling of the targeted precursors, an electrode is placed over the foetal head and five short 37V pulses are given. The usage of five intermittent 37V shocks allows for targeting of more superficial cortical layers (46). As there is a clearly distinguishable signal in all electroporated brains, this set-up shows to be highly effective and reproducible in labeling precursors for projection neurons. By electroporating precursor cells, the composition of the phospholipid bilayer, of which their cell membrane consists, temporarily shifts towards a more permeable phase. This transition results in the creation of a ‘pore’ in the membrane through which a construct can enter the cell. By appliance of the mid-line sutures as anatomical landmarks when injecting the plasmid solution, the lateral ventricle can be targeted. Prior to the start of the expreriment, a modulatory effect of the manipulations intrinsic to IUE was tested. The results described in figure 9 of the results section do not suggest the presence of a confounding effect that can be attributed to the IUE itself. The presence of microglia at the site of injection does not differ from that in the contralateral hemisphere. Based on these results, it can be concluded that the potential presence of tissue damage following the injection is not sufficient to cause microglia-infiltration as part of the natural glial response. Considering the wide use of the technique in countless other studies, these results were expected. Ideally, the effect of the electroporation should

be tested in the electroporated hemisphere itself. With this in mind, a staining for the microglia/macrophage-specific marker Iba1 on brain slices retrieved from the area of interest can be performed. Whether or not this check-up should be standard procedure following an electroporation can be debated. The relevance of ruling out a glial response in the area of interest, possibly interfering with neuronal development, is clear. However, from a practical and financial point of view, this may be inefficient and time-consuming. As mentioned before, when performed with expertise, IUE provides reproducible results. Despite the potential of IUE, data did not reveal an effect of Poly(I:C) on the distribution of layer 4 projection neurons in the cortex. When repeating the experiment in the future, additional measures should be taken in order to drastically decrease standard deviation. In addition to increasing sample size, both the set-up used to electroporate the animals as well as the read-out could be optimized. First of all, sufficient training prior to the start of the study is recommended in order to standardize the timing and accuracy with which the procedure is executed. For this study, total duration of the IUE varied between animals, potentially allowing an effect of the anesthesia. As isofluorane increases viscosity of the blood –and therefore decreases blood flow towards the uterus- the total duration of the procedure may have affected the offspring of different mothers differently. In addition to influencing the abortion rate, this may have also influenced brain development in surviving fetuses as prenatal ischemia has been described to influence cortical development (90). Second, the method of administering the plasmid can be optimized. As described earlier, the plasmid is injected into the target tissue using a small needle, followed by internalization by neuronal precursors upon electroporation. In light of achieving more control over the area the plasmids are injected into, the plasmid solution was injected using a moth-controlled pipetting system. Due to the specific consistency of the different tissues in the foetal head, each tissue compartment requires a different pressure to load the plasmid solution into the area. By injecting the plasmid-containing solution by mouth, the pressure with which the solution is injected can be controlled. However, as this is neither a pressure fixed nor volume fixed method, optimal control over the amount of plasmids injected lacks. It would be desirable to use a volume-fixed system where a standardized volume of the plasmid solution is shot into the target tissue by appliance of compressed air. Finally, results are strongly influenced by the angle at which the paddle electrodes are placed over the brain. However, considering neither the positioning of the electrode on the foetal head nor the size of the foetal brain is fixed, delivery of the electric pulses varies in space between fetuses. As a result of this, the position of the precursors that take up the plasmid highly varies. Therefore, the area in which the signal is maximal does not always align with the targeted area. The frequency at which this problem occurred decreased significantly with practice. As an example, in figure 10, the targeted region is indicated with a green box. Unfortunately, the region which was actually electroporated falls mostly outside of this box. Therefore, measurements were restricted to the electroporated area that overlaps with the target zone, the region of interest. To ensure a more accurate analysis, cell density in each bin should be confirmed using confocal images rather than pictures obtained through fluorescence microscopy. Furthermore, rather than analyzing fixed positions using single photographs, migration speed can be calculated using time lapse imaging (89).

Future perspectives

Although the impact of findings in this study is limited, altered development of projection neurons as a result of early insults could provide important insights with regard to the neuro-immune crosstalk during embryonic development. This could aid our understanding about molecular processes underlying neurodevelopmental disorders.

In the near future, some of the variables in the current set-up of the experiments described in this work should be adjusted in order to increase the accuracy of the experiments prior to drawing an optimal conclusion regarding the impact of Poly(I:C)-induced MIA early during development on the formation of the foetal brain. Furthermore, the different subsets of projection neurons, all born on varying developmental ages, should be targeted.

Subsequently, other neurodevelopmental processes should be taken into consideration. The next step towards cognitive impairment is aberrant brain wiring. Therefore, the effects of MIA on synaptogenesis should be studied. Prenatal exposure to IL-6 results in a dysregulated glutamate/dopamine balance in the hippocampus, resulting in behavioral deficits (55). Several publications have been made describing a positive correlation between neurophyschiatric disorders and genes coding for receptors in the synaptic cleft and their downstream signaling targets (23). In addition, the mechanisms by which early epidemiological insults mark the development of the brain, causing an onset of schizophrenia at adolescence, should be clarified further. The notion that several targets crucial in neurodevelopment are epigenetically labeled, influencing the extent to which they are expressed, is considered valid by a growing audience. Therefore, studying the methylation status of target genes involved in several neurodevelopmental processes after stimulation of the maternal immune system would be a primary challenge.

Finally, the effects of Poly(I:C)-induced MIA on other brain cells should be studied. Colonization of the embryonic brain by microglia coincides with neuronal generation (91). In addition, indications that microglia are influenced by MIA have been provided in literature. During embryonic development, the size of the NPC-pool is regulated by microglial phagocytosis. MIA-induced activation of microglia diminished the number of NPCs (43). Furthermore, microglia promote proliferation of NPCs and programmed cell death of neurons during development. In addition, prenatal activation of microglia caused delayed impairment of glutamatergic function. Deficient microglia-neuron interaction results in impaired functional connectivity in the brain and disturbed social behavior (92). For these reasons, microglia are vital in regulating the density of viable neurons (93). The effects of MIA on the microglia-neuron interaction should be focused on.

Conclusion

Poly(I:C)-inuced MIA during early development did not cause any issues concerning general cortical layering. However, the density of mitotic cells in the foetal cortex increased as a result of MIA. In order to make a definitive conclusion concerning the turnover of projection neurons upon Poly(I:C)-inuced MIA, other markers should be tested. MIA resulted in a reduced mRNA expression of the RELN gene, but rendered the Reelin protein levels untouched. Whether or not downstream targets in the Reelin pathway regulating migrational processes is influenced by Poly(I:C) treatment of gestating dams, did not fall within the scope of this project. Nevertheless, neuronal migration of layer 4 projection neurons into the cortex was not influenced.

These findings only partially support the hypothesis that MIA distorts the embryonic projection neuron development at the level of proliferation and migration. Experimental read-outs should be sensitized in order to make a definitive conclusion. In combination with other research strategies for future research, many questions regarding this topic have yet to be answered.

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