The regulatory role of cytokines on neurodevelopment and behaviour during the early postnatal period : An investigation on the impact of TNF on mouse behaviour in the early postnatal period and a novel approach of data analysis applied to the immune activation mouse model of autism

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THÈSE DE DOCTORAT

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Titre (en français) : Le rôle régulateur des cytokines dans le neurodéveloppement et le comportement au début de la période postnatale

Résumé (de 1700 à 4000 caractères espaces compris)

Plusieurs études ont montré que l'activation du système immunitaire maternel (MIA) pendant la grossesse augmentait le risque de troubles neurologiques et d'anomalies du comportement dans la descendance. Afin d'étudier les mécanismes impliqués, plusieurs auteurs ont comparé le comportement de souris nées de mères injectées pendant la grossesse avec du poly(I:C), une molécule mimant une infection par le virus de la grippe, et celui de souris nées de mères injectées avec une solution saline. Bien que ces études aient permis de confirmer que l'activation du système immunitaire maternel pouvait induire des troubles du comportement, la majorité d'entre elles se sont fondées sur des tests comportementaux effectués chez la souris adulte. Ainsi, il reste à déterminer si la modification des niveaux d'autres cytokines pendant la période périnatale peut avoir une incidence sur le neurodéveloppement précoce et sur le comportement de la jeune souris. Pour répondre à cette question, nous avons caractérisé la descendance de plusieurs cohortes de mères injectées avec du poly(I:C) ou avec une solution saline, pour leur comportement entre 5 et 15 jours après la naissance et pour la concentration de plusieurs cytokines dans le sérum. Parce que le neurodéveloppement et la production de cytokines sont affectés par plusieurs variables, nous avons utilisé une analyse multivariée pour identifier les variables environnementales et biologiques associées au fait d'être le descendant d'une mère injectée avec du poly(I :C) (par opposition au fait d'être le descendant d'une mère injectée avec une solution saline). Nous avons constaté que la diminution du poids et de la température corporelle de la mère après injection de poly(I:C), la taille de la portée, le poids de la souris à 15 jours, le nombre de vocalisations ultrasonores (USV) émises par la souris à 6 jours, la distance parcourue par le souris et le temps passé immobile à 13 jours, ainsi que les concentrations sériques de TNF, IL-5, IL-15 et CXCL10 à 15 jours étaient associés au fait d'être le descendant d'une mère injectée avec du poly(I :C). Pour continuer à explorer le rôle régulateur du TNF, nous avons injecté quotidiennement du TNF recombinant à des souris nouveau-nées entre le jour 1 et le jour 5 après leur naissance, et nous avons étudié leur développement et leur comportement entre le jour 8 et le jour 15. Contrairement à nos attentes, l'injection de TNF à des souris nouveau-nées n'a pas d'impact négatif sur le développement, mais favorise plutôt l'acquisition de réflexes sensorimoteurs et le comportement exploratoire. Pris dans leur l'ensemble, nos résultats confirment que les cytokines jouent un rôle crucial dans le neurodéveloppement et que des variations dans l'abondance de certaines d'entre elles, et notamment du TNF, ont un impact sur l'acquisition de certains réflexes et comportement pendant les premiers jours de la vie. Bien que nos études ne nous aient pas permis d'explorer les mécanismes par lesquels cytokines influent sur le neurodéveloppement, les protocoles que nous avons élaborés et les résultats que

nous avons obtenus fournissent un cadre pour d'autres études visant à mieux comprendre ces mécanismes.

Mots-clés : neurodéveloppement, cytokines, immunité maternelle, inflammation, autisme, protection neuronale, comportement, analyse statistique multivariée

Title (in English): The regulatory role of cytokines on neurodevelopment and behaviour during the early postnatal period

Abstract (from 1700 to 4000 prints including spaces)

Both preclinical and clinical studies have shown that immune activation and inflammation during the early stages of neurodevelopment increase the risk of neurodevelopment disorders and behaviour abnormalities in adults. While the underlying mechanisms have only been partially elucidated, experiments in the maternal immune activation mouse model (MIA) – in which pregnant dams are injected with the viral mimic poly(I:C) – have demonstrated the critical role of two cytokines: interleukin (IL)-6 and IL-17A. However, the vast majority of the studies performed to date have used behavioural tests in adult mice as a read out to study the impact of cytokines on neurodevelopment. Therefore, it is not clear whether altered levels of other cytokines during the perinatal period could impact neurodevelopment and behaviour in infant mice. To address this issue, we have analysed the progeny of several cohorts of poly(I:C)- and saline-injected mothers for behaviour between postnatal day 5 (P5) and P15 and serum cytokine levels at P15. Because both perinatal neurodevelopment and cytokine production are known or believed to be impacted by many environmental variables, we analysed our data using a multivariable statistical model to identify features associated with being born to a poly(I:C)-injected mother (as opposed to being born to a saline-injected mother). We found that the drop of body weight and temperature of the mother after poly(I:C) injection, the litter size, the pup weight at P15, the number of ultrasonic vocalizations (USV) emitted by the pup at P6, the distance travelled by the pup and the time it spent mobile at P13, as well as serum levels of Tumour Necrosis Factor (TNF), IL-5, IL-15 and C-X-C motif chemokine (CXCL)10 were all associated with altered odds of being born to a poly(I:C)-injected mother. To further explore the role of TNF during the early postnatal period, we injected mouse pups daily from P1 to P5 and assessed these animals for both developmental milestones and behaviour from P8 to P15. Unexpectedly, injection of recombinant TNF did not have a detrimental impact on neurodevelopment but rather promoted sensorimotor reflexes acquisition and exploratory behaviour. Altogether, our results confirm that cytokines play a critical role during neurodevelopment and that altered levels of specific cytokines, and in particular TNF, could regulate the acquisition of developmental milestones and behaviour in infant mice. While

we have only obtained preliminary insights into underlying mechanisms, the protocols that we have developed provide a framework for further studies.

Keywords: neurodevelopment, cytokines, maternal immunity, inflammation, autism, neuronal protection, behaviour, multivariable statistical analysis

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I dedicate this work to Greta, my beloved sister, who left us in 2016. You will never be forgotten.

“La steaua care-a răsări	“So far it is athwart the blue
E-o cale-atât de lungă,	To where yon star appears,
Că mii de ani i-au trebuit	That for its light to reach our view
Luminii să ne-ajungă.	Has needed thousand years.
Poate de mult s-a stins în drum	Maybe those ages gone it shed
În depărtări albastre,	Its glow, then languished in the skies,
Iar raza ei abia acum	Yet only now its rays have sped
Luci vederii noastre,	Their journey to our eyes.
Icoana stelei ce-a murit	The icon of the star that died
Încet pe cer se suie:	Slowly the vault ascended;
Era pe când nu s-a zărit,	Time was ere it could first be spied,
Azi o vedem, şi nu e.	We see now what is ended.
Tot astfel când al nostru dor	So is it when our love's aspire
Pieri în noapte-adâncă,	Is hid beneath night's bowl,
Lumina stinsului amor	The gleam of its extinguished fire
Ne urmăreşte încă”	Enkindles yet our soul.”

– Mihai Eminescu

Translated by Corneliu M. Popescu

Table of contents

List of abbreviations

5'—C—phosphate—G—3'	CpG
Activator protein 1	AP1
Alzheimer's disease	AD
Analysis of variance	ANOVA
Applied behaviour analysis	ABA
Asperger's syndrome	AS
Attention Deficit Hyperactivity Disorder	ADHD
Autism Spectrum Disorder	ASD
Blood-brain-barrier	BBB
Bone morphogenetic proteins	BMP
Caesarean-section	C-section
Cardiotrophin-1	CT-1
C-C motif ligand	CCL
Central Nervous System	CNS
Ciliary neurotrophic factor	CNTF
cochlear root neurons	CRN
Colony stimulating factors	CSF
Confidence Interval	CI
C-X-C chemokine receptor	CXCR
C-X-C motif ligand	CXCL
Cyclooxygenase type 2	COX2
degrees Celsius	°C
Diagnostic and Statistical Manual of Mental Disorders, version 5	DSM-V
Embryonic day	E
Fragile X syndrome (gene)	*FMR1*
G protein-coupled receptor	GPCR
Gamma-aminobutyric acid receptor subunit beta-3	GABRB3
Gastrointestinal	GI
Generalized Estimating Equations	GEE
Hypothalamic-Pituitary-Adrenal axis	HPA axis
hour	h
Intellectual quotient	IQ
Interferon	IFN
Interferon Regulatory Factor	IRF
Interleukin	IL
Interleukin-6 receptor	IL-6R
kiloDalton	kDa
kilogram	Kg
kiloHertz	kHz
Knock-out	KO
Leukemia inhibitory factor	LIF
Lower Limit of Detection	LLOD
Lypopolysaccharide	LPS

Major Histocompatibility Complex I	MHC-I
Maternal immune activation	MIA
microgram	µg
microliter	µL
milliliter	ml
millisecond	ms
m-opioid receptor knock-out mice	*Oprm*$^{-/-}$
Natural Killer	NK cell
neurexin	*NRXN*
Neurodevelomental disorder	NDD
neuroligins	*NLGN*
Neuronal stem cells	NSC
N-methyl-D-aspartate	*NMDA*
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-kB
nucleus reticularis pontis caudalis	PnC
number	n
Odd Ratios	OR
Oxytocin receptor	OXTR
Positive And Negative Symptoms Scale	PANSS
Pathogen-associated-molecular patterns	PAMP
Pattern recognition receptors	PRR
Peripheral blood mononuclear cell	PBMC
Pervasive developmental disorder-not otherwise specified	PDD-NOS
Polyinosinic:polycytidylic acid	Poly(I:C)
Postnatal day	P
Prepulse inhibition	*PPI*
Principal component analysis	PCA
Prostaglandin E2	PGE2
p-value	p
radial glial cells	RGS
Rett syndrome (gene)	*MECP2*
Ribonucleic acid	RNA
Room temperature	RT
second	s
Segmented filamentous bacteria	*SFB*
SH3 and multiple ankyrin repeat domains 1 knock-out mice	*Shank1*$^{-/-}$
SH3 and multiple ankyrin repeat domains 2/3 (gene)	*Shank2/3*
soluble Tumour Necrosis Factor	sTNF
Strengths and Difficulties Questionnaire	SDQ
T helper 17 cell	Th17 cell
T helper cell 1	Th1 cell
T helper cell 2	Th2 cell
Toll-like receptor	TLR
Transforming growth factors	TGF
transmembrane form Tumour Necrosis Factor	mTNF
Tuberous sclerosis (gene)	*TSC1/TCS2*

Tumour Growth Factor β	TGFβ
Tumour Necrosis Factor	TNF
Tumour Necrosis Factor receptor 1	TNFR1
Tumour Necrosis Factor receptor 1-knock-out	*Tnfr1*-KO
Tumour Necrosis Factor receptor 2	TNFR2
Ultrasonic vocalisations	USV
United States	US
Variable Inclusion Probability	VIP

Introduction

1. Interactions between the immune system and the brain during immune activation

1.1. Overview

The immune system represents the totality of factors and entities in a given organism, which act in coordination as a defence machinery against invading pathogens (for review see (Chaplin 2010)). It is broadly divided into two main branches, the innate and the adaptive immune systems. The innate immune system includes germline-encoded mechanisms and represents the first step of defence, acting within hours of a given infection. Its main components are physical barriers, such as the epithelial cell and mucus layers, soluble secreted proteins, among which complement proteins, cytokines and lipid mediators, cells, such as mononuclear phagocytes (monocytes, macrophages, dendritic cells) and granulocytes (neutrophils, basophils and eosinophils) and membrane bound receptors which detect microbial presence. Unlike the innate immune system, the adaptive immune system is able to adapt its detection mechanisms with high specificity towards a particular antigen, by using antigen specific receptors found on the cell surfaces of T and B lymphocytes. The adaptive response is slower to react and follows the innate immune response.

1.2. Cytokines and cytokine receptors

The key molecules which regulate and connect the innate and adaptive immune responses are cytokines and the receptors through which they signal **(Illustration 1)**. Cytokines represent a large group of signalling proteins which play key roles in immune protection against invading pathogens, homeostatic maintenance of the organism shaping the nervous system during early neurodevelopment and adulthood. Cytokines are produced by both immune and non-immune cells and have cell type – and location – dependent functions, capable of acting as autocrine and paracrine signalling. Cytokines are grouped into several main classes: interleukins, interferons, chemokines, tumour necrosis factors, transforming growth factors (TGFs) and colony stimulating factors (CSFs). The main producers of peripheral cytokines are macrophages and T cells, and to a lesser extent, monocytes, fibroblasts and endothelial cells. In the central nervous system (CNS), cytokines are mainly

produced by microglia, which are the brain-resident innate immune cells, but also by non-immune cell types, such as neurons and astrocytes (Turner et al. 2014*)*. Cytokines signal through a variety of receptors which differ both structurally and functionally. Proinflammatory cytokines, such as IL-1, IL-6 and TNF signal through type I transmembrane receptors with distinct extracellular and intracellular domains used for signal transduction, while chemokines, such as CXCL12, signal through specific chemokine receptors, belonging to the seven-transmembrane-G protein-coupled receptor (GPCR) family (Turner et al. 2014*)*.

	Family	Members
Adaptive immunity	Common γ chain receptor ligands	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21
	Common β chain (CD131) receptor ligands	IL-3, IL-5, GM-CSF
	Shared IL-2β chain (CD122)	IL-2, IL-15
	Shared receptors	IL-13 (IL-13R–IL-4R complex)
		TSLP (TSLPR–IL-7R complex)
Pro-inflammatory signalling	IL-1	IL-1α, IL-1β, IL-1ra, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37 and IL-1Hy2
	IL-6	IL-6, IL-11, IL-31, CNTF, CT-1, LIF, OPN, OSM
	TNFα	TNFα, TNFβ, BAFF, APRIL
	IL-17	IL-17A-F, IL-25 (IL-17E)
	Type I IFN	IFNα, IFNβ, IFNω, IFNκ, Limitin
	Type II IFN	IFNγ
	Type III IFN	IFNλ1 (IL-29), IFNλ2 (IL-28A), IFNλ3 (IL-28B)
Anti-inflammatory signalling	IL-12	IL-12, IL-23, IL-27, IL-35
	IL-10	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29

Abbreviations: CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; LIF, leukaemia inhibitory factor; OPN, osteopontin; OSM, oncostatin M; TNFα, tumour necrosis factor α; TSLP, thymic stromal lymphopoietin.

Illustration 1. Example of cytokine classification by immune response and family. Adapted from Turner et al. 2014.

1.3. Pathogen recognition

Cytokines and other immune mediators are produced upon immune cell recognition of invading pathogens. Toll-like receptors (TLRs) operate the cellular mechanisms of pathogen recognition. TLRs are germline-encoded pattern recognition receptors (PRRs), capable of recognizing a wide range of pathogens based on their shared molecular structures, termed pathogen-associated-molecular patterns (PAMPs). Upon PAMP recognition, PRRs trigger an intracellular signalling cascade which ends in the nuclear translocation and activation of transcription factors, such as NF-kB, AP1, IRF3 or IRF7, which subsequently mediate the induction of type I interferon (IFN) and pro-inflammatory cytokines. Specific TLRs respond to specific types of pathogens, an effect driven by TLR localization. TLRs are localized both at the plasma membrane and intracellularly, on endosomes, phagosomes and the endoplasmatic reticulum **(Illustration 2)**. Therefore, TLRs, such as TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6 mostly recognise components of microbial membranes, such as lipopolysaccharide (LPS) and flagellin, and are therefore involved in fungal and bacterial

immune responses, while TLR-3, TLR-7, TLR-8 and TLR-9 mostly recognise nucleic acids, such as CpG DNA motifs as well as double- and single-stranded RNA, and are involved in anti-viral responses (O'Neill, Golenbock, and Bowie 2013; Kawai and Akira 2011).

TLRs have also been found to be expressed in the brain in neurons and glial cells. Microglia, the main immune cells in the brain, express the largest repertoire of TLRs. Moreover, TLR expression has been analysed in mouse models during multiple stage of brain development and it was shown that distinct TLR, such as TLR-7 and TLR-9, change their pattern of expression during the different stages of embryonic brain development (Kaul et al. 2012). TLR-3 plays a particularly important role during the early stages of development, as it was found to be the most highly expressed. It acts as an inhibitor of neural progenitor cell proliferation and axonal growth and therefore plays a key role in modulating neurogenesis (Lathia et al. 2008).

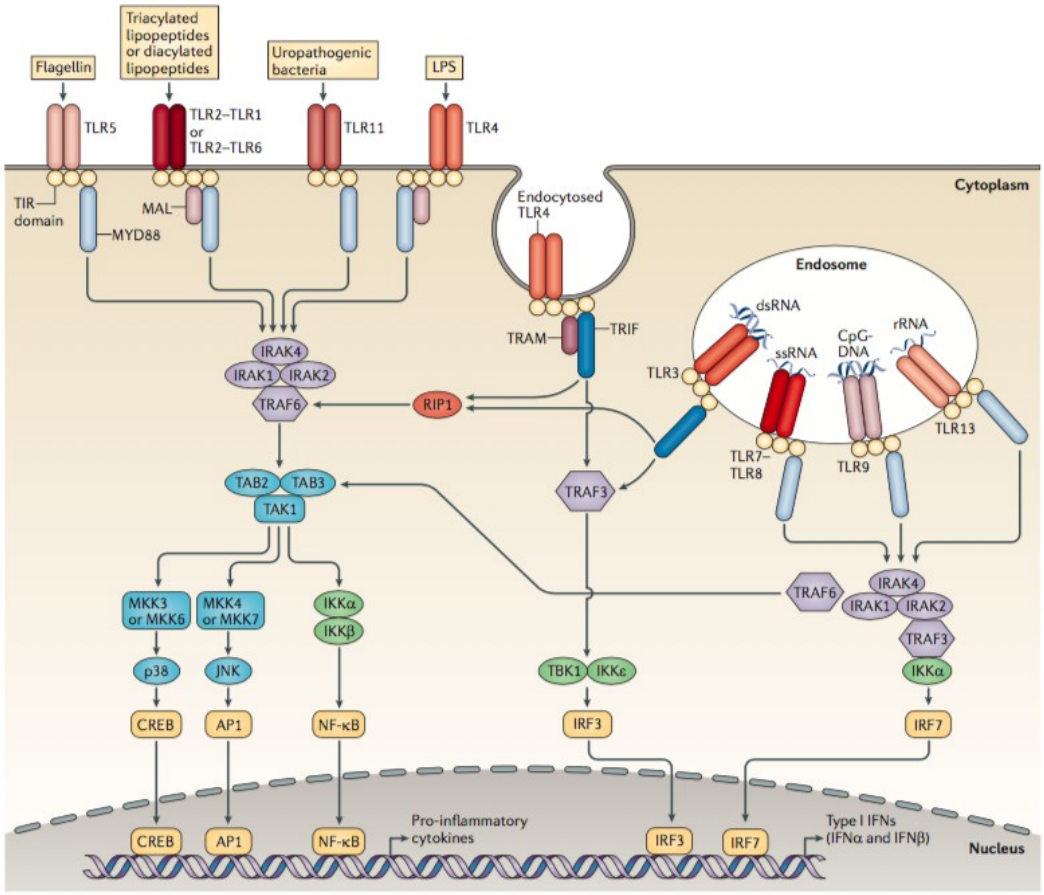


Illustration 2. Representation of the TLR signalling pathways. The different types of TLRs present extra- and intra- cellularly are presented in association with their individual ligands.

Upon ligand binding, several mechanisms assist in the activation of transcription factors, which leads to cytokine production. Adapted from O'Neill, Golenbock, and Bowie 2013.

1.4. Pathogen Associated Molecular Patterns

Molecules which mimic viral and bacterial infections, whether synthetic or naturally occurring, can also bind to TLRs and induce a similar immune response to actual infections. Some of the most commonly used substitutes for inducing infection of viral and bacterial origin are polyinosinic:polycytidylic acid (Poly(I:C)) and LPS, respectively. Poly(I:C) is a synthetic double-stranded RNA analogue used to mimic aspects of a viral infection. It acts by binding to TLR3, which initiates a signalling cascade which, in turn, elicits an acute-phase response in the host, including fever and induction of pro-inflammatory cytokines (IL-1, IL-6, TNF), chemokines, type I IFN and complement proteins (Boksa 2010). LPS is a natural component of the cell wall of Gram-negative bacteria and is used to mimic a bacterial infection. It binds to TLR4 and, similarly to Poly(I:C), leads to upregulation of pro-inflammatory cytokines. Although similar in their mode of action, Poly(I:C) and LPS immune responses can differ in the magnitude of the induced cytokine responses and the type of activated cells. The advantage of using these immunogens to induce immune response is the possibility to experimentally control the time-course and dose of antigen exposure. However, it should be noted that neither Poly(I:C), nor LPS completely mimic the entire time course of a propagating viral or bacterial infection (Harvey and Boksa 2012).

For instance, Poly(I:C) is widely used to mimic maternal viral infection in mice during pregnancy. Epidemiologic evidence suggests that maternal infection is a risk factor for autism spectrum disorders (ASD) and schizophrenia (Hornig et al. 2018; Atladóttir et al. 2010; H. yin Jiang et al. 2016) and it has been modelled in several rodent models. Poly(I:C) injection in pregnant dams during the middle stages of pregnancy, activates the maternal immune system, leading to increased serum levels of the proinflammatory cytokines TNF, IFN-β, IL-1β, IL-6 and IL-17A (Careaga et al. 2018; Mueller et al. 2019; Choi et al. 2016; Kim et al. 2017; E. Y. Hsiao and Patterson 2011). This maternal cytokine dysregulation alters the placental immune environment, in which IL-6 is specifically involved (W. L. Wu et al. 2017), and results in the activation of decidual immune cells, such as macrophages, granulocytes

and uterine NK cells. This increases the placental levels of IL-6, which has downstream effects on the foetal brain, subsequently causing deleterious behaviour in the offspring (Hsiao 2011; Patterson 2011) **(Illustration 3)**.

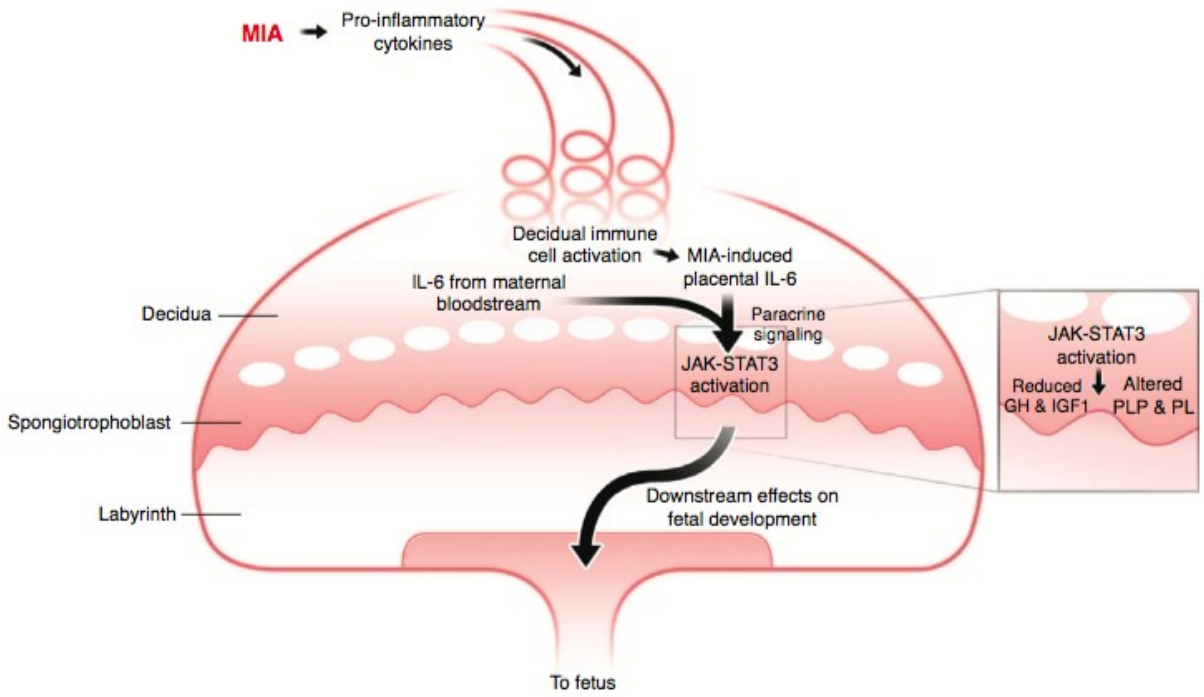


Illustration 3. Representation of the mechanisms involved in the dysregulaton of the placental immune environment, as a result of Poly(I:C) injection in pregnant dams. This immune activation has deleterious effects on the faetal brain. Adapted from Patterson 2011**.**

1.5. The peripheral immune system and the brain

Immune activation can spread to areas which are considered immune privileged in homeostatic conditions, such as the placental environment and the CNS. Moreover, during an initial immune activation, endothelial barriers across the body, such as the placental, intestinal and blood-brain-barriers (BBB) can become more permeabilized, commonly referred to as “leaky”, and allow passage of immune cells and larger immune mediators, such as cytokines, into these environments with particular immunological functions. In the brain, this immunological peripheral influx, often caused by infections, can trigger neuroinflammation, which is associated with sickness behaviour (Konsman, Parnet, and Dantzer 2002), as well as other behavioural alterations, described in the next sections. In addition, cytokines can be directly synthesized in the brain by the glia, as well as neurons, a

process which has been seen after brain insults in the absence of infection, through “sterile” damage (Mayer 2013).

The brain parenchyma part of the CNS is an immune privileged site. However, other parts constituting the CNS, such as the meninges, choroid plexus, circumventricular organs, and ventricles undergo immune response similar to those present in the periphery (Hagberg, Gressens, and Mallard 2012). In homeostatic conditions, the BBB restricts entry of potentially harmful molecules and cells to the parenchyma. During peripheral infections, as well as traumatic sterile injury in the brain, pro-inflammatory cytokines, such as IL-1β, IL-6 or TNF, access the brain through saturable transport systems (W. A. Banks, Kastin, and Broadwell 1995; Gutierrez, Banks, and Kastin 1993). Moreover, peripheral leukocytes, such as macrophages and neutrophils, can cross the BBB through a process mediated by changes in adhesion molecules, and contribute to activate glial cells mediating neuroinflammatory processes (Soares et al. 1995; Kubes and Ward 2006; R. S. b. Clark et al. 1994). Microglia, which are the primary brain-resident immune cells, play an important role in sensing CNS damage, by continuously sampling their immediate environment for pathogens or tissue injury. They are mostly responsible for phagocytosing and eliminating microbes, dead cells and protein aggregates (Colonna and Butovsky 2017). Following injury, they rapidly become activated and start releasing pro-inflammatory mediators, such as cytokines, chemokines and reactive oxygen species. Prolonged microglial activation can induce excitotoxic neuronal death and contribute to progressive CNS disorders (C. Mayer 2013; Ye et al. 2013). Another important class of glial cells in the brain is formed by astrocytes which are also the most abundant cell type in the brain, working mainly to support neuronal and synaptic functions. Following tissue injury, activated astrocytes deposit a proteoglycan matrix which forms glial scars that can lead to inhibition of axonal regeneration and function under chronic injury (Yiu and He 2006). Both types of glial cells secrete cytokines and can harm CNS functioning under specific conditions **(Illustration 4)**.

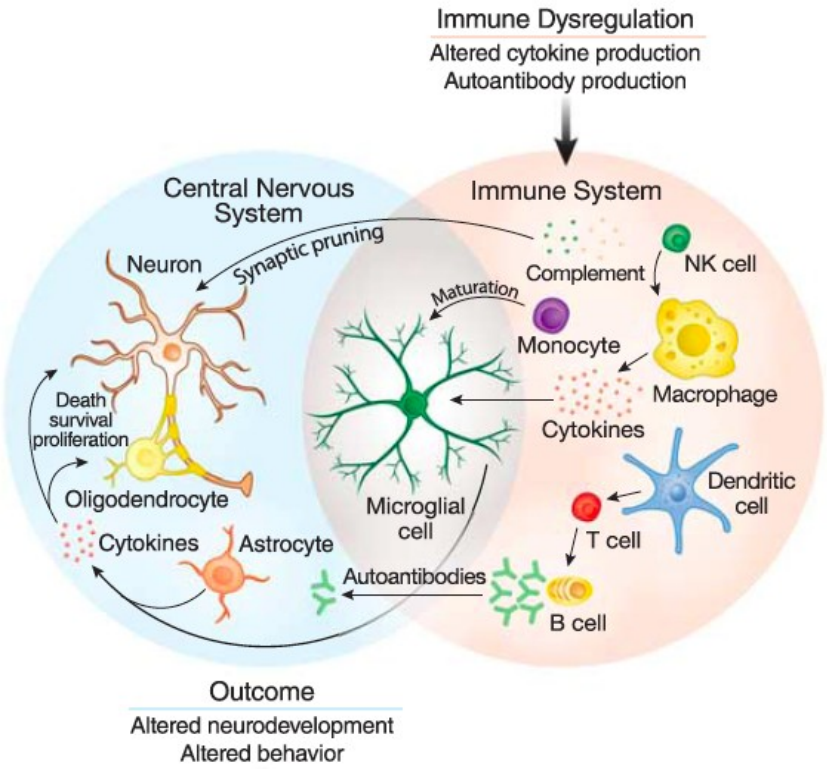


Illustration 4. Summary of the brain-immune interaction in mediating behavioural abnormalities. Immune cells and their mediators act directly on neurons and glia and alter important developmental and functional processes. Adapted from Meltzer and Van De Water 2017.

1.6. Cytokines and behaviour

An important mechanism by which glial cells contribute to lead to pathological processes is through the over-expression of pro-inflammatory cytokines, mostly TNF and IL-1β. Numerous studies have shown that cytokines can control brain function and behaviour, with mostly detrimental effects. Cytokines can notably induce anxiety (Simen et al. 2006; Spadaro and Dunn 1990), sickness behaviour (Konsman, Parnet, and Dantzer 2002; Anisman and Merali 1999), depression (Maes et al. 1993; Dowlati et al. 2010; Réus et al. 2017) and impair cognitive processes (Heyser et al. 1997; Menza et al. 2010). This is further supported by association studies in psychiatric cohorts and in the general population, which have suggested that some pro-inflammatory cytokines are associated with mental health disorders (O'Shea et al. 2014; Kuban et al. 2016; N. M. Jiang et al. 2014), including neurodevelopmental disorders such as ASD (Goines et al. 2011; Spann et al. 2018).

2. How cytokines shape neurodevelopment

Although the immune system is a key player of the host's defence, it also plays a critical role in the maintenance of tissue integrity and general homeostasis. The immune system has also evolved to regulate physiological processes, such as development, reproduction, metabolism and several aspects of CNS development (Sattler 2017).

2.1. The essential role of cytokines during embryonic development

Cytokines can have pro- or anti-inflammatory functions and be neuroprotective or destructive, depending on their timing of expression (age-related), level of expression (acute vs. chronic) and concentrations (Morganti-Kossman 1997). During neurogenesis, radial glial cells (RGCs) derived from neuroepithelial cells (Hatakeyama et al. 2004) act as precursors for all neurons, astrocytes, oligodendrocytes and adult neural stem cells and guide the migration of immature neurons to their final location (Pinto and Götz 2007). The cytokines of particular importance during this process are the gp130/IL-6 family cytokines and the bone morphogenetic proteins (BMPs), part of the TGFβ superfamily. Members of the gp130 family cytokines, such as IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1) regulate RGCs self-renewal (Gregg and Weiss 2005; Hatta et al. 2002; Yoshimatsu et al. 2006), while inhibition of the neural induction repressors BMPs contribute to neural induction (Gaulden and Reiter 2008). Moreover, chemokines, such as the CXCL12 through their receptor CXCR4, promote migration and proliferation of newly generated neurons and glia (Klein et al. 2001; Zhu et al. 2002; Lu, Grove, and Miller 2002), and play an important role in axonal pathfinding (Chalasani et al. 2003). Another important role of cytokines during neurodevelopment is that of regulators of synaptogenesis and synaptic pruning (Sedel et al. 2004; Barker et al. 2001), such as in the case of microglia-derived TNF.

2.2. Microglia: neuroimmune interactions in shaping neuronal circuitry

Apart from cytokines, immune cells, and in particular, microglia, also have specific roles in neurodevelopment during the early stages of neurogenesis, as well as during the postnatal period and adulthood. They are the first glial cells to migrate into the CNS during embryonic brain development. This is an important period of neuronal migration, during which

microglia guide neurons and their axons to form prenatal circuits (Colonna and Butovsky 2017), as well as influence neural precursor cell differentiation (Aarum et al. 2003). Moreover, *in vitro* coculture of microglial and neuronal stem cells (NSCs) show that microglial-secreted factors are necessary for NSC self-renewal (Walton et al. 2006). During postnatal development, microglia modulate synaptic pruning. This activity is achieved by the phagocytosis of dendritic spines that did not receive synaptic inputs (Colonna and Butovsky 2017). Also, microglia phagocyte the debris of surnumerary neurons which had to be eliminated as they were unable to form functional circuits. All these effects contribute to microglial shaping of the neuronal networks during early development.

2.3. Neonatal immune system vs. adult immune system

There is increasing knowledge about the involvement of immune cells and their mediators in early brain development, as well as the immunological differences between the perinatal and adult brain (Garay and McAllister 2010). In comparison to the adult immune system, the neonatal immune system is polarized towards Th2 responses (Maródi 2002; Levy 2007; Wynn and Levy 2010). Moreover, stimulated neonatal serum monocytes secrete less TNFα, a Th1-polarising cytokine, and more IL-6, a Th2-polarising cytokine, than adult monocytes (Angelone et al. 2006). There are also clear age-related differences in immune responses in the brain. In the adult CNS and in particular in the brain parenchyma, the response to inflammatory stimuli, such as pro-inflammatory cytokines or LPS, is characterized by the ability to restrict peripheral leukocyte migration (Andersson, Perry and Gordon, 1992a; Andersson, Perry and Gordon, 1992b). In contrast, during the early stages of mouse CNS development, neutrophil and monocyte are recruited to the brain parenchyma upon endotoxin injection, but the characteristics of this response are age-dependent (Lawson and Perry 1995): immediately after birth, at post-natal day (P) 0, the brain inflammatory response is relatively weak, showing reduced microglial response upon intracerebral LPS administration, as well as slow and reduced neutrophil and monocyte recruitment from the periphery. By P7, the microglial response following LPS injection becomes fast and efficient and there is increased neutrophil recruitment, as compared to P0 (Lawson and Perry 1995).

2.4. The essential role of cytokines during postnatal development

During postnatal development, cytokines in homeostatic conditions have been shown to display a dynamic pattern of expression, both in blood and brain tissue. This pattern is age- and region-specific, which is suggestive of the need for a timely and restricted expression of specific cytokines during neurodevelopment (Garay et al. 2013; Deverman and Patterson 2009; Dziegielewska et al. 2000; Bauer, Kerr, and Patterson 2007). The expression of IL-6, a cytokine involved in neurogenesis, as well as its receptor, IL-6R, have been demonstrated to be tissue-specific in the rat brain, depending on the postnatal developmental stage: *Il6* and *Il6r* mRNAs levels are highest in adult hippocampus, whereas the levels of *Il6* mRNA are highest in all other brain regions during early brain development (Gadient and Otten 1994).

2.5. Specific role of cytokines in neurodevelopment: the example of TNF

Tumour Necrosis Factor (TNF) is a proinflammatory cytokine historically known as a chief orchestrator of the innate immune response (Holbrook et al. 2019). TNF is normally present in minute amounts, however, following an immune challenge, TNF is massively induced in activated macrophages in peripheral tissues. TNF is expressed as a 27 kDa transmembrane form (mTNF) which acts by cell-to-cell contacts, and as a soluble 17 kDa form (sTNF) produced by regulated cleavage of mTNF that is released in tissues and blood (Kriegler et al. 1988). TNF signals through two membrane receptors, TNFR1 and TNFR2. While both sTNF and mTNF activate TNFR1 signalling transduction pathway, only mTNF triggers TNFR2 signaling (Probert 2015). TNF and its receptors also expressed outside the immune compartment, and notably in the CNS.

Evidence of the role of TNF in early neurodevelopment comes from studies in young mice. Slight increases in TNF levels are observed in the hippocampus and in the cortex during the first 2 postnatal weeks of life, a time of active neurogenesis and synaptogenesis (Garay et al. 2013). Moreover, low doses of TNF promoted the survival, proliferation, and neuronal differentiation mouse neonatal neural precursor cells cultures, while higher doses were apoptotic (Bernardino et al. 2008). Furthermore, young *Tnf*-knockout (KO) mice exhibit an accelerated maturation of the dentate gyrus hippocampal region, but with pyramidal neurons harbouring a smaller dendritic arborisation in CA1 and CA3 regions (Golan et al. 2004). Finally,

both *in vitro* and *in vivo* studies have shown that developing pyramidal neurons from the cortex of *Tnf*-KO mice are deficient in synaptic scaling, a form of homeostatic plasticity that enables adjustment of synaptic strength at the neuron-scale in response to sustained activity, which is critical for the activity-dependent refinement of neural circuitry during early development (Stellwagen and Malenka 2006; Kaneko et al. 2008; Ranson et al. 2012) . This suggests a critical role for TNF in shaping the nervous system during early developmental stages.

TNF is also required for CNS functioning during adulthood. In physiological conditions, it is constitutively secreted in minute amounts by neurons and glia (Probert 2015). In these conditions, TNF is required for brain cell maintenance and homeostasis. Notably, TNF promotes proliferation of oligodendrocyte progenitors and remyelination (Arnett et al. 2001). Moreover, TNF is known to enhance excitatory synaptic scaling (Beattie et al. 2002). In this context, TNF secreted by astrocytes controls the exposure of AMPA receptors at the synapse, thereby directly regulating synaptic neurotransmission in the hippocampus, cortex and striatum (Beattie et al. 2002; Lewitus et al. 2014; Santello, Bezzi, and Volterra 2011). In the cerebellum, TNF increases the intrinsic excitability of cerebellar Purkinje cells by controling the release of glial glutamate (Shim et al. 2018). *In vivo,* both *Tnf*-knockout (KO) and *Tnfr1*-KO mice have elevated hippocampal adult neurogenesis, while lack of TNFR2 decreases baseline neurogenesis (Chen and Palmer 2013; Iosif et al. 2006).

2.6. Human studies supporting a role of cytokines in neurodevelopment

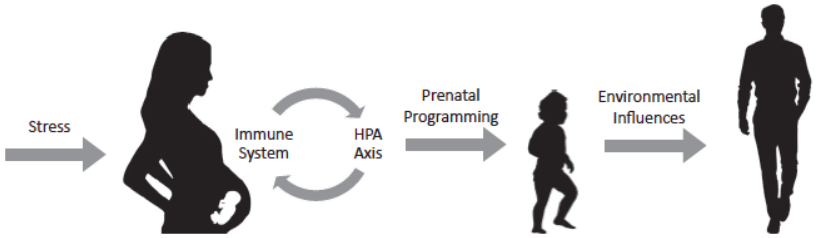
Although many studies link disrupted patterns of cytokines with neurodevelopmental conditions (see next section 3.), some have identified both deleterious and beneficial links between the levels of specific gestational cytokines and neurocognitive behaviour in the general population.

Gestational cytokine levels and neurocognitive behaviour: To assess the influence of maternal cytokine levels on offspring neurocognitive development, one study studied the association between maternal serum cytokine levels (measured longitudinally during the 2nd and 3rd trimesters of pregnancy) and neurocognitive outcme in the offspring at 7 years of

age. The authors first estimated the cumulative exposure to each cytokine and then studied the associations with neurocognitive outcomes at 7 years. Among the cytokines assessed, they found two pro-inflammatory cytokines - TNF and IL-8 - to be associated with negative or positive neurocognitive outcomes, respectively. TNF was associated with problems in visual-motor functioning and lower cognitive scores, whereas IL-8 was associated with better cognitive performance and motor functioning (Ghassabian et al. 2018). While association does not necessarily imply causation, this study draws attention to the possible involvement maternal cytokines in neurodevelopmental processes at early life stages.

Another study investigated whether the socioeconomic environment can influence maternal immune activity during gestation and whether this was associated with adverse behavioural outcome in the offspring during the first year of life (Gilman et al. 2017). Several proinflammatory cytokines were measured in the maternal serum during the 3rd trimester of pregnancy. They found that gestational levels of IL-8 were lower in the most disadvantaged pregnancies experiencing more social-economic distress. Furthermore, maternal socio-economic disadvantage was associated with higher risk of structural and sensorimotor-related neurological abnormalities in the offspring. Finally, they found that decreased maternal IL-8 levels in disadvantaged pregnancies were positively associated with increased risk of neurological abnormalities. Together with previous studies reviewed in Hantsoo *et al.*, 2018, this study suggests the involvement of maternal stress response to adversity, which can translate into maternal immune dysregulation and contribute to increase the offspring's vulnerability to neuropsychiatric disorders.

The following figure **(Illustration 5)** provides more insight into the link between maternal stressors during pregnancy, which include immune dysfunction, and offspring neuropsychiatric development.



MATERNAL STRESS EXPOSURE	IMPACT ON IMMUNE PARAMETERS DURING PREGNANCY	CHILD NEUROPSYCHIATRIC OUTCOMES	ADULT NEUROPSYCHIATRIC OUTCOMES
Stressful Life Events	Elevated IL-1β , IL-5, IL-6, IL-8 in cord blood [67] Elevated TNF-α [80]	Increased psychotic experiences risk [70] Increased ASD, ADHD risk [14, 18–20, 73, 74]	Increased schizophrenia risk [68–70] Greater psychiatric hospitalization risk [75] Increased depression risk [71, 72] No increased risk [76–78]
Chronic Stress	Impaired HPA-immune coordination [58] Elevated IL-1: IL-10 ratio [58] Elevated IL-4, IL-6 [82] Elevated EBV VCA IgG [83] Elevated IL-6, IL-10, CRP [81, 84] Greater stimulated IL-6, IL-1β production [81] Placental inflammation [86, 87] Greater stimulated IL-8, TNF-α from cord blood cells [88] Lower IL-8 [91]	Behavioral problems, mental health morbidity [90] Neurologic abnormalities [91] Depression [92]	
Traumatic Stress	Elevated TNF-α [99] Elevated IgE in cord blood [100]	Difficult or irritable temperament [104, 105] Lower social-emotional intelligence [105] Internalizing, externalizing behaviors [106] ASD-like traits [107]	Increased schizophrenia risk [103] Risk of major depression [108] ADHD symptoms [108]
Adverse Childhood Experiences	Elevated IL-6 [110] Elevated CRP [111] Elevated IL-15 [112] Blunted cortisol response [116]	Behavioral problems [113] Altered brain structure [114] Blunted cortisol response [116]	

Illustration 5. The impact of stressful events on the maternal immune system during gestation, involving negative outcomes for offspring neurodevelopment and behaviour. Adapted from Hantsoo et al. 2018**.**

Cytokine levels at birth and neurocognitive behaviour: Another study that should be mentioned when describing the impact of cytokines on child neurocognitive development is the one conducted by Von Ehrenstein *et al.*, 2012 on the link between cytokine levels at birth, measured in cord blood, and child's intellectual development measured by the intellectual quotient (IQ). The study found that increased levels of IFN-γ and IL-12p70 at birth were associated with reduced odds of low IQ (IQ<70) at 5 years of age. Also, increased cord blood levels of TNF was associated with reduced odds of low IQ (IQ<70) in preterm children. This suggests important links between proinflammatory cytokines and early brain development and that dysregulation of cytokine patterns could contribute to later abnormal child behaviour.

Our team has also recently investigated the association of cytokines at birth with child's behavioural outcome in a cohort of healthy children of 5 years of age. We used data and biological samples from 786 mother-child pairs participating to the French national mother-

child cohort EDEN. Maternal serum was collected in the 2^{d} trimester of pregnancy. At the age of 5, children were assessed for behavioural difficulties using the Strengths and Difficulties Questionnaire (SDQ). Serum samples were analysed for levels of well-characterized effector or regulatory cytokines. We then used the Elastic net model, a penalized logistic regression method, to investigate associations between serum levels of cytokines and each of the five SDQ-assessed behavioural dimensions after adjustment for relevant covariates and confounders, including parental data for various socio-economic parameters, such as the age of delivery, breastfeeding, C-section, parental education, etc. We found five cytokines to be associated with increased odds of developing problems in one or more behavioural dimensions: CXCL10, IL-10 and IL-12p40 with emotions, CCL11 with both conduct problems and peer problems. In contrast, five cytokines were associated with decreased odds of problems in one or more behavioural dimensions: IL-7, IL-15 and TNF-β with emotions, IL-15 and CCL26 with peer problems, IL-15, CCL26 and TNF with prosocial behaviour. **Table 1** summarizes these results. This supports the notion that cytokines at birth could contribute to shape the developing CNS and impact the behavioural outcome of the child later in life.

Table 1. Adjusted associations between cytokines measured in cord blood serum and high-risk of behavioural problems at 5 years of age. Weighted mean Odd Ratios (OR), weighted 95% Confidence Interval (CI) and Variable Inclusion Probability (VIP) for each of the variables selected by the Elastic Net are shown. The VIP was used as a measure of the stability of an association as it can be interpreted as the posterior probability of including a given variable in the model. Only variables with VIPs above 90% are presented.

		Emotional symptoms			Conduct problems			Hyperactivity/Inattention			Peer problems			Prosocial behaviour problems		
	Variables description	ORs	95% CI	VIP	ORs	95% CI	VIP	ORs	95% CI	VIP	ORs	95% CI	VIP	ORs	95% CI	VIP
Maternal variables	Age at delivery (years)							0.954	[0.911,0.995]	97.4%						
	Pre-pregnancy BMI ($kg.m^{-2}$)										1.031	[1.002,1.078]	96.2%	0.97	[0.926,1.001]	92.3%
	Smoking during pregnancy (cigarettes/day)							1.086	[1.01,1.151]	98.8%				1.058	[0.993,1.135]	90.4%
	Alcohol drinking during pregnancy (mean glasses/week)	1.089	[0.982,1.228	91.8%												
Perinatal variables	Birth weight (kg)										1.001	[1,1.001]	97.7%			
	Sex: Male				1.331	[1.158,1.671]	100.0%	1.33	[1.128,1.71]	100.0%				1.222	[1.056,1.461]	100.0%
	Sex: Female				0.753	[0.604,0.866]	100.0%	0.755	[0.589,0.888]	100.0%				0.825	[0.697,0.955]	100.0%
Psychosocial variables	Maternal prenatal anxiety (STAI scale)				1.003	[0.98,1.013]	97.3%									
	Maternal depression during pregnancy (no)				0.726	[0.578,0.915]	97.3%				0.849	[0.658,1.005]	90.6%			
	Maternal depression during pregnancy (yes)				1.374	[1.095,1.736]	97.3%				1.179	[0.998,1.519]	90.6%			
	Maternal education (years)				0.918	[0.831,0.988]	99.9%				0.948	[0.877,1]	92.9%			
	Paternal education (years)							0.876	[0.825,0.942]	100.0%	0.923	[0.851,0.977]	98.1%			
	Number of older siblings	0.53	[0.424,0.698]	99.6%				0.828	[0.651,0.977]	98.1%				1.278	[1.032,1.628]	99.1%
Cord blood cytokines	CCL11				1.002	[1,1.003]	96.3%				1.002	[1.001,1.004]	96.2%			
	CCL26										0.983	[0.962,0.994]	100.0%	0.989	[0.969,1]	95.3%
	CXCL10	1.001	[1,1.002]	91.8%												
	IL-7	0.948	[0.89,0.989]	97.3%												
	IL-10	1.044	[0.999,1.252]	92.0%												
	IL-12p40	1.001	[1,1.002]	95.0%												
	IL-15	0.974	[0.864,0.997]	92.8%							0.977	[0.928,0.997]	90.1%	0.989	[0.948,0.999]	92.0%
	TNF-α													0.905	[0.748,0.996]	97.3%
	TNF-β	0.796	[0.585,0.984]	90.7%												
	Legend		VIP>90%													
			OR>1													
			OR<1													

Cytokine levels in childhood and neurocognitive behaviour: A second study was conducted by our team, investigating the association of cytokines measured at 5 years of age on behaviour, assessed at the same age. We showed several cytokines to be protective, while others, detrimental to specific behavioural dimensions. Moreover, this study also shows the impact of parental and psychosocial variables on child behaviour. We found that IL-6, IL-7, and IL-15 were associated with increased odds of problems in prosocial behaviour, emotions, and peer relationships, respectively. In contrast, eight cytokines were associated with decreased odds of problems in one dimension: IL-8, IL-10, and IL-17A with emotional problems, TNF with conduct problems, CCL2 with hyperactivity/inattention, CXCL10 with peer problems, and CCL3 and IL-16 with abnormal prosocial behaviour. **Table 2** summarizes these results. Without implying causation, these associations support the notion that cytokines regulate brain functions and behaviour.

Table 2: Adjusted associations between cytokines measured in serum and high-risk of behavioral problems at 5 years of age. Weighted mean Odd Ratios (OR), weighted 95% Confidence Interval (CI) and Variable Inclusion Probability (VIP) for each of the variables selected by the Elastic Net in the analysis of MI-R datasets are shown. The VIP was used as a measure of the stability of an association as it can be interpreted as the posterior probability of including a given variable in the model. Only variables with VIPs above 90% are presented.

		Emotional symptoms			Conduct problems			Hyperactivity/Inattention			Peer problems			Prosocial behaviour problem		
Type	Variable ID	OR	95% CI	VIP	OR	95% CI	VIP	OR	95% CI	VIP	OR	95% CI	VIP	OR	95% CI	VIP
Maternal variables	Age at delivery (years)							0.851	[0.69,0.991]	90.6%						
	Gestational Diabetes: Yes	1.333	[1.021,1.826]	93.8%												
	Caffeine consumption during pregnancy (mg/day)	1.193	[1.016,1.457]	93.7%												
Perinatal variables	Breastfeeding : Yes	0.850	[0.708,0.991]	94.8%												
	Gestational age (weeks of amennorhea)										0.798	[0.639,0.983]	92.8%			
	Birth trimester: 1st													0.640	[0.386,0.939]	97.7%
Psychosocial variables	Parental separation (Pregnancy - Age 5):Yes													1.247	[0.974,1.672]	90.2%
	Maternal depression (Pregnancy - Age 5): Yes	1.170	[1.005,1.403]	93.9%	1.25	[1.041,1.559]	97.1%									
	Paternal education (years)							0.733	[0.603,0.891]	99.5%						
	Family income (Age 5)										0.796	[0.633,0.985]	91.3%			
	Family financial difficulties (Pregnancy - Age 5): Yes				1.254	[1.031,1.562]	97.0%	1.229	[1.023,1.544]	94.2%	1.343	[1.065,1.68]	98.0%			
	Number of older siblings	0.693	[0.56,0.878]	99.7%				0.827	[0.667,0.98]	94.3%						
	Home stimulations				0.810	[0.666,0.956]	98.3%				0.851	[0.696,0.992]	92.8%			
Child's variables at 5 years-old	Blood sampling time				0.858	[0.703,0.99]	93.0%	0.869	[0.717,0.994]	88.0%	0.825	[0.673,0.978]	94.1%			
	Allergies: No				0.829	[0.664,0.983]	90.9%	0.871	[0.715,1.016]	85.8%						
	Allergies: Yes				1.202	[1.015,1.496]	90.9%	1.146	[0.989,1.393]	85.8%						
	Sex:Male				1.536	[1.232,1.883]	100.0%	1.35	[1.116,1.699]	99.7%				1.288	[1.063,1.585]	98.8%
	Sex:Female				0.654	[0.532,0.814]	100.0%	0.744	[0.592,0.901]	99.7%				0.781	[0.637,0.94]	98.8%
Cytokines	CCL2							0.830	[0.666,0.982]	92.7%						
	CCL3													0.840	[0.621,0.986]	91.7%
	CXCL10										0.793	[0.616,0.968]	96.0%			
	IL-6													1.201	[1.021,1.499]	93.1%
	IL-7	1.243	[1.045,1.496]	99.0%												
	IL-8	0.835	[0.69,0.977]	98.0%												
	IL-10	0.751	[0.544,0.968]	99.0%												
	IL-16													0.850	[0.677,0.996]	90.4%
	TNF-α				0.776	[0.593,0.963]	98.0%									

Legend: VIP>90%; OR>1; OR<1

3. Cytokines can interfere with neurodevelopment and contribute to neurodevelopmental disorders, including autism spectrum disorders

3.1. Neurodevelopmental disorders - overview

Neurodevelomental disorders (NDDs) are a broad group of heterogenous conditions, usually diagnosed in infancy. NDDs involve early disruptions of brain development and are often associated with cognitive and behavioural impairments, as well as neurological anomalies. The Diagnostic and Statistical Manual of Mental Disorders, version 5 (DSM-V) groups together autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), intellectual disability and specific communication, learning and motor disorders under the term of NDDs. Acquired developmental deficits induce impairments of personal, social, academic, or occupational functioning and NDD have therefore extremely heavy socioeconomic consequences. Early detection of NDD improves the prognosis and quality of life for the child and family. If initiated early, therapies and behavioral interventions can target specific symptoms and bring about substantial improvement.

While NDD aetiology remains poorly understood, the complex interaction between genetic predisposition and pre- or/and perinatal exposure to environmental risks plays a prominent role. Currently, NDD diagnosis relies on batteries of psychocognitive tests. There is some degree of overlap between NDD symptoms, which makes them difficult to accurately diagnose, especially at very young ages. Most NDD symptoms are characterized by a delay, or an absence thereof, in the acquisition of a developmental skill, such as social, language, cognitive or motor abilities and affect in general more males than females in the general population. Moreover, there is a great heterogeneity in the intensity of the symptoms displayed between individuals affected by the same NDD. Also, a diagnosis of a specific NDD is frequently associated with diagnoses of other psychiatric comorbidities (Jeste Spurling 2015; Thapar 2016).

3.2. Autism spectrum disorders

ASD refers to a group of NDDs which encompass autism, Asperger's syndrome (AS) and pervasive developmental disorder-not otherwise specified (PDD-NOS). As described by the DSM-V criteria, ASD is known to have 3 core clinical symptoms, which all ASD patients have exhibit to a certain degree. These core symtpoms include: i) social interaction and communication deficits, ii) the presence of repetitive behaviour (stereotypies) and restricted interests and iii) sensory processing impairment. Furthermore, ASD is associated with a number of comorbidities, such as anxiety, mood fluctuations, ADHD, primary intellectual disability and global developmental delay. These comorbidities affect nearly 75% of ASD patients, where the prevalence and type of comorbidity depends on each individual (Sharma, Gonda, and Tarazi 2018). Moreover, some patients may also exhibit associated systemic symptoms, which include immune dysfunction and GI disorders. Epidemiologically, the ASD prevalence has been increasing over the last two decades, reaching 1 in 68 children affected in the United States (US) with 3 males diagnosed for 1 female (Waye and Cheng 2018; Loomes, Hull, and Mandy 2017). The disease onset begins typically before the age of 3, during which most children are diagnosed, but in some cases the symptoms can be overlooked until school age. To date, available pharmacological treatments for ASD mostly target associated symptoms and typically do not improve social behaviour deficits. Behavioural interventions remain the only treatments that improve ASD core symptoms. Notably, applied behaviour analysis (ABA)-based behavioural interventions proved to be particularly effective if undertaken early. The medications used only manage to alleviate some of the associated symptoms and include atypical antipsychotics, psychostimulants, antidepressants and β-2 adrenergic receptor agonists. In addition, hormonal therapies involving oxytocyin or vasopressin receptor antagonists also have the potential to improve core ASD symptoms related to social behaviour deficits (Sharma, Gonda, and Tarazi 2018).

Like most NDDs, the development of ASD results from complex interactions between genetic and environmental factors (Martens and van Loo 2009; Sharma, Gonda, and Tarazi 2018; Almandil et al. 2019). It is currently believed that genetic variation is responsible for approximately 50% of the risk of developing ASD (Yoo 2015). Identical twin studies have shown that ASD is highly heritable, with the second twin having 36-95% chance of also having the disease. Moreover, ASD symptoms are often expressed in patients with genetic

diseases, such as in the case of Down syndrome, Fragile X syndrome or Tuberous sclerosis (Sharma, Gonda, and Tarazi 2018; Almandil et al. 2019). The genetic makeup of ASD can be classified according to the frequency in genetic variation – common or rare –, mode of inheritance, type of variation – single-nucleotide polymorphisms or copy number variation – and mode of action – dominant or recessive. A few consistently reported genes among the common variants associated with ASD risk are N-methyl-D-aspartate receptor (*NMDA*), gamma-aminobutyric acid receptor subunit beta-3 (*GABRB3*) and oxytocin receptor (*OXTR*). Among rare gene variants involving monogenic autism are included *FMR1* (present in Fragile X syndrome), *MECP2* (present in Rett syndrome) and *TSC1/TCS2* (present in Tuberous sclerosis). Moreover, the most consistently reported genetic abnormalities associated with ASD are mutations in synaptic genes, among which, SH3 and multiple ankyrin repeat domains 3 (*Shank3*) or 2 (*Shank2*), neuroligins (*NLGN*) and neurexin (*NRXN*) (Yoo 2015; Almandil et al. 2019).

Early-life exposure to environmental risk is thought to contribute to the development of ASD. **Illustration 6** describes the different stages of early-life development and gives a good indication of the developmental windows of environmental vulnerability. In utero exposure to environmental pollutants (pesticides, toxins, aerosol particles) or drugs consumed by the mother (e.g. valproate), as well as *in utero* exposure to maternal obesity are acknowledged risk factors for ASD (Hertz-Picciotto, Schmidt, and Krakowiak 2018). Also, maternal immune activation (MIA) caused by infection or auto-immune disorder is an important environmental factor associated with subsequent ASD diagnosis in the offspring (Careaga, Murai, and Bauman 2017; Hsiao 2013).

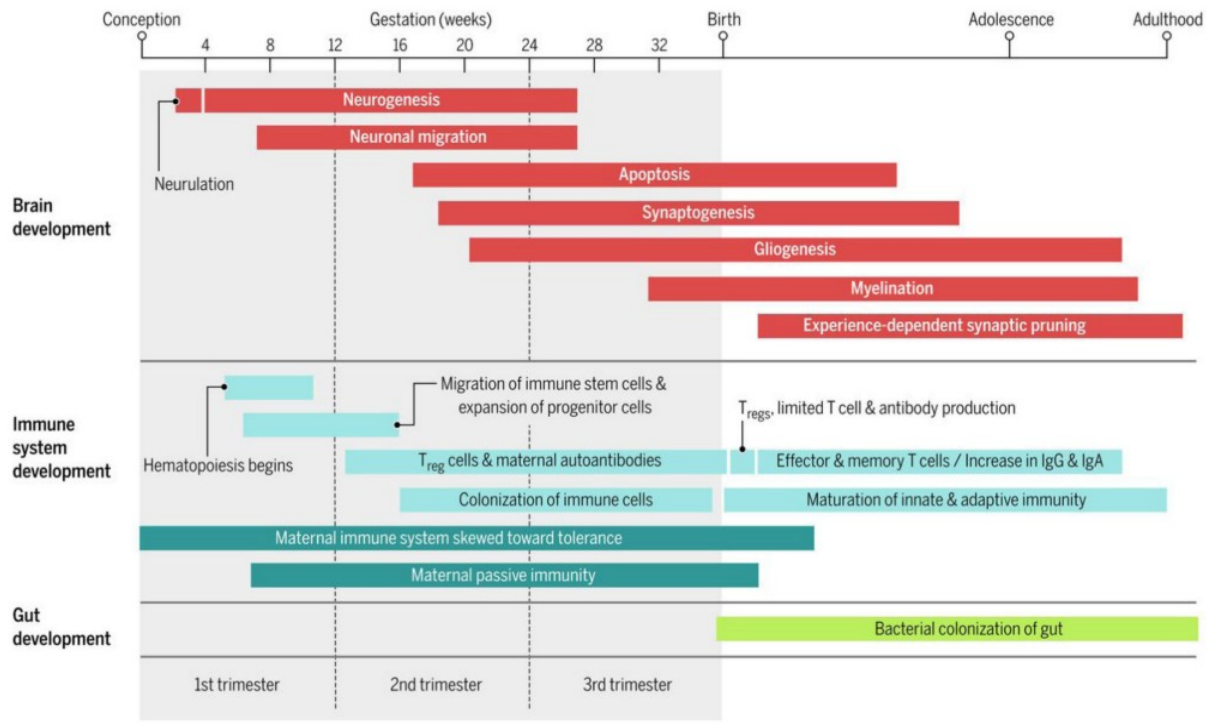


Illustration 6. Timeline of developmental stages involving the brain, immune system and the gut development with microbiota acquisition. Adapted from Estes and McAllister 2016**.**

3.3. Human studies: the case of ASD

Maternal immune activation as a risk factor for ASD: One important environmental factor associated with an increased risk of neurodevelopmental disorders is maternal infection during pregnancy. Many studies have suggested that maternal exposure to various immune stimuli, such as viral and bacterial infections, is associated with abnormal brain development and mental illness in the offspring, particularly schizophrenia and schizophrenia-like disorders **(Illustration 7)**.

There is evidence of other classes of pathogens being able to affect offspring neurodevelopment upon gestational exposure, namely rubella, toxoplasma and maternal genital or reproductive infections (Brown et al. 2001; Brown and Susser 2002; H. J. Sørensen et al. 2009; Penner and Brown 2007; Hyman, Arndt, and Rodier 2005). Moreover, maternal infection was also found to be associated with autism diagnosis in the offspring. This was first described in a group of children with congenital rubella in the wake of the 1964 US rubella pandemic (Chess 1971). Another study on the impact of congenital infections with

Cytomegalovirus and autism diagnosis confirmed the initial association (Stubbs, Ash, and Williams 1984). This effect is now known to be due, not to the exact pathogen, but rather to the intensity of the maternal immune activation (MIA), which occurred during pregnancy (Shi 2003; Patterson 2009; Myka L.Estes 2016). Indeed, prenatal fever or hospitalization following infection, rather than the type of the infection *per se*, was associated with increased ASD risk (Hornig et al. 2018; Atladóttir et al. 2010).

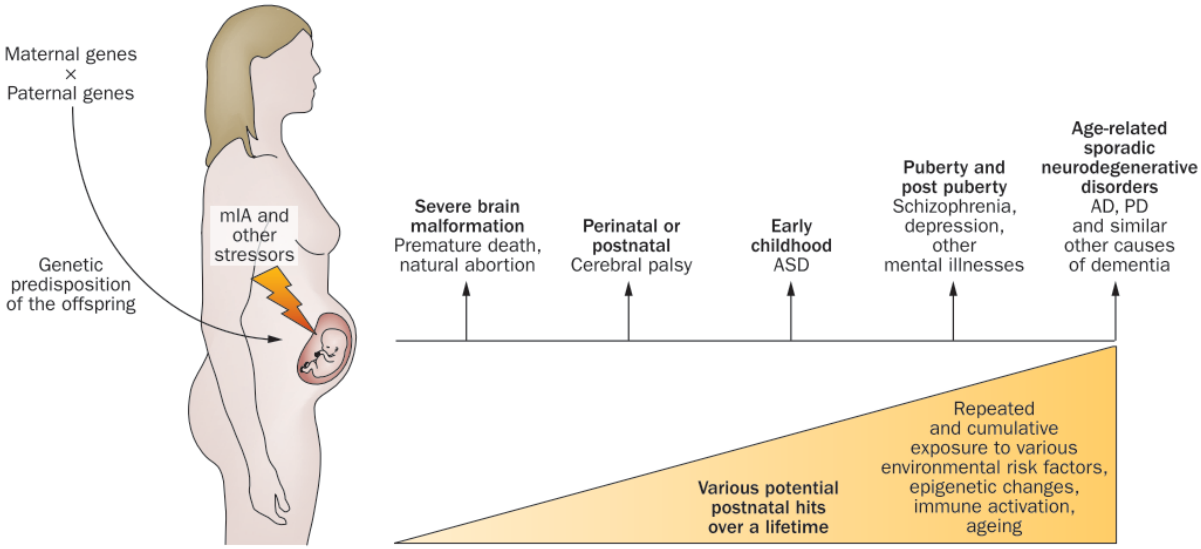


Illustration 7. The maternal immune activation chain of events, leading to the onset of neurodevelopmental disorders. Adapted from Knuesel et al. 2014.

Maternal microbiota risk factors for ASD: Human microbiota has been shown to play an important role in health and disease, potentially acting as a "hit" to pathology onset (Codagnone et al. 2019). Recent studies have provided more insight into the compositional changes undergone by the microbiota during pre- and post-natal development (Torrazza and Neu 2011; Palmer et al. 2007). Interestingly, maternal microbiota is vertically transmitted to the offspring (Funkhouser and Bordenstein 2013). If dysbiotic or unappropriately transmitted to the offspring, microbiota could contribute to trigger developmental abnormalities in the offspring. Moreover, intestinal microbiota can modulate the postnatal development of the immune system and CNS. Its effects are driven by the mode of delivery, vaginal or C-section (Björkstén 2004; E. A. Mayer et al. 2015). Vaginally-born infants adopt a maternal vaginal and faecal flora, while infants born via caesarean display a microbiota similar to that of maternal skin, which is considered to be transmitted postnatally via

newborn handling at the time of birth (Funkhouser and Bordenstein 2013). Interestingly, children born via C-section have been shown to have significantly higher odds of being later diagnosed with ASD diagnosis (Yip et al. 2017). As pregnancy progresses, so does the diversity of intestinal and vaginal flora: intestinal microbiota diversity increasing as pregnancy progresses (Dominguez-Bello et al. 2010), while vaginal microbiota diversity decreases with pregnancy progression (Aagaard et al. 2012). While most studies describing the maternal-foetal microbiome interaction were performed in humans, recent evidence has also been found in rodents, where the maternal microbiome was found to promote the development of autistic-like behaviour in a mouse model of ASD, which will be described in more detail in the next sections.

Association studies between cytokines and ASD in ASD patients: In ASD patients, anomalies in immune function, both in the CNS and in periphery may be responsible for changes in brain connectivity associated with ASD (Illustration 8). In support of this, analyses of the brain transcriptome of individuals with ASD highlighted the enrichment in both synaptic and immune network modules (Voineagu et al. 2011). Moreover, autism has also been linked to neuroinflammation in the brain with microglial and astroglial activation, and increased proinflammatory cytokines in the cerebrospinal fluid of autistic patients (Pardo, Vargas, and Zimmerman 2005; Morgan et al. 2010; Müller and Schwarz 2006; Vargas et al. 2005; Chez et al. 2007). In addition, cytokine dysregulation has also been associated with the pathogenesis of ASD. One study found elevated levels of CCL2 and TGF-β1 in brain tissue from patients with autism (Vargas et al. 2005). The same study also showed a proinflammatory profile of elevated levels of both cytokines and chemokines (amongst which IL-6, IL-8, IFN-γ, CCL, CCL4, CXCL10) in the CSF from autistic patients. Studies on several cohorts reported elevated levels of TNF in the serum and cerebrospinal fluid in children diagnosed with ASD, as compared with healthy children (Ashwood et al. 2011b; Molloy et al. 2006; Vargas et al. 2005; Abdallah et al. 2013; Chez et al. 2007), as well as increased levels in post-mortem brain tissue of ASD patients (Li et al. 2009). Furthermore, the levels of TNF were found to be associated with the severity of autistic symptoms (Chez et al. 2007). Last, increased TNF has also been positively associated with gastrointestinal (GI) dysfunction in LPS-stimulated PBMCs from children with autism (Rose et al. 2018).

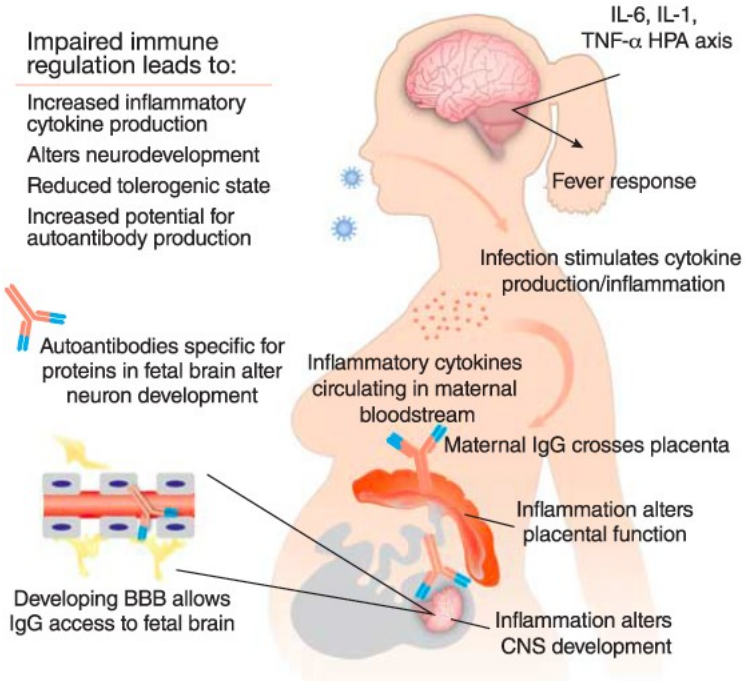


Illustration 8. Overview of the factors leading to maternal immune disruption during gestation. The potential outcome of these events is abnormal faetal brain development leading to behavioural and cognitive impairment. Adapted from Meltzer and Van De Water 2017.

3.4. Mouse models of neurodevelopmental defects triggered by immune activation

The MIA model: Animal studies have further demonstrated that maternal immune activation is a risk factor for neurodevelopmental disorders, such as ASD (Deverman and Patterson 2009). Originally, the MIA model was induced using infection with live human influenza virus into pregnant mice around mid-gestation. The resulting offspring showed abnormal behaviour in tests checking for prepulse inhibition (PPI) of acoustic startle response, exploratory behaviour and social interactions. The authors also observed similar behavioural impairments when they injected Poly(I:C), a viral mimic, described in previous chapters (Shi 2003). This proved that the maternal immune response, rather than the virus itself, was responsible for the behavioural alterations found in offspring. This effect was further characterized in many studies and it was concluded that both bacterial and viral mimics – LPS and Poly(I:C), respectively – can be used as MIA induction models to study ASD pathological processes in mice (Malkova et al. 2012; Hava et al. 2006).

Since then, many studies have reported ASD-like behaviours in offspring of Poly(I:C)-injected mothers between embryonic days 9.5 (E9.5) and 12.5 (E12.5). These behaviours included deficits in PPI of startle, latent inhibition, exploratory behaviour, social interaction and communication, as well as repetitive behaviours (Urs Meyer, Feldon, and Fatemi 2009; Malkova et al. 2012; Shi 2003; Smith et al. 2007). Some of these phenotypes, such as PPI of startle deficits and learned fear, were shown to be transmitted across generations, reaching as high as the third generation of mice (Weber-Stadlbauer et al. 2017). Moreover, the timing of exposure was shown to be important, as Poly(I:C) injection at earlier stages of pregnancy – from E6 to E17 – induces different alterations with notably more severe alterations in learning and memory processes, and surges in cytokine levels in the maternal serum and faetal brain (U. Meyer 2006; Urs Meyer, Yee, and Feldon 2007).

Recent studies have been published on the difficulties to generate and maintain the MIA model induced using maternal injection of Poly(I:C). Specifically, it was reported that changing cage setup from open to individually ventilated cages can affect the efficacy of the MIA model (Mueller et al. 2018). Moreover, the batch of Poly(I:C) was also fund to be extremely important, as different batches have different molecular weights of Poly(I:C), which can affect their immunogenicity (Zhou et al. 2013). Low molecular weight Poly(I:C) induces a decreased immune response in the level of expression of proinflammatory cytokines in the maternal serum in Sprague-Dawley rats (Careaga et al. 2018) and C57BL6/N mice (Mueller et al. 2019), in the mouse placenta and foetal brain (Mueller et al. 2019) as well as decreased sickness behaviour in rats (Careaga et al. 2018) and mice (Mueller et al. 2019). These results managed to explain, in part, the occasional lack of consensus present among research groups, in the different MIA parameters related to behavioural, immunological and neurological outcomes. Moreover, there have been recent discussions about data reproducibility on the MIA model inter- and intra- institutionally and how we could best take into account various aspects of study design (Roderick and Kentner 2018; Kentner et al. 2018; Careaga, Murai, and Bauman 2017). In addition, animal behaviour is known to be influenced by a variety of external factors, fact which we also personally experienced. These range from the housing environmental conditions (ventilated or non-ventilated cages), temperature, hydrometric levels, exposure to noise, frequency of

handling, sex of the person handling the mice (Sorge et al. 2014) – , as well as seasonal changes, despite the controlled environment of animal facilities.

There is strong evidence that cytokine dysregulation plays an important role in the MIA behavioural phenotypes mentioned above. Pregnant MIA dams exhibit increased levels of the proinflammatory cytokines TNF, IFN-β, and IL-1β 3 h post poly(I:C) administration, and increased serum levels of IL-17A two days later (Choi *et al.*, 2016). Moreover, IL-6 (Smith et al. 2007) and IL-17A (Choi *et al.*, 2016) were also elevated in the placenta and brain of offspring born from poly(I:C)-injected mothers. Administration of IL-6 to pregnant dams recapitulated the behavioural effects on the progeny obtained with poly(I:C) injection (Smith et al. 2007), as did direct intracerebroventricular administration of IL-17A to the foetus, in the absence of maternal inflammation (Choi *et al.*, 2016). Furthermore, blockade of maternal T helper 17 (T_H17) cells or of maternal IL-17A abrogated neurobehavioural anomalies in the MIA offspring (Choi *et al.*, 2016). In addition, pretreatment of pregnant mothers with neutralizing antibodies against either IL-6 or placenta-specific inactivation of the *Il6r* gene rescued MIA-associated behavioural abnormalities (Smith et al. 2007; W. L. Wu et al. 2017). This led to the understanding that maternal Th17 cells and IL-6 signaling play important roles in the induction of neurobehavioural alterations in MIA offspring.

Brain alterations were also observed in MIA rodent models. In particular, maternal Poly(I:C) injection in several mouse strains and Wistar rats lead to decreased dendritic spine density and deficits in inhibitory and excitatory synaptic transmission, as well as decreased Purkinje cells in the cerebellum of resulting offspring (Pendyala et al. 2017; Coiro 2015; Ito et al. 2011; Patrich et al. 2016). Moreover, influenza virus infection or Poly(I:C) injection in pregnant dams induced a reduced brain white and gray matter volume, as well as ventricle to brain ratio in the offspring (Gumusoglu and Stevens 2018; Shi et al. 2010).

The maternal microbiota was also found to contribute to the development of autistic-like behaviour in the MIA model. One study demonstrated that the presence of a specific maternal intestinal commensal bacteria - segmented filamentous bacteria (SFB) - was required for the development of the MIA phenotype in the resulting offspring (Kim et al. 2017). SFB lead to the differentiation of T_H17 cells and the subsequent production of

inflammatory cytokines IL-1β, IL-23, IL-6 and, most importantly, IL-17A, which drove MIA behavioural abnormalities. Moreover, Poly(I:C) injection altered offspring microbiota composition and induced further gut dysregulation, among which, increased intestinal permeability, which is associated with an altered immune response (J. R. Turner 2009), and altered cytokine and chemokine profiles, with a specific increase in IL-6 levels (Hsiao et al. 2013).

Despite these immunological and neuronal changes, not much is known about the way cytokines alter brain development in conditions of maternal infection. MIA could, possibly, work by altering the cytokines which are involved in neural connectivity and function (Estes 2016). As previously mentioned in the first chapters of this thesis, cytokines play a key role in neurogenesis and synaptic plasticity and function, where their levels of expression are subjected to age-dependent changes. These cytokines might modify the expression of the major histocompatibility complex I (MHC-I) molecules on offspring neurons during maternal infection, as they are known for their capacity to regulate the levels of MHC-I in (Glynn et al. 2011). MHC-I is present on cortical neurons before and during synaptogenesis in normal conditions, where it acts as a negative regulator of synapse formation and density, required for synaptic pruning (Shatz 2009; Glynn et al. 2011). Alterations in synaptic formation and pruning are associated with the pathogenesis of ASD (Sekar et al. 2016; Tang et al. 2014). MHC-I is highly upregulated on neurons in MIA offspring, which is thought to be required for the reduction in MIA neural connectivity (Elmer et al. 2013).

Neurodevelopmental defects induced by perinatal inflammation: Prenatal or early postnatal immune activation, like in the case of maternal infection, intrauterine infections or neonatal infections, has been associated with many NDDs, among which cerebral palsy, preterm birth and low birth weight. Cerebral palsy is most often characterised by white matter injury and damage to certain areas in the brain, including the cortex, basal ganglia and the thalamus. Moreover, white matter injury prevents olygodendrocyte progenitor cell maturation and causes hypomyelination (Harvey and Boksa 2012). Inflammation is an important risk factor for white matter injury, involving a strong astrogliosis and microgliosis response. Brain inflammation can be triggered by systemic infections, which allows for blood-brain-barrier disruption and passage of immune cells and their mediators to the brain

parenchyma. Systemic administration of IL-1β in neonates has been shown to induce serum and brain induction of pro-inflammatory cytokines IL-1β and TNF, as well as production of cyclooxygenase type 2 (COX2) and prostaglandin E2 (PGE2) (Favrais et al. 2007; Ådén et al. 2010). Overall, IL-1β injection in early life proved to be sufficient to induce neonatal white matter injury, associated with myelinopathy, axonopathy, arrest of olygodendrocyte maturation, as well as memory impairment (Favrais et al. 2011). This neurological deficit was blocked by a COX2 inhibitor, which, not only prevented IL-1β – mediated effects, such as PGE2 production and upregulation of proinflammatory cytokines, but also promoted IL-10 expression in the brain (Favrais et al. 2007). In addition, TNF deletion or blockade by etanercept after co-administration with IL-1β and ibotenate (excitotoxic), succeeded in reducing brain damage by 50% (Ådén et al. 2010), likely due to breakdown of the BBB upon insult, described in a previous study (Coffey, Perry, and Rawlins 1990). This suggests that both TNF and COX2 inhibitors could represent new strategies for neonates with an inflammatory-mediated risk of brain damage.

4. Scientific hypotheses and objectives

Based on previous work published in the field, we have made the hypothesis that neurodevelopment and behavioural outcomes early in life are regulated, either positively or negatively, by specific cytokines both before and immediately after birth.

To test this hypothesis, I used mouse models and my specific aims were:

- To investigate the impact of increased TNF levels between 1 and 5 days after birth on behavioural outcomes during the mouse early postnatal period;
- To identify cytokines associated with altered behavioural outcomes two weeks after birth after adjustment for covariates known or believed to impact either mouse behaviour or cytokines levels.

To reach my first objective, I injected recombinant TNF or PBS daily from P1 to P5 into mouse pups. I then monitored each mouse for several developmental milestones, reflexes and behavioural tests during the first two weeks of age. I also collected serum samples at P16 and measured the level of several cytokines. While serum TNF levels increased in TNF-injected mice at P5, this phenomenon was transient and TNF- and PBS- injected mice did not differ in the level of TNF and other pro-inflammatory cytokines at P16. Compared to control mice injected with PBS, mice injected with TNF acquired the righting and the acoustic startle reflexes more rapidly and exhibited an increased exploratory behaviour at two weeks of age.

To reach my second objective, I have used the extensively described murine MIA model in which pregnant mice are injected at E12.5 with either Poly(I:C) or saline. In these experiments, for each individual offspring I noted several parameters. These included parental age, previous maternal pregnancies and the maternal weight and temperature change before and after Poly(I:C) injection, which served as proxies for the impact of Poly(I:C) on physiological parameters. In addition, I recorded the litter size at birth, the pup weight at two weeks of age (P15), several USV parameters at 8 days of age (P8), locomotor activity (distance travelled and the time spent immobile) at P13, as well as the serum levels of 16 cytokines at P15 (CCL2, CCL3, CCL20, CXCL1, CXCL2, CXCL10, IFN-gamma, IL-1-beta, IL-5, IL-6, IL-15, IL-16, IL-17A, IL-27p28/IL-30, IL-33 and TNF). We then used a penalized

regression model to identify variables associated with being the progeny of Poly(I:C)-injected mothers. In agreement with previous studies, we found that offspring from Poly(I:C)-injected mothers produced fewer USVs at P8, and were less motile at P15 compared to control pups. Most importantly, we identified several variables that were associated with belonging to the Poly(I:C)-injected group. These include weight loss and decreased body temperature experienced by the mother after Poly(I:C) injection, number of pups per litter, pup weight at P15 and serum levels of TNF, IL-5, IL-15, and CXCL10 at P15.

We have therefore chosen to analyse our data using a multivariable analysis based on penalised regression for the study on the MIA model, so as to include as many MIA confounders as possible and evaluate their importance to the overall result. All other studies on the MIA model, as well as most published mouse work, have only been analysed using a univariate approach, which limited data understanding and interpretation. However, several recent studies (Pinto and Götz 2007; Koshiba et al. 2013; Arandas et al. 2017; Göbl et al. 2015) provide evidence that researchers have started to appreciate the importance of complex statistical methods for the interpretation of their data. Some of the types of multivariable analysis employed by these studies are penalized regression models, principal component analysis, cluster analysis and correspondence analysis.

Manuscript #1

Title: TNF promotes sensorimotor reflexes acquisition and exploratory behaviour during early mouse postnatal development

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Abstract
Neuroinflammation is characterized by the presence of pathological levels of pro-inflammatory cytokines in the Central Nervous System and is often associated with cognitive impairments and behavioral problems. In apparent contrast with their detrimental role in neuroinflammation, specific cytokines have also been demonstrated to be required for normal brain function in homeostatic conditions, and more specifically to play a beneficial role in neurogenesis, synaptic pruning and synaptic scaling. Despite these latter studies, whether and how pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF) impact neurodevelopment during the immediate postnatal period remains to be elucidated. To address this issue, we have injected mouse pups daily with recombinant TNF from postnatal day (P)1 to P5. Compared to control pups injected with saline, mice injected with TNF acquired the righting and the acoustic startle reflexes more rapidly and exhibited an increased exploratory behavior two weeks after birth. Our results confirm that cytokines, and notably TNF, could play a beneficial role during the early mouse development.

1. Introduction

Tumor Necrosis Factor (TNF) is a proinflammatory cytokine historically known as a chief orchestrator of the innate immune response (Holbrook et al. 2019). TNF is normally present in minute amounts, however, following an immune challenge, TNF is massively induced in activated macrophages in peripheral tissues. TNF is expressed as a 27 kDa transmembrane form (mTNF) which acts by cell-to-cell contacts, and as a soluble 17 kDa form (sTNF) produced by regulated cleavage of mTNF that is released in tissues and blood (Kriegler et al. 1988). TNF signals through two membrane receptors, TNFR1 and TNFR2. While both sTNF and mTNF activate TNFR1 signaling transduction pathway, only mTNF triggers TNFR2 signaling (Probert 2015).

TNF and its receptors also expressed outside the immune compartment, and notably in the brain. Indeed, cells of the brain parenchyma (neurons, neural stem cells, oligodendrocytes, astrocytes and microglia), as well as endothelial cells of the blood-brain-barrier express TNF and some of these cells also express its receptors (Probert 2015). Immune activation, systemic or central inflammation, injuries to the brain (excitotoxicity, neurodegeneration, ischemia) trigger the synthesis of TNF by neurons, astrocytes and microglia and activation of its receptors (Dantzer et al. 2008). This surge in TNF levels in the brain is thought to condition the induction of neuronal damages consecutive to neuroinflammation (Probert 2015). The importance of TNF in neuroinflammatory processes is further supported by animal studies in which immune activation is used to provoke a surge in both peripheral and central proinflammatory cytokines, including TNF, neuroinflammation, as well as anxiodepressive-like behaviours (anhedonia, hypolocomotion, cognitive deficits, social behaviour deficits), collectively known as sickness behaviour (Dantzer et al. 2008). In adult rodents, the sole intracerebroventricular injection of TNF was able to induce sickness behaviour (Connor et al. 1998; Kaster et al. 2012), while central blockade of TNF with etanercept alleviated sickness behaviour induced by lipopolysaccharides (LPS) (Camara et al. 2015). Also, the acute intraperitoneal injection of TNF induced sickness behaviour associated with a spiked increase in the brain levels of TNF, IL-6 and CCL2, as well as astrocytic and microglial activation (Biesmans et al. 2015; Hayley et al. 1999). Finally, TNF was shown to mediate synaptic and learning deficits after systemic poly(I:C) immune challenge, by increasing dendritic spines elimination in the cortex and altering motor learning processes (Garré et al. 2017).

These neuroinflammatory conditions, in which massive induction of TNF in the brain appears deleterious for brain function and behaviour, differ, and by far, from physiological conditions, in which TNF is constitutively secreted in minute amounts by neurons and glia (Probert 2015). In these conditions, TNF is required for brain cell maintenance and homeostasis. Notably, TNF promotes proliferation of oligodendrocyte progenitors and remyelination (Arnett et al. 2001). Also, TNF enhances excitatory synaptic scaling, a form of homeostatic plasticity that enables adjustment of synaptic strength at the neuron-scale in response to sustained activity (Beattie et al. 2002). In this context, TNF secreted by astrocytes controls the exposure of AMPA receptors at the synapse, thereby directly regulating synaptic neurotransmission in the hippocampus, cortex and striatum (Beattie et al. 2002; Lewitus et al. 2014; Santello, Bezzi, and Volterra 2011; Shim et al. 2018). In the cerebellum, TNF increases the intrinsic excitability of cerebellar Purkinje cells by controling the release of glial glutamate (Shim et al. 2018). *In vivo,* both *Tnf*-knockout (KO) and *Tnfr1*-KO mice have elevated hippocampal adult neurogenesis, while lack of TNFR2 decreases baseline neurogenesis (Iosif et al. 2006; Chen and Palmer 2013). Further evidence for a neuromodulatory role for TNF signalling also came from behavioural studies in *Tnf*-knockout (KO), *Tnfr1*-KO and *Tnfr2*-KO mice. Depending on the studies, *Tnf*-

KO mice display mixed response in a number of tests assessing anxiety or cognition (Golan et al. 2004; Yamada et al. 2000; Camara et al. 2013). These discrepancies were recently explained by the fact that it is indeed the absence of maternal TNF, rather than the absence of TNF in offspring, which conditioned reduced fear response in the offspring (Zupan et al. 2017), as well as enhanced spatial memory associated with increased hippocampal neurogenesis (Liu et al. 2014). Regarding TNF receptors, both *Tnfr1*-KO or *Tnfr2*-KO mice display less despair in the forced swim test, while *Tnfr1*-KO mice exhibit hedonic response in the sucrose drinking test and decreased fear conditioning compared with wildtype littermates (Simen et al. 2006). Collectively, these data point towards a possible role for TNF in the regulation of behaviours linked to emotions and cognition.

In addition to studies in adults, a considerable body of evidence also support early neurodevelopmental roles for TNF. Slight increases in TNF levels are observed in the hippocampus and in the cortex during the first 2 postnatal weeks of life, a time of active neurogenesis and synaptogenesis (Garay et al. 2013). Moreover, low doses of TNF promoted the survival, proliferation, and neuronal differentiation mouse neonatal neural precursor cells cultures, while higher doses were apoptotic (Bernardino et al. 2008). Furthermore, young *Tnf*-KO mice exhibit an accelerated maturation of the dentate gyrus hippocampal region, but with pyramidal neurons harbouring a smaller dendritic arborisation in CA1 and CA3 regions (Golan et al. 2004). Finally, both *in vitro* and *in vivo* studies have shown that developing pyramidal neurons from the cortex of *Tnf*-KO mice are deficient in synaptic scaling, that is critical for the activity-dependent refinement of neural circuitry during early development (Stellwagen and Malenka 2006; Kaneko et al. 2008; Ranson et al. 2012).

This suggests a critical role for TNF in shaping the nervous system during early developmental stages. However, the impact of TNF on behaviour in the early postnatal period has not been investigated so far. Here we show that repeated perinatal systemic injections of TNF in the perinatal period promotes the acquisition of developmental reflexes and exploratory behaviour in pups.

2. Materials and Methods

2.1 Animals and treatments

Adult OF1 female mice were purchased at 15.5 days of gestation (embryonic day (E) 15.5 post conception) from Charles River (L'Arbresles, France). Mice were housed in a temperature (22-24°C) and hygrometry (70-80%)-controlled room with a 12 h light/dark cycle (lights on from 8:00 a.m. to 8:00 p.m.) with *ad libitum* access to water and food (standard chow, reference 4RF25, Mucedola). Pregnant dams were housed into individual cages and remained in the same cages until the end of the experiment. The day and time of birth were recorded and only pups born on E19.5 were used. The day of birth was labelled postnatal day 0 (P0). At P1, all pups were sexed and only male pups were used for the experiments described here forth.

At P1, each cage containing one mother was randomly assigned a litter of 11-14 male pups. Each litter was divided into two distinct treatment groups, to control for the litter effect. In the pilot study to determine the optimal TNF dose to be used, pups were divided into five experimental groups (containing 6 pups per group) treated intraperitoneally with either recombinant murine soluble TNF (Biolegend, ref.575204) diluted in sterile PBS, or PBS as vehicle for the control group. Intraperitoneal injection was performed under the iliac fossa, on the right side of the pup, between 10-12 a.m. We administered 5 µL of solution per pup, with the concentration of 0.25, 1, 5 and 20 µg/Kg calculated according to a standard pup weight for each day of the treatment. Pups were treated once a day from P1 to P5.

Two independent cohorts were then generated using the highest TNF dose of 20 µg/Kg, one containing 19 pups and the second 45 pups. For each cohort, pups were divided into two experimental groups treated intraperitoneally with either TNF or vehicle for the control group. To control for the litter effect, each litter corresponding to one mother contained both animals treated with TNF and control animals treated with Vehicle. Altogether 33 pups were injected with TNF and 31 with PBS. Intraperitoneal exposure was performed by administering 5 µL of solution per pup, with the concentration calculated according to a standard pup weight for every day of the treatment. Pups were treated once a day for 5 days, from P1 to P5.

2.2. Developmental and behavioural tests

The acquisition of developmental milestones, reflex ontology and behavioural development of mouse pups were assessed using procedures derived from Fox's battery of tests and Wahlsten's adaption of Fox's tests (Fox 1965; Wahlsten 1974), as described in (Heyser 2004; Feather-Schussler and Ferguson 2016). Pups were tested individually, preferably in the morning (9 a.m.-1 p.m). To limit stress due to maternal separation, the time spent by the pup away from the mother and home cage was limited to the duration of each test. At the end of each test the pup was immediately put back in its home cage.

Ear development: The day of the opening of the ear canal, defined as a fully detached outer ear membrane, was recorded in pups aged P3 to P5. A fixed score of 0, 1 or 2, according to the number of ears developed per animal was assigned.

Eyelid opening: The day of the eyelid opening, defined as any visible break in the membrane covering the eye, was recorded in pups aged P12 to P16. A fixed score of 0, 1 or 2, according to the number of eyes opened per animal was assigned.

Righting reflex: Pups were examined for the righting reflex every two days between P2 and P6. Each pup was placed on its back on a flat, hard surface and kept immobile for 5 s. The pup was then released and the time taken to return to the upright position was recorded. Animals unable to perform the righting after 1 min were assigned a score of 60 s. Each pup was only tested once per day.

Acoustic startle reflex: Pups aged P10 to P14 were examined every day and the day of reflex acquisition was determined. A cell counter was use to generate an auditive stimulus and the animal's startle response was recorded. A fixed score of either 0 or 1 was assigned. A score of 1 was given to pups which displayed a startle reaction; and a score of 0, to pups which did not exhibit a startle response.

Ambulation test: The ambulation ability of pups was assessed every day between P6 and P12 to monitor the acquisition of walking proficiency. Each pup was placed on a flat, hard surface and the walking pattern was assessed over 1 min, according to previous scoring criteria (Feather-Schussler and Ferguson 2016): 0 = no movement, 1 = crawling with asymmetric limb movement, 2 = slow crawling but symmetric limb movement, and 3 = fast crawling/walking.

Olfactory Orientation: Pups aged P9 were tested for their ability to sense and follow the maternal smell using only olfactory cues. Each pup was separated from its litter and placed into the center of a rectangular-shaped transparent plastic box divided into three equal-sized zones: the "maternal" zone on one side, the central zone and the "clean" zone on the opposite side from the maternal zone. The maternal zone contained freshly sampled maternal bedding, while the clean zone contained clean bedding. A Plexiglas odour separator was placed above the central zone to prevent the maternal smell from permeating. The movement of the pup was video-recorded over a period of 10 min. The ANY-maze videotracking software (reference) was used to determine: the latency to the first entry in the maternal bedding zone and the time spent in each of the three zones. The pups were returned to their original cages after the completion of the test.

Exploratory behavior: At P13, each pup was separated from its litter and placed into a rectangular-shaped transparent plastic box. The movement of the pup was recorded over a period of 10 min and the videos were analyzed using the ANY-maze videotracking software to determine the total distance travelled and the time the pups spent in a mobile state. The exploratory index was then obtained by calculating the ratio between these two parameters. By relating the distance travelled to the time spent in an active state, this index indicated whether the pup explored the setup more extensively. Pups were returned to their original cages after the completion of the test.

2.3. Sacrifice and sample collection

Pups were sacrificed at age P5 in the pilot study and at P16 in the other two experiments. Pups were anaesthetized using a lethal injection of anaesthetic. The blood was collected by cardiac puncture into clean 1.5 ml Eppendorf tubes. To obtain serum, the blood was allowed to cloat for 1 h at room

temperature (RT) and then centrifuged (10,000g, 4°C, 20 min), transferred to clean 0.5 ml Eppendorf tubes and immediately froze in liquid nitrogen and stored at -80°C until further analysis. Brains were removed from the skull, immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

2.4. Serum cytokine measurements

We used the the V-PLEX® Mouse TNF Kit or the V-PLEX® Mouse Cytokine 29-Plex Kit electrochemiluminescence-based assays from (Meso Scale Diagnostics, Rockville, USA). Assays were performed according to the manufacturer's instructions. The reagents were stored at 4°C until used, when they were allowed to reach RT immediately prior to usage. All buffers used were provided with the kit, with the exception of the Wash Buffer, which was produced in-house using sterile PBS 1x and 0.05% Tween-20. Lyophilized standards (containing cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, KC/GRO, TNF, IL-9, MCP-1, IL-33, IL-27p28/IL-30, IL-15, IL-17A/F, MIP-1α, IP-10, MIP-2, MIP-3α, IL-22, IL-23, IL-17C; IL-31, IL-21, IL-16, IL-17A, IL-17E/IL-25) were reconstituted to master stock solutions. Eight concentrations of the standard were made by fourfold serial dilutions of the master stock. The majority of serum samples were diluted 1:4, with the exception of several samples with low remaining serum volume, for which dilutions between 1:6 to 1:11 were used. All samples were diluted on ice on pre-plates. The background level was determined using a buffer blank. Eight serial dilutions of standards and buffer only (in duplicates) were run together with samples run in singlicate using the Sector Imager 2400 plate reader (Meso Scale Diagnostics, Rockville, USA). Concentrations of cytokines in each sample were interpolated from standard curves generated with a five-parameter logistic regression equation in Discovery Workbench 3.0 software (Meso Scale Diagnostics, Rockville, USA). The software allows for a graphic visualization of the placement of all samples on the standard curve for each cytokine. IL-2, IL-4, IL-12p70, IL-9, IL-17A/F, IL-22, IL-23, IL-17C, IL-31, IL-21, IL-17E, IL-25 were below the Lower Limit of Detection (LLOD) in more than 20% of the samples and were therefore not retained for downstream analyses. For the other cytokines, cytokine levels below the lower limit of detection (LLOD), we imputed a value equal to half the LLOD value indicated by the manufacturer.

2.5. Statistics

Univariate analysis: For univariate comparison analysis of 2 groups, the normality of the data was first assessed using the Kolgomorov-Smirnov test. For normal data, the 2-tailed unpaired Student's T-test was used. For non-normal data, raw data was log-transformed to meet normality criteria prior to Student's T-test. If normality was not reached after the log transformation, the non-parametric Mann-Whitney U-test was used. For multiple group comparisons, a 2-way ANOVA with *post hoc* Sidak's correction for multiple comparisons was used. Statistical significance was represented as a minimum p-value (p) lower than 0.05. All univariate analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, USA).

Repeated measures multivariate analysis, modelling the effect of treatment: Measurements of the developmental, behavioural and cytokines variables were made repeatedly on pups from two independent cohorts at different time points. This experimental design introduces dependencies, measurements are correlated within individuals and we cannot exclude random differences between cohorts and time of measurement. These dependencies must be accounted for in order to make correct inferences when comparing individuals that have received TNF injections and controls. We decided to

use the marginal model generalized estimating equation (GEE) approach to model each variable marginally, considering treatment, cohort and time as covariates (Burton, Gurrin, and Sly 2005; Samur, Coskunfirat, and Saka 2014).

2.6. Study approvals

Animal housing and experimentation were conducted in facilities certified by regional authorities (Direction Départementale de Protection des Populations des Alpes-Maritimes, accreditation #C-06-152-5). The study was in accordance to procedures approved by the Ministère de l'Enseignement Supérieur et de la Recherche (APAFIS#19129-201902071212672).

3. Results

3.1. Perinatal TNF injections from P1 to P5 yield increased serum levels of TNF at P5

To determine the optimal dose of TNF to use in the study, we performed a pilot experiment in which mouse pups were injected intraperitoneally daily from P1 to P5 with various doses of TNF ranging from 0.25 to 20 µg/Kg. All pups survived, even when injected with the highest TNF dose, and there was no sign of inflammation (redness, swelling) on the injected flank. There was no significant effect of TNF treatment on body weight in all groups, suggesting that TNF treatment did not impair early growth (Fig. 1A).

Compared to saline-injected mice, pups injected with low doses of TNF (0.25, 1 µg/Kg) did not exhibit changes in circulating levels of TNF at P5. For pups injected with higher doses, 5 and 20 µg/Kg, there was a dose-dependent increase in serum TNF levels at P6, with 2-fold and 11-fold increase, respectively (Fig. 1B). Based on these results, we chose to use the highest dose of TNF in further experiments, which yielded high levels of TNF levels while preserving general growth.

3.2. TNF-injected pups acquire sensorimotor reflexes more rapidly

We monitored the growth, cytokine circulating levels at P16, the acquisition of developmental milestones (ear eversion, eyelid opening) and sensorimotor reflexes (righting reflex, acoustic startle reflex) in pups injected from P1 to P5 with 20 µg/Kg of TNF and in control pups (Fig. 2A). For each variable under scrutiny, we used GEE to test the effects of treatment considering a repeated measures dependency on the measurements, the effect of time and of having pups from two distinct cohorts. Regarding body weight gain from P2 to P16, there were significant effects of time and cohort variables, but no significant impact of TNF treatment (Fig. 2B, C), suggesting that TNF did not impact general growth.

We assessed serum samples at P16 for the levels of TNF and other cytokines after repeated injections of TNF at 20 µg/Kg from P1 to P5. Vehicle- and TNF-injected pups exhibited similar levels of TNF as well as all other tested cytokines including IL-1β, IL-6, IFN-γ, IL-5 and CXCL1 (Supplementary Fig. 1). Of note, positive correlations between the levels of IL-5 and IL-6, as well as CXCL1 and IL-6 were observed (Supplementary Fig. 2). This suggested that perinatal injections of TNF from P1 to P5 is not sufficient to induce a sustained systemic inflammation by P16.

Furthermore, TNF treatment did not modify the timing of developmental milestones such as ear aversion (Fig. 2D, E) or eyelid opening (Fig. 2F, G), while there were significant effects of time and cohort variables. This suggested that TNF did not induce gross developmental changes.

However, TNF treatment had a significant effect on the acquisitions of early reflexes (Fig. 2H-K). Both the righting reflex (Fig. 2H, I) and the acoustic startle reflex (Fig. 2J, K) occurred at earlier stages in TNF-injected pups compared to control animals, as shown by the significant effect of time and treatment variables. At P2, TNF-injected pups took almost twice less time to right up than control pups (Fig. 2H). By P14, all the TNF-injected mice had acquired the acoustic startle reflex, while only 50% of the control pups had acquired it (Fig. 2J).

3.3. TNF-injected pups exhibit enhanced exploratory behaviour

We further characterized the impact of perinatal TNF treatment on: olfactory orientation at P9, walking proficiency (ambulation, from P6 to P12) and locomotor activity at P13 (Fig. 3A). In the olfactory orientation test, TNF- and saline-injected mice performed equally well as measured by the latency or time to first reach the maternal bedding (Fig. 3B) and the equal time spent the three zones of the set-up (Fig. 3C). This indicated that sensorimotor processing was likely similar in both groups for this task.

When assessing walking proficiency in TNF-injected and control pups, there was also a significant effect of the variables time and cohort (Fig. 3D, E). However, there was no effect of TNF treatment, as TNF- and saline-injected pups exhibited similar development overtime of walking proficiency, with the acquisition of a mature walking pattern by P12 in both groups (Fig. 3D, E).

We then monitored the exploratory behaviour of TNF-injected and control pups when individually placed in a novel environment (i.e. an unfamiliar clean cage without bedding) for 10 min. TNF-injected pups spent more time mobile (Fig. 2F, G) and travelled a longer distance during a 10 min session (Fig. 3H), when compared to vehicle-injected pups, but there was no effect of time or cohort variables on these parameters. Given that by P12 both TNF-injected and control pups had acquired a mature walking pattern (Fig. 3D, E), these data can directly be interpreted as an increased exploratory behaviour in TNF-injected pups, as shown by their increase in the exploratory index compared to control pups (Fig. 3I).

4. Discussion

4.1. Beneficial effects of TNF on reflex acquisition and exploratory behaviour during early mouse development

One major finding of this study is that TNF accelerated the acquisition of sensorimotor reflexes (both righting and acoustic startle reflexes) in pups, as well as promoted exploratory behaviour in a novel environment. While underlying mechanism remain to be investigated, it was demonstrated in 3-days old rat pups that radiolabelled soluble TNF injected peripherally crossed the brain-blood-barrier (BBB) using a saturable transport system (Gutierrez, Banks, and Kastin 1993). Interestingly, TNF injection did not yield a disruption of the BBB and TNF efflux from the brain was slow, suggesting that peripheral TNF was sequestered in the brain (Gutierrez, Banks, and Kastin 1993). Therefore, it is likely that at least a fraction of the recombinant TNF that we injected peripherally into mouse pups from P1 to P5 crossed the BBB and eventually resulted in increased levels of TNF in brain.

Previous studies have shown that sTNF triggers the TNFR1 signalling transduction pathway and that all cells of the brain parenchyma, including neurons, astrocytes, oligodendrocytes and microglia, as well as epithelial cells of the BBB express TNFR1 (Probert 2015; Holbrook et al. 2019). Therefore, the beneficial effect of TNF on the acquisition of sensorimotor reflexes and the exploratory behaviour may be explained by a direct effect of TNF on several cell types in the brain.

The righting reflex rectifies the orientation of the pup when it is placed supine on its back. In early developmental stages, the reflex essentially relies on somatosensory inputs (tactile stimuli on the body and head), and does not involve visual inputs since eyelids are not opened or vestibular inputs, as the vestibular system is not mature yet at P2 (Jamon 2014). The cerebellum is a key region for integrating somatosensory inputs and correct posture via motor neurons of the spinal cord which control muscular movements to right the body. It may be envisioned that TNF-injected pups may have undergone an earlier development of the cerebellar or spinal neurons coordinating the righting reflex at early stages. TNF was recently shown to enhance the intrinsic excitability of cerebellar Purkinje cells in the juvenile rat cerebellum (Shim et al. 2018). This could contribute to earlier acquisition of the righting reflex by a better integration of propriotactile cues and a quicker coordination of movement.

The startle response is an unconscious defensive response to a sudden stimulus, in our case acoustic. In mouse pups, the startle reflex yields a sudden extension of the head and fore and hind limbs which are then withdrawn to reach a crouched position. The neural pathway involved is well described and involves first a synapse from the auditory nerve fibers in the ear to the cochlear root neurons (CRN), the first acoustic neurons of the central nervous system. The CRN axons then synapse on neurons in the nucleus reticularis pontis caudalis (PnC), located in the pons of the brainstem. Finally, the PnC axons synapse to spinal motor neurons to induce the startle (M. Davis 1984). It is possible that TNF-injected pups have developed earlier axonal projections of cochlear root neurons or PnC neurons, causing the reflex to occur earlier.

At P13, pups have hardly opened their eyelids and spatial information processing is still immature (Ricceri, Colozza, and Calamandrei 2000). At this stage, pups mostly rely on sensory inputs from palpation with their whiskers for guidance of exploratory motor behaviour (Arakawa and Erzurumlu 2015). In the juvenile male rat, TNF increases the intrinsic excitability of cerebellar Purkinje cells (Shim et al. 2018). Furthermore, a recent study has demonstrated that whisker reflex adaptation was facilitated by potentiation of cerebellar Purkinje cells (Romano et al. 2018). It is possible that perinatal injection of

TNF could modify the course of development of Purkinje cells, contributing to increase palpation whisking-driven exploratory behaviour at this stage.

4.2. Physiological relevance of our experimental paradigm

Our study is mainly based on gain-of-function experiments in which mouse pups are injected daily from P1 to P5 with recombinant TNF. We show that this yielded an 11-fold increase in circulating TNF levels at P5 and improves reflexes acquisition and exploratory behaviour. We consider that this increase is modest, as compared to the massive increase over several hundred folds in peripheral TNF observed upon systemic inflammation or immune activation and in which TNF likely plays a deleterious role. Previously, Gressens and his colleagues have shown that perinatal treatment with the pro-inflammatory cytokine IL-1β, twice daily from P1 to P5 (at 10 µg/Kg), induced systemic inflammation, neuroinflammation and microstructure lesions in the white matter, impaired oligodendrocytes maturation, with long-lasting behavioural consequences (Favrais et al. 2011). Furthermore, they showed that repeated perinatal injections of IL-1β, IL-6, IL-9 and TNF (twice a day at 20 µg/Kg) sensitised the brain to further excitotoxic brain lesions at P5 (Dommergues et al. 2000). Proinflammatory cytokines and interleukin-9 exacerbate excitotoxic lesions of the newborn murine neopallium. It is possible that in physiological conditions TNF exerts beneficial effects on brain development and behaviour while in situations of brain injury or systemic inflammation it could contribute to greater neuronal vulnerability to excitotoxic lesions.

4.3. Experimental design and statistical analysis

Environmental factors, maternal effect and litter effects are acknowledged confounding variables in developmental and behavioural studies. To overcome the maternal prenatal effect, we randomly assigned a new mother to pups at P1, so that each litter was constituted by pups coming from different mothers. To overcome the litter effect, litters were culled to 11-12 individuals to limit litter size effect. Also, the two conditions tested (TNF or vehicle treatment), were represented in each individual litter. In doing so, we obtained both TNF-treated and control pups bread by the same mother, thus excluding the confounding effect of differential maternal care or milk quality, that could contribute to developmental or behavioural differences.

To model the effect of the variables “time” and “cohort” which, besides the variable “treatment”, that could impact developmental and behavioural outcomes, we used the Generalized Estimating Equations (GEE) model. This modelling strategy is particularly adapted to the study of outcomes that were measured repeatedly overtime on the same individuals. The focus of the GEE is on estimating "population-averaged" effects, rather than the regression parameters that would enable prediction of the effect of changing one or more covariates on a given individual.

4.4. Limitations of the study

One limitation of this study is that it is restricted to males. A large body of evidence suggests that males are more at risk than females for neurodevelopmental disorders (E. P. Davis and Pfaff 2014). Likewise, several pre-clinical studies in rodents have shown a sexual dimorphism in neurodevelopmental mechanisms and in sensitivity to developmental brain insults (DiPietro and

Voegtline 2017; Liddelow et al. 2017; Chung and Auger 2013; Davies and Wilkinson 2006). Therefore, it remains to be determined whether TNF injection also accelerate the acquisition of reflexes and promotes exploratory behaviour in female pups. Also, we have not assessed whether TNF perinatal injection induced long-lasting behavioural changes beyond P14, and notably in adult animals.

Another limitation is that our experimental model is the OF1 outbred stock obtained from "a closed population of genetically variable animals that is bred to maintain maximum heterozygosity" as defined in (Chia et al. 2005). The use of an outbred stock presents both advantages and disadvantages. On one hand, the OF1 outbred stock is larger, more robust and produces more pups per litter than any inbred mouse strain (Chia et al. 2005). Furthermore, OF1 mothers provide high quality maternal care and are more resilient to environmental stressors, such as recurrent manipulation of the progeny imposed by our experimental design. On the other hand, outbred stocks bear recessive mutations that may affect experimental results and lower treatment effect size. This genetic variation may affect behavioural responses, since a stock may contain a mixture of homozygous, heterozygous and wildtype pups (Chia et al. 2005). However, outbred stock present the advantage to mimic more closely human populations. The fact that we randomized pups from P1 to for new litters minimizes the possible confounding effect of genetics in our model. Nevertheless, the possibility to generalize our data to inbred mouse strains such as C57Bl/6J should be explored in follow-up studies. This would allow also enable to use mice knock-out for *Tnf*, *Tnfr1* or *Tnfr2* which are currently unavailable in OF1 stocks. Further functional studies in the OF1 mouse stock will involve neutralizing antibodies targeting TNF or injection of soluble TNF receptors to perform loss-of-function experiments.

5. Conclusions & future directions

Our study has revealed the beneficial early-life impact of perinatal TNF on reflexes acquisition, as well as exploratory behaviour. This reveals a possible new facet of TNF function during early mouse development and complement previous behavioural studies performed in adult animals. In a follow-up study, we will measure cytokine levels in the brain of TNF-injected animals at P5 to evaluate to which extent exogenous TNF is able to cross the BBB. Also, we will monitor TNF levels in the brain later at P16 to verify whether perinatal TNF exposure yields sustained increased in brain TNF. Also, we will investigate the consequences of perinatal TNF-injections in the brain at P16, using RNAseq transcriptomics. Finally, we are foreseeing loss-of-function experiments targeting TNF. This should provide insight in the molecular pathways impacted by early exposure to TNF that could possibly explain the behavioural phenotypes we describe. Future work is required to understand brain molecular and cellular changes induced by TNF treatment. Due to the impact of TNF on neurogenesis and synaptic scaling, future studies should focus on understanding whether the beneficial developmental and behavioural effects of TNF we describe in this study are due to astrocytic, microglial or neuronal pathways.

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Figures

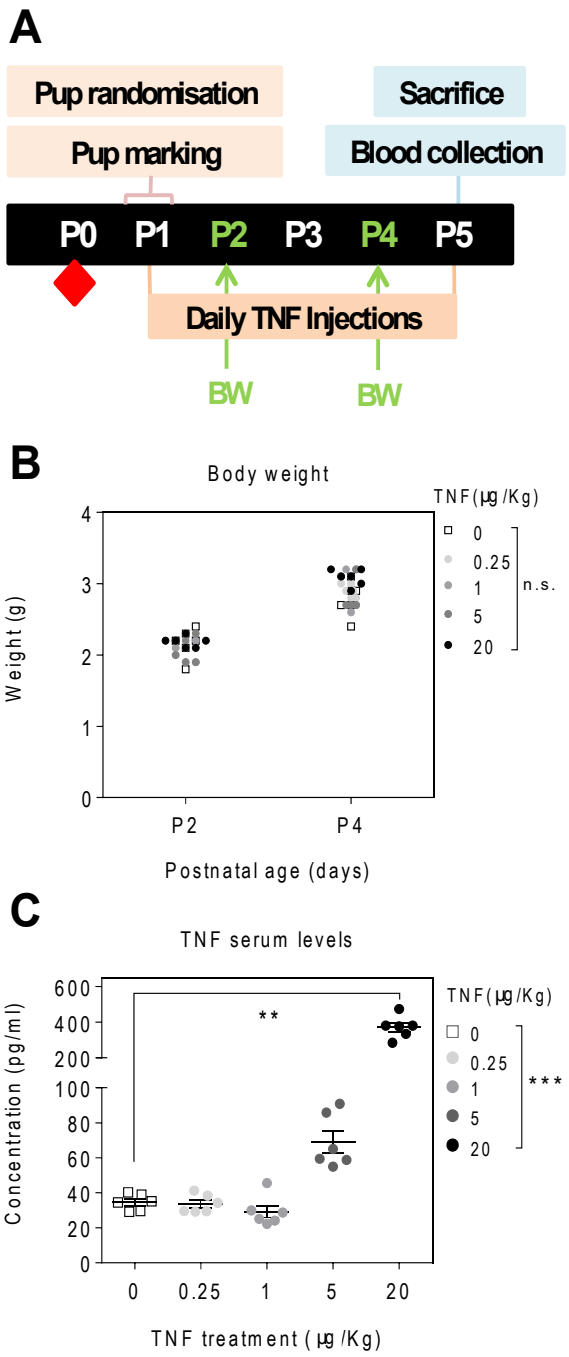


Figure 1. Pilot experiment used to define the optimal experimental conditions of TNF perinatal injection.

(A) Timeline of the experiment. OF-1 mouse pups born on P0 were injected intraperitoneally with four different doses of TNF (0.25, 1, 5, 20 µg/kg) or PBS (0) from P1 to P5, after which the blood was collected.

(B) Body weight of pups at P2 and P4. Data are presented as means ± SEM; n = 6 mice/group; 2-way ANOVA: p(Treatment) = 0.4368, p(Postnatal age) < 0.0001, p(Interaction) = 0.0002; Tukey's post hoc tests for treatment-wise comparisons: ns.

(C) TNF serum levels at P5 in the pups injected with either PBS or the four different doses of TNF described above. Data are presented as means ± SEM; n = 6 mice/group; Kruskal-Wallis test: p(Treatment) = 0.0001; Dunn's post hoc multiple comparison test for treatment effect, ***, p < 0.001. BW, body weight; P, postnatal day.

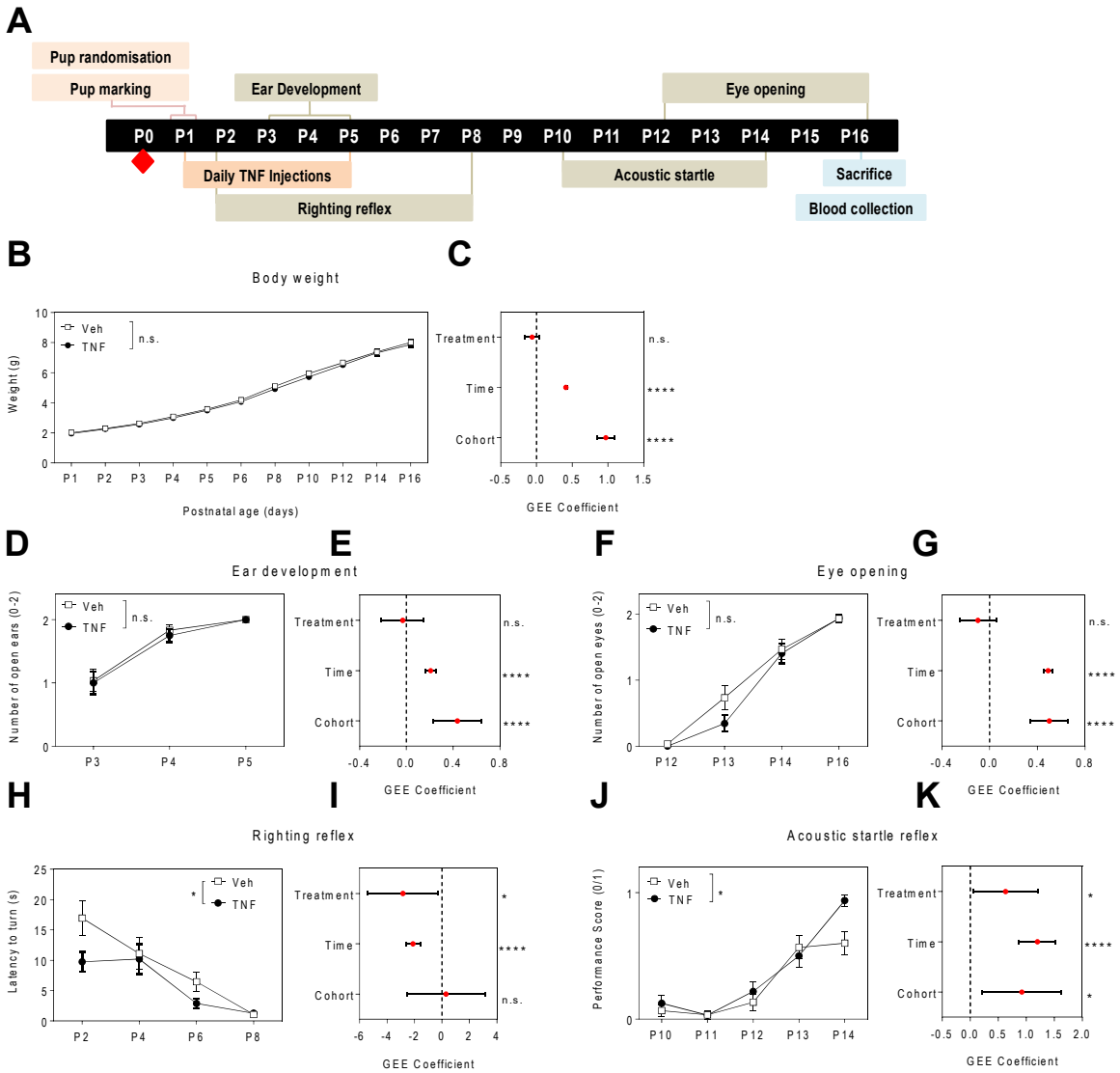

A
Pup randomisation
Pup marking
Ear Development
Eye opening
P0 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14 P15 P16
Daily TNF Injections
Righting reflex
Acoustic startle
Sacrifice
Blood collection
B
Body weight
Veh
TNF
n.s.
Weight (g)
Postnatal age (days)
C
Treatment
Time
Cohort
n.s.
****
GEE Coefficient
D
Ear development
Number of open ears (0-2)
E
F
Eye opening
Number of open eyes (0-2)
G
H
Righting reflex
Latency to turn (s)
I
J
Acoustic startle reflex
Performance Score (0/1)
K

Figure 2. Changes in developmental milestones in pups up to 2 weeks of age upon TNF injection.

(A) Timeline representation of the experimental plan. OF1 mouse pups born on P0 were injected intraperitoneally with TNF (20 µg/kg) or PBS (Veh) everyday for five days. The developmental milestones tested were Ear Development, Eye opening, Righting reflex and Acoustic startle reflex. The pups were sacrificed at P16 and the blood was collected.

(B-C) Body weight of pups aged P1 to P16. (B) Data are presented as means ± SEM; n = 29 PBS, n = 31 TNF. (C) GEE coefficients and p-values associated with the cohort, treatment and time as covariates in the protocol: p(Cohort) < 0.0001, p(Treatment) = 0.2183, p(Time) < 0.0001.

(D-E) Ear development of pups aged P3 to P5. (D) A fix score of 0, 1 or 2 was set according to the number of ears everted/animal. Data are presented as means ± SEM; n = 31 PBS, n = 32 TNF. (E) GEE coefficients and p-values associated with the cohort, treatment and time as covariates in the protocol: p(Cohort) < 0.0001, p(Treatment) = 0.729, p(Time) < 0.0001.

(F-G) Eye opening of pups aged P12 to P16. The data represents a fixed score of 0, 1 or 2, according to the number of eyes opened/animal. (F) Data are presented as means ± SEM; n = 31 PBS, n = 32 TNF. (G) GEE coefficients and p-values associated with the cohort, treatment and time as covariates in the protocol: p(Cohort) < 0.0001, p(Treatment) = 0.212, p(Time) < 0.0001.

(H-I) Righting reflex of pups aged P2 to P8, performed under 1 minute. (H) Data are presented as means ± SEM; n = 31 PBS, n = 32 TNF. (I) GEE coefficients and p-values associated with the cohort, treatment and time as covariates in the protocol: p(Cohort) = 0.840, p(Treatment) = 0.0308, p(Time) < 0.0001.

(J-K) Acoustic startle reflex of pups aged P10 to P14. The data represents a fixed score of either 0 or 1, where 1 is given to pups which perform the test and 0, to pups which do not perform the test. (J) Data are presented as means ± SEM; n = 31 PBS, n = 32 TNF; (K) GEE coefficients and p-values associated with the cohort, treatment and time as covariates in the protocol: p(Cohort) = 0.0101, p(Treatment) = 0.0332, p(Time) < 0.0001. *, p < 0.05, ****, p < 0.0001. P, postnatal day.

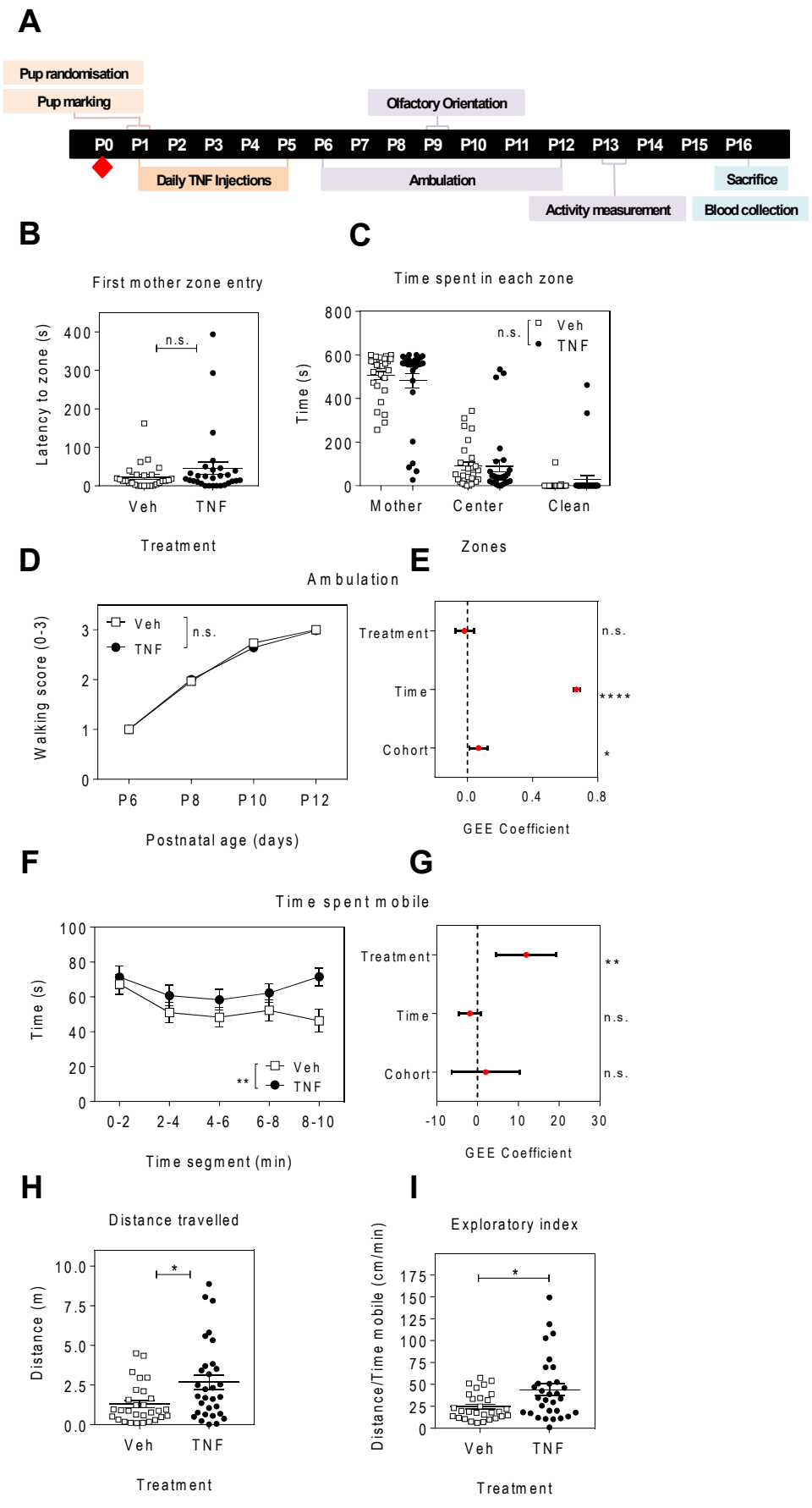

A
Pup randomisation
Pup marking
Olfactory Orientation
P0 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14 P15 P16
Daily TNF Injections
Ambulation
Sacrifice
Activity measurement
Blood collection
B
First mother zone entry
Latency to zone (s)
n.s.
Veh
TNF
Treatment
C
Time spent in each zone
Time (s)
n.s.
Veh
TNF
Mother
Center
Clean
Zones
D
Ambulation
Walking score (0-3)
Veh
TNF
n.s.
P6
P8
P10
P12
Postnatal age (days)
E
Treatment
Time
Cohort
n.s.
****
*
GEE Coefficient
F
Time spent mobile
Time (s)
Veh
TNF
**
0-2
2-4
4-6
6-8
8-10
Time segment (min)
G
Treatment
Time
Cohort
**
n.s.
n.s.
GEE Coefficient
H
Distance travelled
Distance (m)
*
Veh
TNF
Treatment
I
Exploratory index
Distance/Time mobile (cm/min)
*
Veh
TNF
Treatment

Figure 3. TNF perinatal injection does not impact olfactory orientation but enhances exploratory behaviour.

(A) Timeline representation of the experimental plan. OF1 mouse pups born on P0 were injected intraperitoneally with TNF (20 µg/kg) or PBS (Veh) everyday for five days. The behaviours tested were Olfactory Orientation, Ambulation and Locomotor activity. The pups were sacrificed at P16 and the blood was collected. (B-C) Olfactory Orientation test performed over 10 minutes in pups aged P9.

(B) Latency to the first entry in the maternal bedding zone. Data are presented as means ± SEM; n = 27 PBS, n = 29 TNF; Mann-Whitney test: ns.

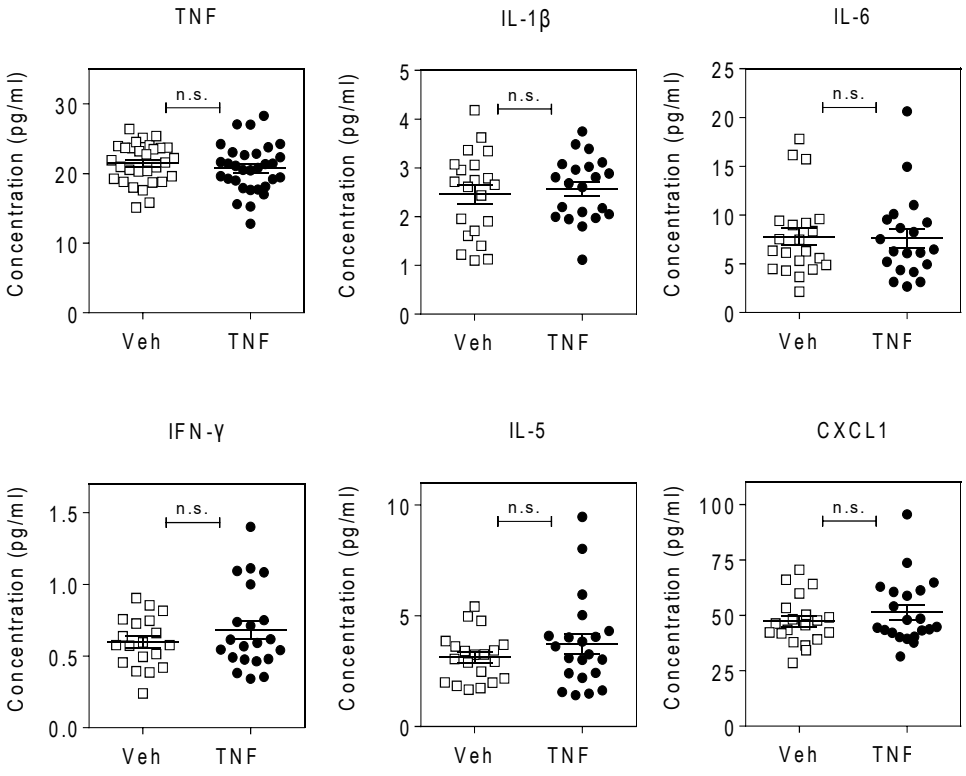
(C) Time spent in each of the 3 zones that the cage was divided into: the Maternal bedding zone, the Center zone and the Clean bedding zone. Data are presented as means ± SEM; n = 27 PBS, n = 29 TNF; 2-way ANOVA: p(Treatment) = 0.9602, p(Postnatal age) < 0.0001, p(Interaction) = 0.7046; Sidak's post hoc tests for treatment-wise comparisons: ns. (D-E) Ambulation test performed over 1 minute in pups aged P6 to P12.

(D) Data are presented as means ± SEM; n = 31 PBS, n = 32 TNF. (E) GEE coefficients and p-values associated with the cohort, treatment and time as covariates in the protocol: p(Cohort) = 0.0216, p(Treatment) = 0.5604, p(Time) < 0.0001; *, p < 0.05, ****, p < 0.0001.

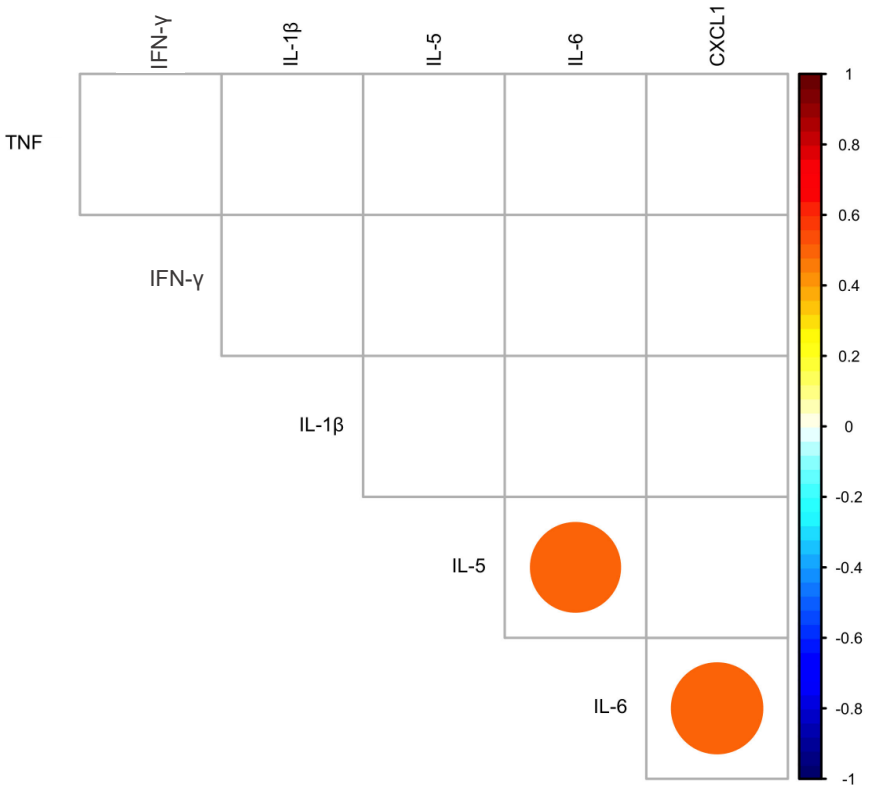
(F-G) Time spent mobile in pups aged P13, represented as time spent mobile for a period of 10 minutes, shown in 2-minute time segments. (F) Data are presented as means ± SEM; n = 29 PBS, n = 31 TNF. (G) GEE coefficients and p-values associated with the cohort, treatment and time as covariates in the protocol: p(Cohort) = 0.6345, p(Treatment) = 0.0014, p(Time) < 0.1632; **, p < 0.01.

(H) Distance travelled in pups aged P13, represented as the total distance travelled over 10 minutes. Data are presented as means ± SEM; n = 29 PBS, n = 31 TNF; Mann-Whitney test: p(Treatment) = 0.0157

(I) Exploratory index in pups aged P13, represented as the ratio between the total distance travelled and time mobile. Data are presented as means ± SEM; n = 29 PBS, n = 31 TNF; Mann-Whitney test: p(Treatment) = 0.0266. *, p < 0.05, ****, p < 0.0001. P, postnatal day.



Supplementary Figure 1. Serum cytokine levels in TNF– injected and control (Veh) pups at P16. The cytokines tested were TNF, IL-1β, IL-6, IFN-γ, IL-5 and CXCL1. For TNF, the data are presented as means ± SEM; n = 30 PBS, n = 32 TNF; Mann-Whitney test: ns. For the rest of the cytokines, the data are presented as means ± SEM; n = 21 PBS, n = 21 TNF, with 1-2 verified outliers/treatment excluded for each cytokine; all Mann-Whitney tests: ns.



Supplementary Figure 2. Correlation analysis between serum biomarker levels in pups at P16. Spearman's r correlation coefficient rank test with Benjamini-Hochberg correction for multiple testing. Statistically significant correlations are presented as a heatmap of r coefficients. Dot area is proportional to r coefficient. Correlation IL-6 vs. IL-5: p = 0.0011; Correlation IL-6 vs CXCL1: p = 0.0085. **, p < 0.01.

Manuscript #2

Title: Integration of behavioural and biological variables using penalized regression: an application to the maternal immune activation model of autism

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Abstract

The maternal immune activation (MIA) mouse model of environmentally-induced autism has been extensively studied in recent years. Alterations in the brain, behaviour, immune system and microbiome have been reported in offspring of poly(I:C)-injected pregnant dams. However, apart from a few studies on the changing levels of cytokines with increasing age of offspring, little is known about the connection between the immune system and behavioural outcomes in the early postnatal period in the MIA model. Moreover, recent discussions emerged among research groups, regarding the difficulties to generate a robust and reproducible MIA model, due to the model's susceptibility to changes in the induction conditions. This led to our belief that changes in data analysis should be made to accommodate this fluctuation in MIA parameters. In this study, we show results analysed based on two statistical approaches, in order to demonstrate the importance of confounders and the limitations posed by the exclusive usage of univariate analysis. Inspired by analysis of clinical cohort data, we focused our analysis on a multivariable statistical model based on penalised regression to test for the role of specific confounders on the MIA model.

1. Introduction

Epidemiologic studies have identified that *in utero* exposure to maternal immune activation (MIA) is involved in the etiology of psychiatric disorders of neurodevelopmental origin (Estes and McAllister 2016). Notably, maternal infection and inflammation have been repeatedly associated as risk factors for schizophrenia and autism spectrum disorders (ASD) (Hornig et al. 2018; Jiang et al. 2016). The development of MIA animal models enabled to uncover some of the mechanisms by which MIA induces neuronal and behavioral alterations in the progeny (Brown and Meyer 2018). A commonly used MIA model is based on gestational administration of polyriboinosinic-polyribocytidilic acid (poly(I:C)), a synthetic double-stranded RNA that mimic viral infection and provokes a maternal acute phase response involving a surge in proinflammatory cytokines. Notably, both the cytokines interleukin (IL)-6 and IL-17A appear critical for the induction of subsequent neuronal and behavioral anomalies in the progeny (Brown and Meyer 2018).
Recent studies have raised concerns regarding the reproducibility of the MIA model and stressed the importance of extensively reporting the MIA experimental conditions and outcomes used, as well as adequate statistical handling of data (Kentner et al. 2018; Mueller et al. 2019). It remains that, to our knowledge, univariate statistics are still the gold standard in the field to identify the influence of variables of interest in the MIA model. Although laboratory studies of animal behaviour remove numerous confounding variables commonly associated with human cohort studies, unaccounted environmental factors that may influence behavioral performance in laboratory mice still remain (Macrì et al. 2013; Toth 2015; André et al. 2018). Overlooking these effects might be one of the reasons multivariable methods are not more commonly applied in animal experimental data analysis. Given the diversity of variables (parental age, maternal immune activation response, growth, cytokines and behaviour) potentially influencing outcome in the MIA model, we reasoned that, alike epidemiological studies performed in human cohorts, multivariable modelling involving regression analysis would be more likely appropriate to accommodate the obtained datasets.

In this study, we investigated the association between cytokines and belonging to the MIA offspring, while considering potentially influencing factors, such as a range of parental parameters, litter size, pup body weight and behavioural. Regression methods are precisely employed when we are trying to understand the relationship between variables, in which some of the independent variables (or covariates) cannot be controlled by the experimenter. Regression expresses the outcome variable as a combination of the independent variables under scrutiny. As such, in order to understand the most important factors driving the MIA model during early postnatal life we employed a regularized (or penalized) logistic regression. The performance of a logistic regression depends on several factors, such as the strength of associations between covariates and outcome, collinearity between covariates, the number of individuals in the smaller of the two outcome groups in relation to the number of regression coefficients to be estimated, and on sample size (Peng and So 2002; van Smeden et al. 2018). The regularized regression framework algorithms were developed to allow estimation of coefficients in high dimension regression problems, where the number of variables is as nearly as large, or larger, than the number of observations, a scenario for which classical algorithms do not provide satisfactory solutions. Penalties are applied to the coefficients to avoid extreme values that may cause overfitting, which, very conveniently, translates into performing variable selection by shrinking some of

the coefficients exactly to zero. It also addresses the issue of multicollinearity (correlation) between variables (Zou and Hastie 2005).

Here we used both univariate statistics and penalized regression to identify parental, biological and behavioural variables critically associated with *in utero* exposure to MIA. We show that multivariable modelling enables to highlight the contribution of variables, and notably of cytokines, that were not identified using classical univariate statistics.

2. Materials and Methods

2.1. Animals

Eight-week-old male and female C57Bl/6N founder mice were purchased from Taconic Biosciences (Lille Skensved, Denmark). The colony was first amplified to generate a pool of females and males to be used for timed matings. All mice were housed in open medium cages, in a temperature (22-24°C) and hygrometry (70-80%)-controlled room, with a 12-h light/dark cycle (lights on from 8:00 a.m. to 8:00 p.m.) with food (standard chow, reference 4RF25, Mucedola) and water *ad libitum*. Each cage was equipped with wooden litter, cotton to nidify as well as a one plastic house. Mice were housed by groups of 3 to 5 animals per cage. For timed the matings, we used females aged 2-5 months and males aged 2-7 months.

2.2. Experimental procedure

On the day of mating, the weight of the females was recorded. Females were mated overnight with males for 16h (6 p.m.-10 a.m. next day considered embryonic day (E) 0.5), by groups of 3 females for 1 male. After mating, the male was removed and females left undisturbed, with the exception of weekly cage cleaning. Females which gained at least 3 g between E0.5 and E11.5 were considered pregnant and the identity and age of the father was recorded. Pregnant dams were randomly assigned to one of two groups injected with poly(I:C) or vehicle (saline). At E12.5, body temperature was measured using a rectal probe before injection. Pregnant dams were injected with poly(I:C) for the MIA group or saline for the control group (see 2.3. for details on injections). Body temperature was recorded again 3 and 6 h post injection. Body weight was recorded 24 h post injection. At E16.5, the pregnant dams were separated into individual cages, where they would stay undisturbed until birth at E19.5 termed postnatal day 0 (P0). Cages with newborn pups were left undisturbed, except for the behavioural tests, and the cages remained unchanged until the end of the experiments at P15. We only used male pups but female pups remained in the litter to preserve numbers and sex balance in each litter. Male pups were individually marked with an odourless permanent marker and remarked every 2 days to keep track of their identity throughout the tests. All male pups underwent the same sequence of behavioural assessments at the same developmental time points, usually between 9-12 a.m. We followed a total of n = 27 male pups born from mothers injected with saline (from 8 litters spread across 3 independent cohorts) and n = 40 male pups born from mothers injected with poly(I:C) (from 12 litters spread across 4 independent cohorts). Offspring coming from poly(I:C) – injected mothers will be referred to as poly(I:C) throughout the study, while pups from saline – injected mothers will be referred to as Vehicle. Each pup was considered as a statistical experimental unit.

2.3. MIA induction in pregnant dams using poly(I:C)

Due to previously reported differences between lots of poly(I:C) (Mueller et al. 2019; Careaga et al. 2018), the same lot was used throughout the study. The poly(I:C) used in this study was obtained as a lyophilized stock (reference P9582, Sigma-Aldrich, lot# 077M4039V) that contained 10% pure poly(I:C), with the rest consisting of buffer salts. Poly(I:C) was dissolved and vortexed at full speed in sterile double-distilled water at room temperature (RT) to generate a stock solution based on pure

poly(I:C) weight. No heat was used during the solubilisation process. Once the poly(I:C) completely dissolved, stock solution was aliquoted and stored at -20°C until use. For each new cohort, a new aliquote was thawn, checked for absence of crystallisation and diluted to the working concentration in saline solution (0.9% NaCl). At E12.5 and between 9-10 p.m., pregnant dams received a single intraperitoneal injection of poly(I:C) at a dose of 5 mg/kg (5µL/g body weight of a 1µg/µL solution), based on pure poly(I:C) weight. Control females were injected in the same conditions with 5µl/g body weight of saline.

2.4. Behavioural tests

2.4.1. Recording and analysis of ultrasonic vocalisations (USV) emitted by pups

At P6, pups from mothers injected with poly(I:C) or saline were isolated from the mother and littermates for 5 min and placed into a soundproof chamber containing a microphone used for ultrasound recordings. The pup was placed in a large open Petri dish padded with cotton, changed between each pup to prevent olfactory recognition of previous pups. The ambient temperature in the soundproof chamber was set to 26°C using a vivarium heating mat to prevent body temperature loss. USVs were recorded for 5 min using the UltraSoundGate Condenser Microphone (Avisoft Bioacoustics, Germany), sensitive to frequencies ranging between 0 - 250 kHz, connected to AvisoftUltraSoundGate 116 USB Audio device (Avisoft Bioacoustics), which was in turn connected to a computer. Vocalisations were recorded as described in (Ferhat et al. 2016), after which each pup was immediately placed back into the maternal cage. At each recording session (by groups of 2 to 4 pups), poly(I:C) pups were recorded simultaneously to control pups in parallel chambers. Sonograms were analysed as described in (Ferhat et al. 2016; Malkova et al. 2012) with the AvisoftSASLab Pro software (version 5.2.12, Avisoft Bioacoustics) based on automated recognition of USV using in-built parameters. The cut-off frequency was set to 25 kHz to reduce background noise outside the frequency range of interest. For element separation during automatic detection, we used whistle tracking and the following parameters: 7 ms hold-time and a minimum duration of 2 ms. We then manually curated the misidentified USV and rectified their duration. Number of USV and total USV duration were then automatically extracted from curated sonograms. USV syllable classification was performed using 10 distinct classes based on pitch contour shapes, originally described in (Scattoni et al. 2008). All USVs were assigned to one of the 10 classes and the percentage of each class was calculated.

2.4.2. Locomotor activity

Each pup was placed individually into a clean small plastic cage (surface) and its movements recorded during 10 min. Videos were analysed *a posteriori* using the ANY-maze videotracking software (Dublin, Ireland) which extracted the time spent mobile and total distance travelled for each individual. The exploratory index was calculated as the ratio between the distance travelled and the time spent mobile for each pup. The pups were returned to their original cages after the completion of the test.

2.5. Cytokine measurements

At P15, pups were anaesthetized using a lethal dose of anaesthetic. After cardiac puncture, the blood of individual mice was collected into 1.5 ml Eppendorf tubes. To obtain serum, the blood was centrifuged (10,000g, 4°C, 20 min), the resulting supernatant transferred to clean 1.5 ml Eppendorf tubes,

immediately frozen in liquid nitrogen and stored at -80°C until further analysis. We used the the V-PLEX® Mouse TNF Kit or the V-PLEX® Mouse Cytokine 29-Plex Kit electrochemiluminescence-based assays from (Meso Scale Diagnostics, Rockville, USA). Assays were performed according to the manufacturer's instructions. The reagents were stored at 4°C until used, when they were allowed to reach RT immediately prior to usage. All buffers used were provided with the kit, with the exception of the Wash Buffer, which was produced in-house using sterile PBS 1x and 0.05% Tween-20. Lyophilized standards (containing cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, CXCL1, TNF-α, IL-9, CCL2, IL-33, IL-27p28/IL-30, IL-15, IL-17A/F, CCL3, CXCL10, CXCL2, CCL20, IL-22, IL-23, IL-17C; IL-31, IL-21, IL-16, IL-17A, IL-17E/IL-25) were reconstituted to master stock solutions. Eight concentrations of the standard were made by fourfold serial dilutions of the master stock. The majority of serum samples were diluted 1:4, with the exception of several samples with low remaining serum volume, for which dilutions between 1:6 to 1:11 were used. All samples were diluted on ice on pre-plates. The background level was determined using a buffer blank. Eight serial dilutions of standards and buffer only (in duplicates) were run together with samples run in singlicate using the Sector Imager 2400 plate reader (Meso Scale Diagnostics, Rockville, USA). Concentrations of cytokines in each sample were interpolated from standard curves generated with a five-parameter logistic regression equation in Discovery Workbench 3.0 software (Meso Scale Diagnostics, Rockville, USA). The software allows for a graphic visualization of the placement of all samples on the standard curve for each cytokine. IL-2, IL-4, IL-12p70, IL-9, IL-17A/F, IL-22, IL-23, IL-17C, IL-31, IL-21, IL-17E, IL-25 check whether it is the same for TNF pups and MIA were below the Lower Limit of Detection (LLOD) in more than 20% of the samples and were therefore not retained for downstream analyses. For the other cytokines, cytokine levels below the lower limit of detection (LLOD), we imputed a value equal to half the LLOD value indicated by the manufacturer.

2.6. Statistics

2.6.1. Comparison analyses

Normality of the data was checked using the Kolgomorov-Smirnov test. Variables for which the normality of data assumption was not rejected were then compared using the 2-tailed unpaired Student's T-test. For non-normal data, and if a logarithmic transformation would not allow to meet the normality criteria, we used the non-parametric Mann-Whitney U test. For multiple group comparisons, a 2-way ANOVA with post hoc Sidak's correction for multiple comparisons was used. For frequency distributions comparisons, a Chi-square test was used. Statistical significance was set to a p-value (p) threshold of 0.05. All univariate analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, USA).

2.6.2. Correlation analyses

For correlation studies, the strength of association between levels of biomarkers was assessed using the Spearman's r correlation coefficient rank test, with Benjamini-Hochberg correction for multiple testing.

2.6.3. Multivariable analysis

A penalized logistic regression was used to model the association between parental and pups variables, cytokine levels at P15 and the outcome "belonging to the MIA class". We computed mean Odd Ratios

(ORs), percentile bootstrap 95% confidence intervals (CI) and Variable Inclusion Probabilities (VIP) for each variable. Optimal λ and α hyperparameters were chosen, via 10-fold cross-validation, to minimize the logarithmic loss. Traditional methods do not provide valid confidence intervals, or p-values, for testing the significance of penalized regression coefficients. As an alternative, we used the non-parametric bootstrap, that can be used for inference in applications of penalized regression (Abram et al. 2016). The bootstrap step involved 500 resamplings of the dataset to create 500 different samples of the same size. The VIP was computed as the percentage of the bootstrap resamples in which each variable was selected by the penalized regression. Depending on the study, determining an appropriate threshold for the VIP can be challenging. In their seminal paper, Bunea et al. 2011 recommended to use a "conservative threshold of 50%" because their goal was "not to miss any possibly relevant predictors". However, this 50% threshold increases the risk of false positives. In this study we chose to set the VIP threshold to 80%, this cut-off was decided as a compromise, it decreases the risk of a false positive among the cytokines while permitting the inclusion of variables previously known or expected to be associated with the MIA model.

2.7. Study approvals

Animal housing and experimentation were conducted in facilities certified by regional authorities (Direction Départementale de Protection des Populations des Alpes-Maritimes, accreditation #C-06-152-5). The study was in accordance to procedures approved by the Ministère de l'Enseignement Supérieur et de la Recherche (Approval PEA #571).

3. Results

3.1. Univariate comparison analysis of variables between MIA and control offspring

3.1.1. Our experimental conditions enable induction of MIA in pregnant dams

Maternal and paternal ages displayed similar distributions among the MIA and control groups (Fig. S1). As proxies of maternal immune activation, we used two outcomes measured immediately after p(I:C) or saline injection in pregnant dams: change in temperature before the injection and 3h later and change in the body weight before the injection and 24h later. Poly(I:C) – treated dams showed a significant decrease in body temperature 3 h post injection (Fig. 1B), and a significant weight loss 24 h post injection (Fig. 1C), as compared to control females injected with saline. This agrees with previous studies showing hypothermia and weight loss post p(I:C) injection (Mueller et al. 2019) and validates our experimental conditions to induce MIA.

3.1.2. MIA offspring display overweight at P15

The average number of pups in the litter at birth and at P3 did not differ between classes, suggesting that each litter had access to equal quantities of milk. However, pups born to MIA mothers weighed on average 11% of their body weight more that control pups (Fig. 1D).

3.1.3. MIA offspring display quantitative and qualitative communication deficits at P6

Communication of MIA offspring was assessed in pups at P6, by recording USV emission in response to pup isolation from the maternal nest. We used two different approaches: a quantitative (Fig. 1E-G) and a qualitative assessment (Fig. 1H). MIA offspring emitted significantly less USVs as compared to control offspring (Fig. 1E). Also, USV total duration was also significantly reduced (Fig. 1F). However, USV mean duration was unaffected (Fig. 1G). We then performed a qualitative analysis of USV by studying the USV repertoire that were classified in 10 classes of distinct syllables based on their structure and shape. There was a significant effect of exposure to MIA on the syllable patterns distribution (Fig.1H). MIA offspring displayed an increase of 34.5% and 27.2%, in the single frequency syllable types Chevron and Short, respectively, as compared to control offspring. Moreover, MIA offspring showed a decrease of 21.6% and 46.5% respectively, in the Frequency-step and Harmonic multiple frequency syllables, as compared to control offspring. Altogether, there was an overall reduction of multiple frequency syllables and increase in single frequency syllables in MIA pups offspring. This agrees with a previous study on MIA offspring that previously described both quantitative and qualitative deficits in USV communication deficits in pups during the first two weeks of life (Malkova et al. 2012).

3.1.4. MIA offspring show reduced locomotor activity at P13

Locomotor activity of pups was assessed in MIA and control pups at P13 (Fig.1 I-K). Compared to control offspring, MIA offspring travelled a shorter distance (Fig. 1I) and spent less time mobile (Fig. 1J). However, there was no change in the exploratory index between the two groups (Fig. 1K). Although locomotor activity was not assessed previously in the early developmental stages, our results are in line with previous studies describing hypolocomotion in the adult MIA offspring (Smith et al. 2007).

3.1.5. MIA offspring does not display significant alterations in cytokine levels at P15

The levels of circulating cytokines were measured in serum at P15 and were not significantly impacted in the MIA offspring (Fig. S2). This agrees with a previous study showing that cytokine levels are unaltered in the serum of MIA pups at P14 (Garay et al. 2013). To highlight possible relationships between cytokines, we computed the Spearman's correlation coefficient of cytokine pairs on pooled datasets from MIA and control offspring (Fig. S2). We identified low to moderate positive and negative correlations between cytokine pairs. The strongest positive correlations were between IL-16 and IL-33 (ρ = 0.76), IL-1β and IL-6 (ρ = 0.60), IL-1β and IL-27p28 (ρ = 0.57), IL-27p28 and IL-15 (ρ = 0.52) and CCL20 with CXCL10 (ρ = 0.51). The strongest negative correlations were between CXCL10 and IL-1β (ρ = - 0.57), CXCL10 and IL-27p28 (ρ = - 0.53) and CXCL10 with IL-15 (ρ = - 0.46).

3.2. Multivariable analysis: identification of cytokines associated with the MIA class adjusting for covariates

In order to select and measure the strength of cytokines associated with the MIA class, we performed a multivariable analysis with the binary outcome of belonging to the MIA offspring (MIA class) or to control offspring (control class) (Tab. 1). Additional variables such as parental variables (age, change in maternal body weight and temperature upon MIA induction), pups variables (litter size, weight, behavioural variables such as number of USVs emitted, distance travelled and time mobile), were also included in the model as covariates. Variables with a VIP higher than 80% were considered to be stably associated with the outcome.

Odds ratios were calculated from the estimated coefficients and used to compare the relative odds of belonging to the MIA class, given the variable values. The odds ratio represents the constant effect of each variable, on the likelihood that the outcome will occur. Importantly, maternal change in body temperature and change in body weight following MIA induction, number of pups per litter, number of USVs emitted, distance travelled and time spent mobile were associated with smaller odds of belonging to the MIA class. In contrast, the pup body weight at P15 was associated with higher odds of belonging to the MIA class. Of note, the confidence intervals shown in Tab. 1 are the likely range of the true value of the estimated odds ratio. Regarding, the variable "pup weight", the interval is wide. The width of the confidence interval depends to a large extent on the sample size and we believe that to be the reason why we obtained such wide intervals for this variable.

Regarding cytokines, four appeared as stably associated with the MIA outcome. Serum levels of IL-15 and TNF were associated with higher odds of belonging to the MIA group. We expect to see an increase (in average) in the odds of belonging to the MIA class, for a one-unit increase in the TNF and IL-15, respectively. Conversely, serum levels of CXCL10 and IL-5 were associated with lower odds of belonging to the MIA class. Similarly, we expect to see a decrease (in average) in the odds of belonging to the MIA class, for a one-unit increase in CXCL10 and IL-5 respectively. TNF and IL-5 average odd-ratios were respectively the highest (1.91) and lowest (0.823), the magnitude of the effects mediated by these cytokines is likely to be stronger than for CXCL10 and IL-15. Therefore, TNF and IL-5 appear as the strongest candidates for association with the MIA class.

4. Discussion

4.1. Penalized regression identifies accurately parental, biological and behavioural variables associated with the MIA class

Penalized regression highlighted that maternal weight loss and hypothermia are negatively associated with the MIA class, suggesting that the intensity of MIA increases the odds of belonging to the MIA class. The necessity to verify MIA induction was recently raised (Kentner et al. 2018) and we complied with these requirements. Parental age did not appear influential, neither did primiparity. However, increase in the variable number of pups per litter decreased the odds of belonging to the MIA class, suggesting that litter size could be affected by MIA, even though comparison analysis did yield significant difference in litter size from MIA mothers.

Increases in the variable body weight are associated with increased odds of belonging to the MIA class. This overweight in MIA offspring could be due to a metabolic unbalance possibly caused by intestinal microbiota dysbiosis or increased gut permeability reported in MIA mouse and rat pups (Hsiao et al. 2013; Clark et al. 2019; Codagnone et al. 2019; Pacheco-López et al. 2013). This could impact assimilation of food nutrients and provoke early fat accumulation in MIA pups. One previous study reported no differences in the body weight between poly(I:C) MIA and control animals during peri-adolescence and adulthood, while showing increased visceral and subcutaneous fat in adult offspring (Pacheco-López et al. 2013). However their sample size was reduced to n=7/group and the lack of significant effects might be due to limited power. Previous studies have also reported decreased weight in pups when born from poly(I:C)-injected mothers: between P3 to P10 in MIA mouse pups (Arsenault et al., 2014) and at P21 in male, but not female MIA rat pups at P21 (Murray et al., 2018). The differences between our result and previous results could be due to differences in the timing and mode of MIA induction (poly(I:C) administration, timing, species used).

Regarding behavioural variables, an increase in USV numbers upon isolation from the mother decreases the odds of belonging to the MIA class. USV are produced in response to isolation from the mother, which calls for mother pup retrieval, as well as to hunger onset and thermal changes in their environment, all of which require maternal care (Hofer, Shair, and Brunelli 2002). Alterations of ultrasound patterns have been found in genetic and environmental mouse models of neurodevelopmental disorders, such as autism and schizophrenia (Scattoni, Crawley, and Ricceri 2009) and can be interpreted as a deficit in pup attachment behaviour. Our results on USV, both at the quantitative and qualitative level, are fully are in line with a previous study (Malkova et al. 2012), showing that exposure to MIA promotes communication deficit in young offspring, but also in adults during social encounter (Hsiao et al. 2013). However, other studies from one group report increased numbers of USVs produced by MIA pups at P9 (Kim et al. 2017; Shin Yim et al. 2017; Choi et al. 2016), as well as no differences between MIA and control pups at P9 (Morais et al. 2018). These inconsistencies are likely due to differences between the protocols used, in relation to the timing and concentration of the injected poly(I:C), as well as the animal supplier and the presence or lack of animal habituation prior to the USV test. Our data also agree with a number of studies on ASD genetic models, such as the *Tsc2*$^{+/-}$ tuberous sclerosis model (Young et al. 2010), , the *Shank1*$^{-/-}$ mouse (Sungur, Schwarting, and Wöhr 2016) or *Oprm*$^{-/-}$ mice deficient for the μ-opioid receptor (*Oprm*$^{-/-}$) (J. A. Becker et al. 2014; Moles, Kieffer, and D'Amato 2004) and the BTBR mouse model of idiopathic ASD (Scattoni et al. 2008), which have all been shown to display reduced USV emission during early development.

Increase in locomotor activity and time mobile was associated with decreased odds of belonging to the MIA class. In the MIA model, locomotor activity was only assessed in the open- field test in adolescent or adult mice and not in pups. Previous studies report decreased performance of MIA offspring in the open-field test, notably with a reduction in the total distance travelled (Chow, Yan, and Wu 2016; Wu et al. 2015; Shin Yim et al. 2017; Kim et al. 2017; Hsiao and Patterson 2011; Hsueh et al. 2018; Shi et al. 2003), in line with our findings.

4.2. Penalized regression reveals that novel cytokines are associated with *in utero* exposure to MIA

Penalized regression highlighted that increased levels of IL-15 and TNF were stably associated with higher odds of belonging to the MIA group, while levels of CXCL10 and IL-5 were associated with lower odds of belonging to the MIA class. TNF and IL-5 average odd-ratios were respectively the highest (1.91) and lowest (0.823), the magnitude of the effects mediated by these cytokines is likely to be stronger than for CXCL10 and IL-15. To our knowledge, IL-6 and IL-17A were the only cytokines previously connected to the neurobehavioural alterations in the MIA model, but only in the antenatal, birth and very early postnatal period (Choi et al. 2016; Smith et al. 2007; Wu et al. 2017). Little was known about the possible contribution of other cytokines at 2-week-old (P15), and neither TNF-α, nor IL-5 were previously connected to MIA, at least at P15.

TNF is expressed early in brain development (Garay et al. 2013; Vitkovic, Bockaert, and Jacque 2000) and plays an important role in neurogenesis, synaptogenesis and synaptic scaling (Beattie et al. 2002; Stellwagen and Malenka 2006). A recent meta-analysis reported elevated levels of TNF in the blood were associated with ASD diagnosis (Saghazadeh et al. 2019a). It is possible that abnormally elevated levels of TNF levels could perturb normal neurodevelopmental processes and contribute to early behavioural alterations in MIA offspring. Upon MIA induction, TNF was increased in the serum, placenta and amniotic fluid of pregnant dams immediately after poly(I:C) injection (Garay et al. 2013; Meyer 2006; Gilmore, Jarskog, and Vadlamudi 2005), but not in the foetal brain, the serum or brain at P1, P7 or P14 (Garay *et al.*, 2013;Gilmore, Jarskog and Vadlamudi, 2005; Meyer, 2006). It is possible that small sample size (n<10) in these studies and inappropriate statistical analysis (only univariate comparison analysis was used) hindered the effects of MIA on TNF-α levels in the offspring.

IL-5 is a cytokine with important roles in mucosal immunity involved in autoimmunity disorders such as allergies and asthma (Travers and Rothenberg 2015). IL-5 was reduced in the cortex and hippocampus of MIA offspring at P7 and in the hippocampus at P14, but not in the serum (Garay et al. 2013). Whether reduced serum levels of IL-5 translated into reduced IL-5 in the brain of MIA offspring should be further tested in our model. These results are *a priori* not in line with a recent meta-analysis which reported that IL-5 levels were positively associated with ASD diagnosis (Saghazadeh et al. 2019b). However, it is possible that IL-5 levels might fluctuate transiently in the MIA offspring, during a specific developmental window.

4.3. Penalized regression framework to analyse datasets derived from animal studies

Although widely used to identify variables differing between groups, univariate statistics focus on one variable at a time and do not provide information regarding causal inference or relationships between the variables of interest. Depending on the elected modelling strategy, multivariable analysis

can provide such information to a certain extent. In animal studies, multivariate analyses are mainly limited to the use of unsupervised clustering methods and rely heavily on principal component analysis (PCA). PCA has been successfully applied to the study of "omics" datasets, for example for bacterial 16S rDNA sequencing datasets (Buffington et al. 2016; Sgritta et al. 2019) or analysis of behavioural data (J. B. Becker and Chartoff 2019; Zupan et al. 2017). In this case, PCA can successfully detect groups of related samples, but, being unsupervised by essence, it fails to include prior knowledge regarding the experimental conditions associated with each groups. Furthermore, the biological features responsible for group separation remain difficult to identify. Finally, PCA lack discriminatory potential, as it does not eliminate variables that contribute little towards explaining variation between groups.

Some of these issues can be overcome by the use of supervised approaches, which integrate information about the class of the sample (e.g., disease vs. control, untreated vs. treated), and enable to maximize inter-class discrimination, to identify the discriminant features and also to interpret results within the framework of causal inference. Regression analysis are supervised methods commonly used in the epidemiology field, in which confounding effect is a major concern in causal studies because it results in biased estimation of exposure effects. Confounders are covariates ancillary to the dependant or independant variables of interest which can, if not taken into account, contribute to suggest a causal effect where there is none, to hide a true effect. Traditional confounders are the sex or age for example. In animal studies, these factors can easily be controlled for and classes matched in this respect. However, this cannot account for all the possible inter-classes variability. This is particularly an issue in the case of environmental mouse models of human pathologies such as the MIA model. Recent work has shown that the amplitude of the MIA response conditioned the magnitude of the future neurobehavioural deficits in the offspring (Kentner et al. 2018; Mueller et al. 2019), stressing that this factor should be taken into account when comparing MIA and control offspring. To overcome this issue, we thought to implement a multivariable method to perform a statistical ''correction'', that would take into account all the covariates available on our two classes, producing corrected estimates of the effect of exposure to MIA for each of the variables of interest, and in particular cytokines.

For the study of our dataset, we elected penalized regression to model associations between our dependent variable "belonging to the MIA class" and our independent variables which corresponded to serum cytokine levels, adjusting for covariates known to impact the outcome and/or the cytokine levels (parental variables, pups biological and behavioural variables). When identifying variables associated with a given outcome (in our case belonging to the MIA class), penalized estimation algorithms deliberately introduce a bias to reduce variability of the estimates. It was recently shown that the model selection procedure introduces biases and variance in estimates, independently of the sample size (Pfeiffer, Redd, and Carroll 2017), and to-date no algorithm is able to overcome this issue. This raises the issue of the stability of the selection procedure. To overcome this issue, we implemented a resampling step, which provided useful information on the model stability and the variables' importance over an iteration process.

Nevertheless, we wish to point that implementing penalized regression will not overcome the issue of a low sample size (De Bin et al. 2016). Penalized regression was initially optimized for prediction in the context of high-dimensionality datasets, in which the number of variables is much higher than the number of observations. However, these algorithms can also be used for causal inference and be applied to "normal" sized datasets ($n<100$), as it is usually the case for datasets derived from animal studies. A classical regression framework would also suffer from the same issue with the study sample size compounded by the multicollinearity. The results we present here constitute

therefore an exploratory analysis with some limitations. It remains that, while univariate comparison analysis failed to highlight differences in cytokine levels between MIA and control offspring, penalized regression enabled to determine that the cytokines TNF and IL-15 were associated with higher odds of belonging to MIA class, while CXCL10 and IL-5, were associated with lower odds. We believe that penalized regression enabled us to improve the estimation efficiency of causal effect by eliminating the contribution of covariates. This highlights the power of penalized regression to identify variables associated with our outcome of interest. We believe that our study not only provides valuable information on the characteristics of the MIA model in relation to the postnatal consequences of *in utero* exposure to MIA, but also highlight the importance of extending the use of statistics to include relevant confounding variables to explain any given result.

5. Conclusions

Here, to obtain a high-confidence dataset derived from the follow-up of MIA and control offspring, we have followed the recently recommended guidelines to optimize the MIA model in our laboratory (Kentner et al. 2018; Mueller et al. 2019). As such, we used C57Bl/6N mice from Taconic, focused on a single batch of poly(I:C), controlling for the induction of MIA by monitoring body temperature and body weight change in the mother upon poly(I:C) injection. Also, we have collected on each individual pup a number of biological variables (body weight, serum cytokine levels) and behavioural variables (USV communication, locomotor activity). Then, we performed two parallel statistical analysis: first, a classical comparison analysis between pups born from MIA or control mothers and second, a multivariable analysis using a penalized regression framework. Only the multivariable approach was able to identify cytokines stably associated with the MIA class at 2-weeks old. This clearly exemplifies the power of multivariable approaches, traditionally used in epidemiological studies on human cohorts, to study datasets derived from animal studies. Multivariable analyses should contribute largely to a better understanding of the interrelationships between variables, as opposed to the limitations in data interpretation imposed by univariate statistics.

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Figures

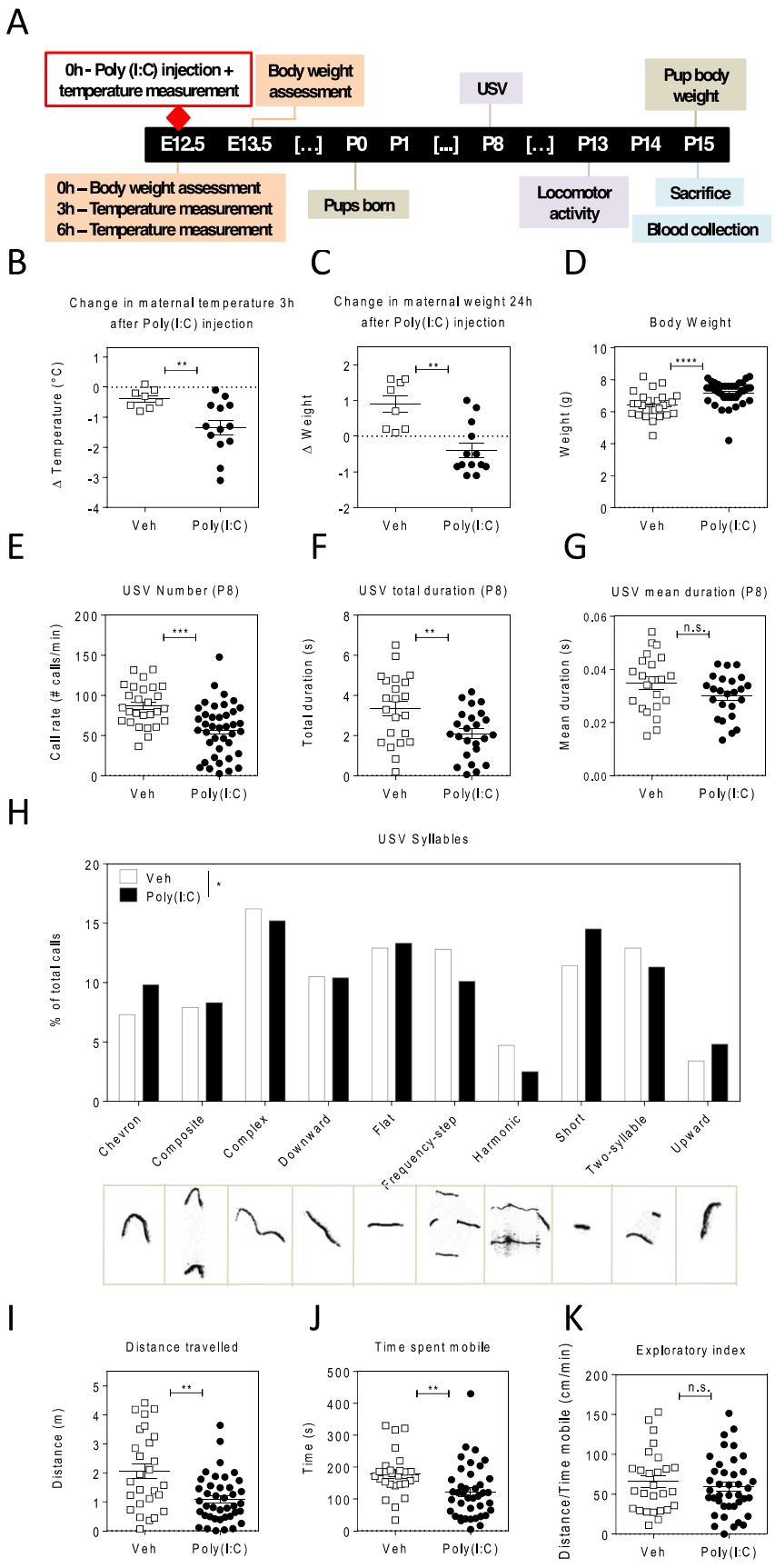

A
0h - Poly (I:C) injection + temperature measurement
Body weight assessment
USV
Pup body weight
E12.5 E13.5 [..] P0 P1 [...] P8 [..] P13 P14 P15
0h – Body weight assessment
3h – Temperature measurement
6h – Temperature measurement
Pups born
Locomotor activity
Sacrifice
Blood collection
B
Change in maternal temperature 3h after Poly(I:C) injection
Δ Temperature (°C)
Veh
Poly(I:C)
C
Change in maternal weight 24h after Poly(I:C) injection
Δ Weight
D
Body Weight
Weight (g)
E
USV Number (P8)
Call rate (# calls/min)
F
USV total duration (P8)
Total duration (s)
G
USV mean duration (P8)
Mean duration (s)
n.s.
H
USV Syllables
% of total calls
Chevron
Composite
Complex
Downward
Flat
Frequency-step
Harmonic
Short
Two-syllable
Upward
I
Distance travelled
Distance (m)
J
Time spent mobile
Time (s)
K
Exploratory index
Distance/Time mobile (cm/min)

Figure 2. Univariate comparison analysis of parental and litter variables between Poly(I:C) and Veh. groups.

(A) Timeline of the experiments.

(B) Change in maternal temperature 3 hrs after Poly(I:C) or Veh. injection. Data are presented as means ± SEM; n = 8 Veh., n = 13 Poly(I:C); 2-tailed Student's T-test: **, $p < 0.01$.

(C) Change in maternal weight 24 hrs after Poly(I:C) or Veh. injection. Data are presented as means ± SEM; n = 8 Veh., n = 13 Poly(I:C); Mann-Whitney U test: **, $p < 0.01$.

(D) Pup body weight at P15. Data are presented as means ± SEM; n = 27 Veh., n = 40 Poly(I:C); Mann-Whitney U test: ****, $p < 0.0001$.

(E-H) Communication assessment of Poly(I:C) and Veh. pups over 5 min. at P8

(E) USV call rate. Data are presented as means ± SEM; n = 27 Veh., n = 40 Poly(I:C); 2-tailed Student's T-test: ***, $p < 0.0001$.

(F) USV total duration (total duration of USV emitted/minute). Data are presented as means ± SEM; n = 22 Veh., n = 24 Poly(I:C); 2-tailed Student's T-test: **, $p < 0.01$.

(G) USV mean duration (total duration of USV emitted/number of USV). Data are presented as means ± SEM; n = 22 Veh., n = 24 Poly(I:C); 2-tailed Student's T-test: ns.

(H) USV syllables divided into 10 classes according to their shape and presented as a percentage of the total number (syllable classes represented under the graph, in the direct order of presentation). Data are presented as percentage; n = 22 Veh., n = 24 Poly(I:C); Chi-square test: *, $p < 0.05$.

(I-K) Locomotor activity measurement of pups over 10 min.

(I) Distance travelled by pups. Data are presented as means ± SEM; n = 27 Veh., n = 40 Poly(I:C); Mann-Whitney U test: **, $p < 0.01$.

(J) Time the pups spent mobile. Data are presented as means ± SEM; n = 27 Veh., n = 40 Poly(I:C); Mann-Whitney U test: **, $p < 0.01$.

(K) Pup exploratory index, represented as the ratio between the total distance travelled and time mobile. Data are presented as means ± SEM; n = 27 Veh., n = 40 Poly(I:C); 2 -tailed Student's T-test: ns.

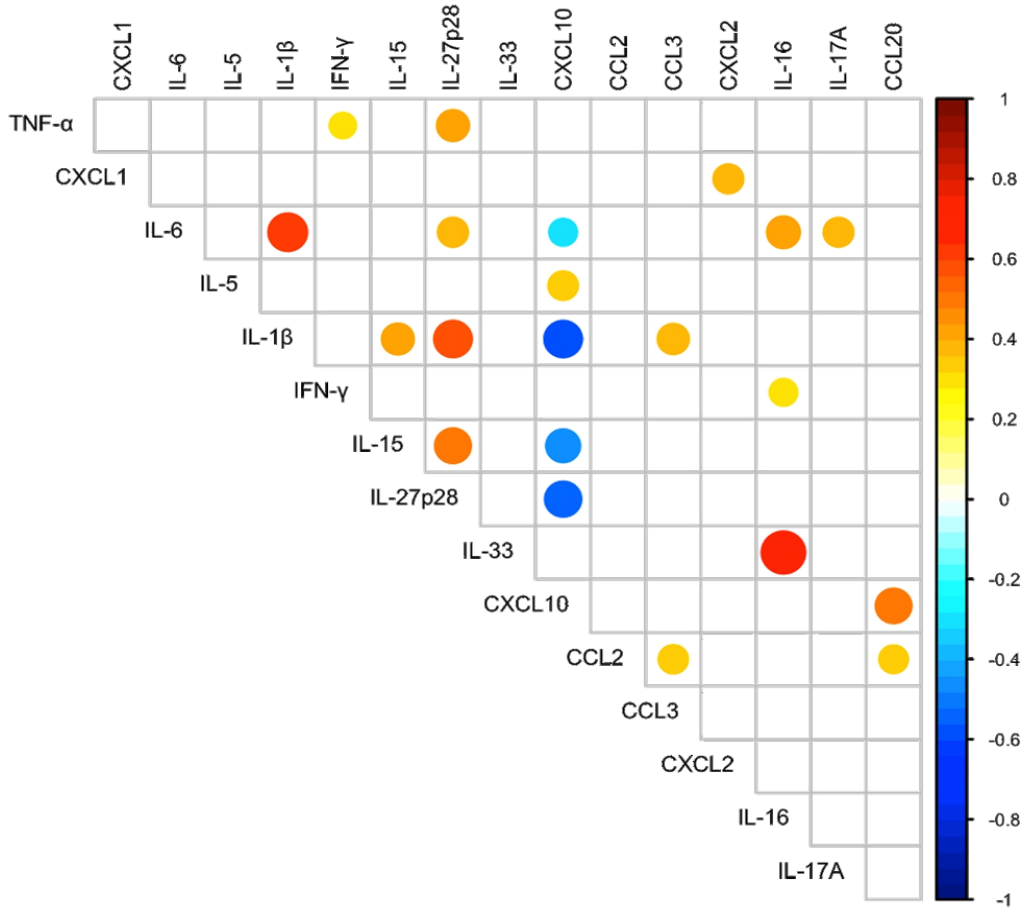
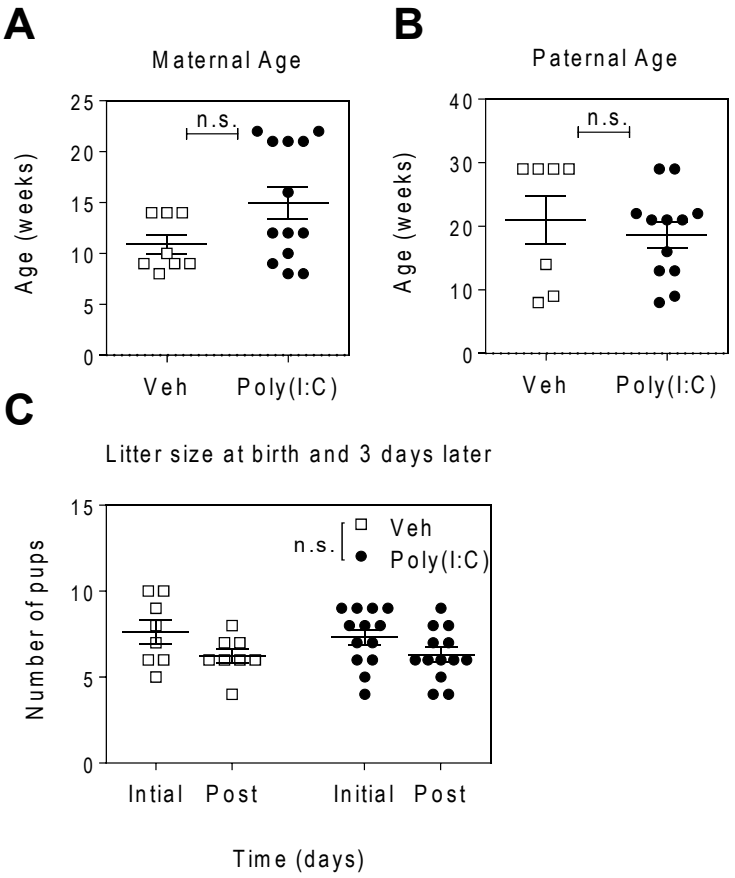


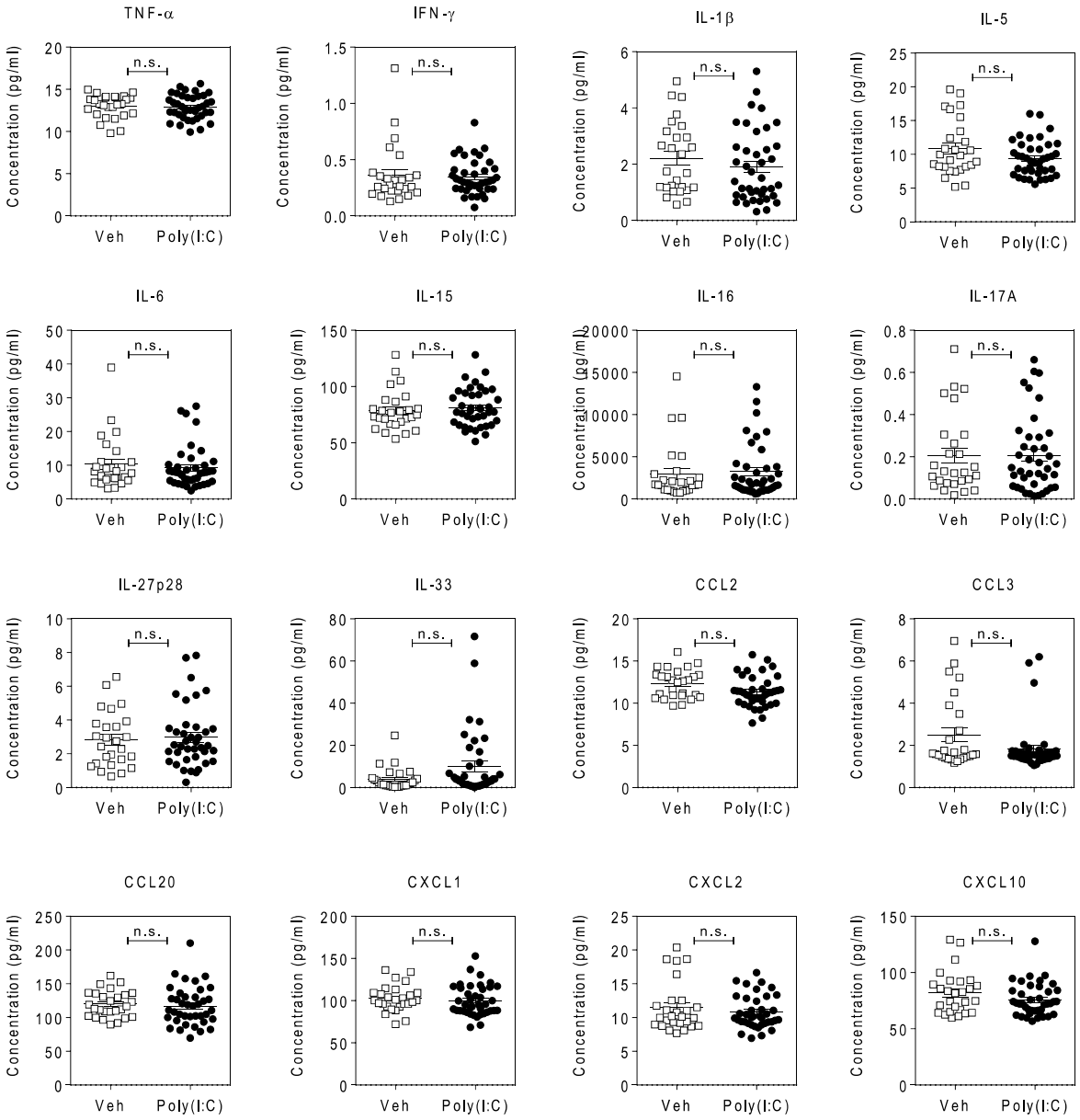
Figure 2. Correlation analysis for serum levels of cytokine in P15 pups. Heatmap of the pairwise Spearman's rank r correlation coefficient between all cytokine pairs. Spearman's coefficients are color-coded and proportional to dot area. Only significant correlations are displayed (adjusted p-value < 0.05).

Table 1. Adjusted associations between parental and pup's variables and belonging to the MIA class, i.e. born from Poly(I:C)-injected mothers. Mean Odd Ratios (OR), percentile bootstrap 95% Confidence Interval (CI) and Variable Inclusion Probability (VIP) for each of the variables selected by penalized regression are shown. The VIP was used as a measure of the stability of an association as it can be interpreted as the posterior probability of including a given variable in the model. Variables with VIPs above 80% were considered as stably associated with the MIA class. In the ORs column, positive (shaded red) and negative (shaded blue) associations are indicated.

	Variable description	Assessment time	mean OR	95%CI	VIP
Parental variables	Maternal age (weeks)	E0.5	1.139	[1.009,1.411]	65.7%
	Previous pregnancy (Yes)	E0.5	1.215	[1.009,2.098]	17.2%
	Maternal Δweight (g)	E13.5	**0.044**	**[0.001,0.514]**	**99.4%**
	Maternal Δtemperature (°C)	E12.5	**0.217**	**[0.032,0.826]**	**92.1%**
	Paternal age (weeks)	E0.5	1.03	[0.928,1.197]	57.3%
Pups variables	**Litter size (# pups)**	P4	**0.334**	**[0.061,0.898]**	**93.0%**
	Pup weight (g)	P15	**7.568**	**[1.481,127.402]**	**98.7%**
	Emitted USV (#)	P8	**0.992**	**[0.983,0.998]**	**97.0%**
	Distance travelled (m)	P13	**0.523**	**[0.183,0.956]**	**82.6%**
	Time mobile (s)	P13	**0.989**	**[0.972,0.999]**	**90.8%**
Cytokines	**TNF-α**	P15	**1.91**	**[1.042,6.41]**	**89.7%**
	CXCL10	P15	0.97	[0.9,1.014]	73.2%
	IL-6	P15	0.965	[0.831,1.134]	58.1%
	IL-5	P15	**0.823**	**[0.588,0.993]**	**85.6%**
	IL-1β	P15	1.338	[0.807,2.88]	57.2%
	IFN-γ	P15	0.786	[0.069,86.478]	59.8%
	IL-15	P15	**1.035**	**[0.998,1.118]**	**85.6%**
	IL-27p28/IL-309	P15	1.344	[0.975,2.32]	74.7%
	IL-33	P15	1.052	[1.002,1.175]	58.0%
	CXCL10	P15	**0.956**	**[0.881,0.995]**	**91.5%**
	CCL2	P15	0.848	[0.48,1.25]	57.4%
	CCL3	P15	0.704	[0.283,1.065]	63.9%
	CXCL2	P15	0.867	[0.501,1.096]	70.5%
	IL-16	P15	1	[1,1]	36.3%
	IL-17A	P15	0.866	[0.007,107.497]	53.4%
	CCL20	P15	1.007	[1,1.04]	43.9%
		Legend		OR>1	
				OR<1	
				VIP>80%	



Supplementary Figure 1. Univariate comparison analysis of parental and litter variables between Poly(I:C) and Veh. groups. (A) Maternal age at birth of Poly(I:C) and Veh. pups. Data are presented as means ± SEM; n = 8 Veh., n = 13 Poly(I:C); Mann-Whitney U test: ns. (B) Paternal age at birth of Poly(I:C) and Veh. pups. Data are presented as means ± SEM; n = 7 Veh., n = 12 Poly(I:C); Mann-Whitney U test: ns. (C) Litter size at birth (P0, initial) and 3 days later (P3, post). Data are presented as means ± SEM; n = 8 Veh. (initial and post), n = 13 Poly(I:C) (initial and post); 2-way ANOVA: p(Treatment) = 0.8413, p(Time) = 0.0011, p(Interaction) = 0.5508; Sidak's post hoc tests for treatment-wise comparisons: n. s., $p > 0.05$.



Supplementary Figure 2. Serum cytokine and chemokine levels in Poly(I:C) and Veh. pups at P15. The cytokines tested were TNF-α, IFN-γ, IL-1β, IL-5, IL-6, IL-15, IL-16, IL-17A, IL-27p28, IL-33, CCL2, CCL3, CCL20, CXCL1, CXCL2, CXCL10. All data are presented as means ± SEM. For TNF-α, IL-1β, IL-5, IL-6, IL-15, IL-16, IL-17A, IL-33, CCL2, CCL3, CXCL1, CXCL10; n = 27 Veh., n = 40 Poly(I:C). For IFN-γ; n = 26 Veh., n = 40 Poly(I:C). For IL-27p28; n = 26 Veh., n = 39 Poly(I:C). For CCL20 and CXCL2; n = 27 Veh., n = 39 Poly(I:C). The differences in the number of pups born from vehicle (Veh) or poly(I:C)-injected mothers were due to outlier exclusion; all Mann-Whitney U tests: n. s., $p > 0.05$.

Discussion

Overview

My work during my PhD focused on the role of cytokines in the early mouse neurodevelopment. We aimed to understand if disruption of the cytokine pattern of expression early during neurodevelopment can interfere, either positively or negatively, with developmental trajectories and behavioural outcomes. We were particularly interested in the period between the middle of gestation (E12.5) and first two weeks of postnatal life (P15), as it encompasses critical windows for neurodevelopment, as shown in **Table 3** and **Illustration 9**.

Table 3

A web resource (http://translatingtime.net/ (Clancy 2007)) compares developmental windows between different species. By comparing mice with humans at different stages of development (Table 4), we observe that at birth, mice are far less advanced in their development than human newborns: mouse birth at E19.5 is equivalent to human embryo E120 (4 months).

	**Developmental parameter**	**Mouse age**	**Human age (days post-conception)**	**Human age (month)**
Age comparison	Prenatal (poly(I:C) injection for MIA paper)	E12.5	50	≈2 months
	Birth	E19.5	120	4 months
	Postnatal (TNF serum sampling)	P5	181	6 months
	Postnatal (sacrifice)	P15	379	≈ 12 months
Developmental milestone acqusition	Eye opening	P11	280	≈ 9 months
	Ears open (auditory canal fully open)	P11	285	≈ 9.5 months
	Olfactory myelination onset	P9	251	≈ 8.5 months
Brain development	Purkinje cell peak	E11	40	≈ 1.5 months
	Neurogenesis (cortical layer VI start – cortical layer II/III end)	E11 – P3	48 – 191	≈ 1.5 months – 6 months
	Cortical subventricular zone onset	E12	57	≈ 2 months
	Cortical axons reach thalamus	E17	94	3 months
	Striatum myelination onset	P12	339	≈ 11 months
	Plasticity critical period start	P16	417	14 months
	Plasticity critical period end	P26	677	22.5 months
	Prefrontal cortex peak synaptic density	P25	641	≈ 21 months

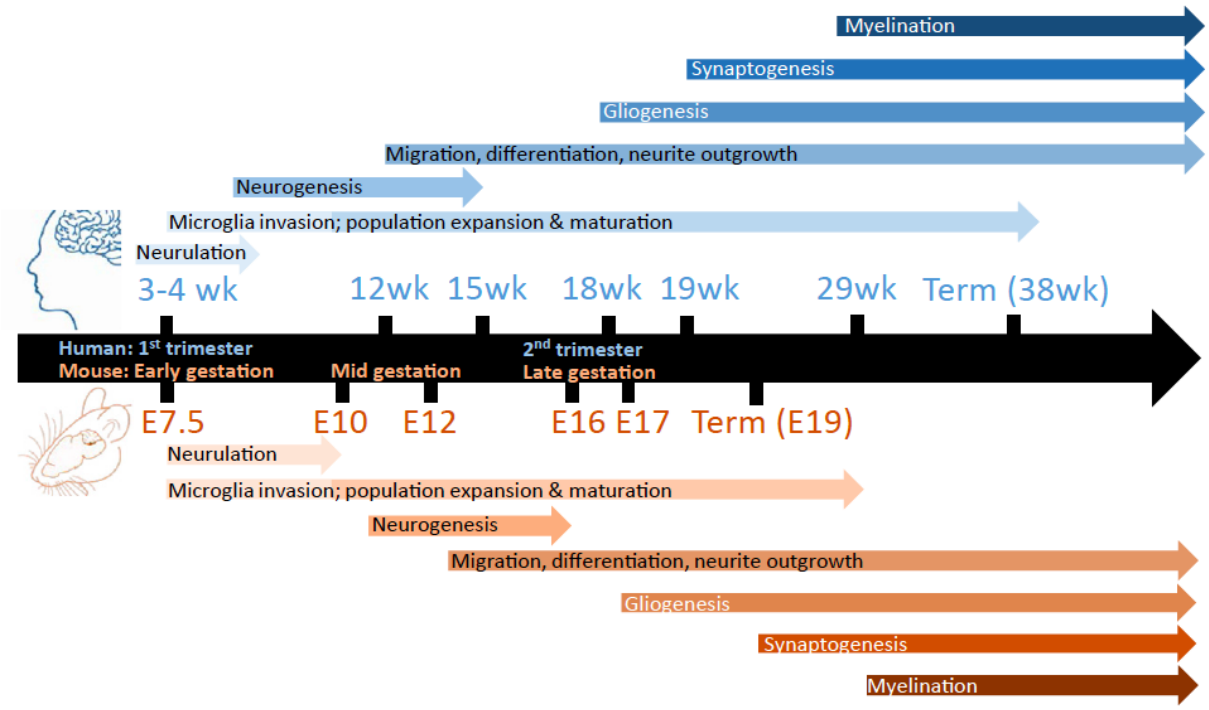


Illustration 9. Comparison between the stages of neurodevelopment in mice and humans. Particularly at the time of birth, mice seem to be less developed that humans. Adapted from Gumusoglu and Stevens, 2018.

My PhD work was also based on a growing body of literature showing that, during this period, cytokines can impact behavioural outcome in mice:

- Gressens and his colleagues developed a mouse model of perinatal inflammation, based on repeated injections of the proinflammatory cytokine IL-1β between P1-P5. They showed that IL-1β induced neuroinflammation, microglial activation, neonatal brain injury that translated into behavioural alterations and notably cognitive impairments (Hagberg et al. 2015). Perinatal inflammation induces white and grey matter lesions (Favrais et al. 2011; Stolp et al. 2019). Dampening of the inflammatory response by treatment with etanercept limited the induced brain damages (Ådén et al. 2010).
- In mice, a single injection of IL-6 at embryonic day 12.5 (E12.5) during pregnancy mimicked the MIA induction by Poly(I:C) injection and induced ASD-like behavioural abnormalities in the adult offspring, such as stereotypies, impaired communication and social interactions (Smith et al. 2007). Intra-cerebroventricular injection of IL-17A in foetuses at the same stage provoked similar abnormalities (Choi *et al.*, 2016). The importance of

maternal IL-6 and IL-17A as drivers of the deleterious effects of poly(I:C) MIA model was also shown by loss-of-function experiments. Blocking either of these cytokines prior to MIA by neutralizing antibodies or by a knock-out strategy alleviates the behavioural symptoms in the adult progeny (Choi et al. 2016; Hsiao and Patterson 2011; Wu et al. 2017).

Based on these studies, we became interested in the impact of inflammation and immune activation on early brain development. Most of the behavioural phenotypes induced by exposure to immune activation, either *in utero* (MIA model) or in the perinatal period (IL-1β perinatal inflammation model) were identified in adult animals. In this context, we reasoned that preclinical studies using NDD models and restricted to adult stages may be biased by confounding factors stemming from cumulative deleterious and compensatory effects occurring at the organism scale throughout the life of the individual. The identified phenotypes may therefore poorly reflect the neurodevelopmental changes undergone by the animal during early peri- and post-natal stages life.

My PhD work aimed to contribute to fill this gap by studying the early consequences of perinatal TNF injections (Manuscript #1) and adding a longitudinal dimension to the study of the MIA model during early postnatal life (Manuscript #2).

1. How does TNF impact neurodevelopment?

Our work suggests that increased TNF levels during the early postnatal period promote the acquisition of the righting and acoustic startle reflexes, as well as an increased exploratory behaviour at two weeks of age. While underlying mechanisms remain to be investigated, it is noteworthy that TNF can cross the BBB in rat neonates via a saturable transport system (Gutierrez, Banks, and Kastin 1993). Therefore, it is likely that at least some of the recombinant TNF molecules that we injected into mouse pups were transported to the brain where they could directly act on their target cell type(s). Previous studies have shown that soluble TNF triggers the TNFR-1 signalling transduction pathway and that all cells of the brain parenchyma, including neurons, astrocytes, oligodendrocytes and microglia, as well as epithelial cells of the BBB express TNFR1 (Probert 2015; Holbrook et al. 2019). Therefore, the beneficial effect of TNF on the acquisition of sensorimotor reflexes and the exploratory behaviour may be explained by a direct effect of TNF on several cell types in the brain. Based on our result of pup earlier acquisition of the righting and startle reflexes upon TNF injections, it may be envisioned that TNF-injected pups may have undergone an earlier development of the cerebellar or spinal neurons, which coordinate the righting reflex at early stages, as well as axonal projections of cochlear root neurons or the neurons in the nucleus reticularis pontis caudalis (PnC), involved in the acoustic startle reflex (M. Davis 1984). Moreover, perinatal injection of TNF could have modified the course of development of Purkinje cells, contributing to increased whisker-induced motor learning and exploratory behaviour (Arakawa and Erzurumlu 2015; Romano et al. 2018).

While we believe that the impact of TNF on reflex acquisition and exploratory behaviour is dependent on a direct action of this injected cytokine on brain cells, indirect mechanisms such as the activation of the Hypothalamic–pituitary–adrenal (HPA) axis or the induction of prostaglandins production cannot be ruled out. Indeed, it was previously demonstrated that increased peripheral levels of TNF could stimulate the HPA axis, eventually resulting in increased production of glucocorticoids by the adrenal glands (TURNBULL 1999; Mulla and Buckingham 1999). Glucocorticoid receptors are present in every cells in the nervous system, including neurons, microglia and astrocytes (Madalena and Lerch 2017). Therefore, increased levels of glucocorticoids in the brain could potentially act on microglia and modify

its current state, possibly towards anti-inflammation, thereby changing the delicate cytokine balance during early neurodevelopment. In this case, we could argue that endocrine changes in the brain due to peripheral inflammatory cues, could be controlled by the brain, using a compensatory mechanism of self-regulation. Alternatively, TNF was previously shown to stimulate the production of prostaglandins from the hypothalamus (Navarra et al. 1992; Mulla and Buckingham 1999), and to induce COX2 expression and subsequent PGE2 release from brain micro-vessel endothelial cells (Mark, Trickler, and Miller 2001) and fibroblasts (Nakao et al. 2002). Prostaglandins are lipid molecules which act on the hypothalamus during infections, in order to trigger an immune inflammatory response against pathogens. Therefore, TNF could possibly act by inducing the production of PGE2 by the endothelial cells of the BBB as proposed in a previous study (Francis et al. 2004).

Of note, our data obtained in mouse pups injected peinatally with TNF highlight a positive role for TNF during neurodevelopment. Strikingly, this is in line with the association studies we performed within the frame of the EDEN cohort. Indeed, we found that TNF levels in serum at birth (measured in cord blood) and in the serum at 5 years were negatively associated with prosocial behaviour problems and conduct problems at 5, respectively. This suggests positive effects of TNF on early development both in humans and mice.

2. What is the physiological relevance of our experimental model in which pups are injected with recombinant TNF?

Because LPS is a potent induced of pro-inflammatory cytokines including IL-6, IL-1 and TNF, many authors have investigated the impact of pro-inflammatory cytokines on neurodevelopment and behaviour by injecting LPS either during gestation and studying the offspring, or directly to mouse pups or adult mice. Are our results consistent with these latter studies? Finding information on the impact of different serum TNF concentrations on the brain proved to be particularly challenging, so I was forced to somewhat extrapolate some findings to suit the following explanation. One study by (William A. Banks et al. 2015) performed in adult mice examined the integrity of the BBB in response to treatment with different levels of LPS, ranging from 0.3 to 3 mg/kg, i.p.. They found that the BBB was resistant to disruption by LPS in all concentrations below 3 mg/kg, where it showed disruption only in specific brain areas, such as the frontal cortex, cerebellum, thalamus and pons-medulla. Furthermore, BBB disruption was observed 24h, but not 4h after the LPS injection. Moreover, brain levels of TNF were not changed between LPS- and saline-injected mice, but increased levels of other cytokines, such as IL-1α, IL-1β, IL-4, IL-9 and IL-10 were observed. Finally, the study concluded that the LPS-induced disruption of the BBB was mediated by COX, and not by glia-induced neuroinflammation. Due the difficulty of finding relevant data about TNF serum levels in response to LPS injection, I extrapolated the results found in two papers where LPS was injected into adult mice and the levels of TNF in serum were reported: the first study injected 10 mg/kg LPS and, which induced a serum TNF increase of approx. 2700 pg/ml 1h post treatment (Rosas-Ballina et al. 2008), while the second study injected a dose of LPS of 7.5 mg/kg (their equivalent for LD_{50}), which induced a serum TNF increase of approx. 750 pg/ml 1h post treatment (Huston et al. 2006). From these two studies, by reducing the LPS dose to the one found in Banks *et al.*, 2015 – 3 mg/kg –, which induced BBB disruption, we get a TNF dose of 300 – 800 pg/ml. This gives a slight indication of the TNF range above which we could expect BBB disruption, assuming that TNF is expressed somewhat linearly, which is questionable. The serum TNF concentration we obtained in our study by injecting 20 µg/kg TNF was of approx. 400 pg/ml, which would position our study within the conditions of BBB disruption. Additionally, we soon plan to

investigate the serum levels of TNF in the brain in pups at P5 and P16, to further elucidate if TNF has crossed the BBB.

One particularly relevant study on the effects of early-life inflammation on mouse behaviour showed that a 100 μg/kg LPS injection into mice at P14 altered their anxiety- and depressive – like behaviour, as well as impaired spatial memory performance, latter only as a result to a second LPS challenge (Dinel et al. 2014). Interestingly, there was no effect on spatial memory formation in adolescence and adulthood. Moreover, TNF plasma levels were increased from 0 to approx. 330 pg/ml, along with increases in the hypothalamus, hippocampus, prefrontal cortex and amydala. In addition, the plasma and brain levels of IL-6, IL-1β and IL-10 were also greatly increased. Therefore, we cannot conclude that the behavioural effects we observed were due to the sole increase in TNF levels. The main message of this study is that peripheral immune activation, affects mouse behaviour differently at distinct stages of development, and does not necessarily cause behavioural impairments, but that it could rather impact positively neurodevelopment.

3. Vulnerability vs. resilience to psychological stress

There has been recent discussion about neurodevelopmental resilience to stress-related emotional disorders, such as in the case of NDDs. A study by Réus *et al.*, 2017 shows that maternal care deprivation-induced early life stress increased offspring inflammation and oxidative stress, which lead to behavioural changes that persist into adulthood. In addition, studies on patients with chronic inflammatory conditions, which displayed increased levels of proinflammatory cytokines, were found to have a high prevalence of major depressive disorder, confirming the role of the immune system in increasing the vulnerability to stress-induced psychiatric illness (Ménard et al. 2017). Similarly, there are links between changes in the immune system and the development of resilience to stress and psychiatric disorders.

Research on human patients with mood disorder and rodent models of stress-induced anxiodepressive behaviours revealed particular differences in the patterns of immune and neuroendocrine responses, between individuals presenting vulnerability vs. resilience to stress (Ménard et al. 2017). From the immunological point of view, it seems that individuals stress-susceptible display an upregulation of pro-inflammatory cytokines, increased monocyte and granulocyte infiltration, as well as microglial activation (Powell et al. 2013; Heidt et al. 2014; Avitsur et al. 2005). On the other hand, stress-resilient individuals exhibit low levels of pro-inflammatory cytokines, T cell immunization upon exposure to CNS-related antigens and microglia hypo-reactivity with low inflammasome activation (Cohen et al. 2006; Brachman et al. 2015). Moreover, similar patterns can be seen between stress-susceptible and resilient individuals in relation to neuroendocrine mechanisms. Psychological stress is associated with activation of the HPA axis and increased circulating levels of glucocorticoids, which could bind to microglial receptors for a lengthened time and polarize microglia towards a chronic pro-inflammatory state (Malik and Spencer 2019). On the other hand, psychological resilience was found to be sex-dependent in its HPA response, associated to increased levels of oxytocin, an hormone which decreases HPA axis activation, as well as to increased quality of life, such as proper maternal care, among other factors (Ménard et al. 2017; Malik and Spencer 2019).

I would like to expand on the previously mentioned point of sex-dependent resilience to psychological stress, adapted to the context of our work. We only studied the consequences of perinatal TNF injections in male individuals, which is one of the limitations of this study. Our reasoning behind that was that males are more at risk than females for neurodevelopmental disorders (E. P. Davis and Pfaff 2014). Likewise, several pre-clinical studies in rodents have shown a sexual dimorphism in neurodevelopmental mechanisms and in sensitivity to developmental brain insults (DiPietro and Voegtline 2017; Chung and Auger 2013; Davies and Wilkinson 2006). Taking into consideration sexual dimorphisms in the stress response present in animal models, it would have been interesting to compare the effect of neonatal TNF injections between males and females pups and observe possible differences. Therefore, it remains to be determined whether TNF injection can also accelerate the acquisition of reflexes and promotes exploratory behaviour in female pups.

On a different note, related to increased resilience to stress in the context of adequate maternal care, we should particularly stress that we used mouse pups of the OF1 outbread strain. The use of an outbred stock presents both advantages and disadvantages. On one hand, the OF1 outbred stock is larger, more robust and produces more pups per litter than any inbred mouse strain (Chia et al. 2005). Furthermore, OF1 mothers provide high quality maternal care and are more resilient to environmental stressors, such as recurrent manipulation of the progeny imposed by our experimental design. On the other hand, outbred stocks bear recessive mutations that may affect experimental results and lower treatment effect size. This genetic variation may affect behavioural responses, since a stock may contain a mixture of homozygous, heterozygous and wildtype pups (Chia et al. 2005). However, outbred stocks present the advantage to mimic more closely human populations. The fact that we randomized pups from P1 to for new litters minimizes the possible confounding effect of genetics in our model. Furthermore, given the possible increased resilience to stress of the OF1 stock, the subtle behavioural alterations in this mouse model are likely to be real and due to a clearly identifiable factor, such as TNF, in our case.

As the old saying goes, “What doesn’t kill you makes you stronger”. I would like to add that PhD students seem to be generally the resilient type, likely experiencing a decreased immune activation. All jokes aside, a study from a few years ago proposed a three-hit model

of vulnerability and resilience. It includes a base of genetic susceptibility, followed by early-life conditions, also considered to be programmed phenotypes, and finishing with late-life conditions (Daskalakis et al. 2013). This concept aims to explain the effect of cumulative stress on an individual's ability to function properly. Interestingly, this concept describes the mechanism of vulnerability acquisition, as an accumulation of failed attempts to cope with adversity, while resilience can be seen as the adaptive capacity acquired through past exposure to adversity in early-life. This article stresses the importance and long-lasting effects of an animal's early-life housing and experimentation conditions. Factors, such as early animal handling, nesting conditions (standard environment vs. enriched environment), as well as variations in maternal care are discussed. The latter was of particular importance to us and we took measure to ensure we controlled for the maternal effect. Therefore, we randomly assigned a new mother to pups at P1, so that each litter was constituted by pups coming from different mothers. To overcome the litter effect, litters were culled to 11-12 individuals to limit litter size effect. Also, the two conditions tested (TNF or vehicle treatment), were represented in each individual litter. In doing so, we obtained both TNF-treated and control pups bred by the same mother, thus excluding the confounding effect of differential maternal care or milk quality, that could contribute to developmental or behavioural differences.

4. What else could we do to further investigate the impact of TNF on neurodevelopment?

I am aware that many more experiments are required to elucidate the regulatory roles of TNF on neurodevelopment, and I have planned to perform these experiments in the upcoming months. Firstly, I will investigate the early changes that are induced in the brain of mouse pups injected with recombinant TNF. To this aim, I will inject mouse pups daily with TNF from P1 to P5 and collect the brains after the last injection. I will use half of the brain to prepare protein extracts and I will analyse them for the levels of TNF and other pro-inflammatory cytokines using the V-plex® (Meso Scale Diagnostics) immunoassay. I will use the other half to prepare cellular suspensions that I will analyse by flow cytometry for the frequency and phenotype of astrocytes, glial cells and neurons. Secondly, I will characterize the long-term impact of TNF injection on brain cells by comparing the transcriptome profiles of the brains of TNF- and PBS- injected mice at P16. Thirdly, I will explore another dimension of behaviour that could be impacted by TNF-treatment, by analysing both quantitatively and qualitatively the USVs emitted by pups at P8, as I have performed in the MIA model. This should provide insights in the communication ability of TNF-injected pups. Fourthly, I will let TNF- and PBS-injected pups grow until the age of 8 weeks and I will characterize the possible behavioural alterations in adulthood, using behavioural tests focusing on motor coordination, social interactions, anxiety and cognition.

5. What is the impact of poly(I:C) injection on pregnant dams?

We have found that that poly(I:C) injection into pregnant dams induced both hypothermia and a decrease in body weight compared to injections with PBS. Based on our observations, as well as data from previous studies (Chow, Yan, and Wu 2016), in normal conditions, a C57BL/6 pregnant dam experiences an increase in body weight of 0.5 – 1 g per day, depending on the number of pups she is carrying, as well as the mother's age and previous pregnancies. Moreover, weight loss is a hallmark of sickness behaviour (C. Murray et al. 2015; Dantzer et al. 2008; Konsman, Parnet, and Dantzer 2002). When a pregnant dam is losing weight, this could suggest either a lack of food intake for the duration of sickness, or a loss in the number of pups being carried, as a result of spontaneous abortion. However, it is difficult to assess the presence or extent of such an effect, as mice tend to cannibalise unborn offspring and rarely leave evidence of pup death in the cage. Previous studies have also assessed the poly(I:C) effect on pregnant dams and reported similar findings, together with increased maternal serum levels of IL-6 and other pro-inflammatory cytokines (TNF, IL-1β, IFN-β/γ, IL17A, CXCL1) (C. Murray et al. 2015; Careaga et al. 2018; Mueller et al. 2019; Lins et al. 2018; Garay et al. 2013; W. L. Wu et al. 2015; Chow, Yan, and Wu 2016). Moreover, cytokine imbalances caused by poly(I:C) treatment were also reported to be present in the placenta and foetal brain (Murray et al. 2015; Mueller et al. 2019; Garay et al. 2013; Hsiao and Patterson 2011). We have not measured cytokine alterations in maternal serum due to the stress induced in the mother, cause by tail blood collection, which could affect pup viability and litter efficiency. Since the MIA model is highly dependent on stable environmental conditions and could therefore be easily disturbed, as well as the fact that we are only using male pups through the course of our study, we decided not to risk inducing spontaneous abortion or a potential reduction in litter size.

6. Why do pups born to poly(I:C)-injected mothers gain weight more rapidly than control pups?

In contrast to our results, Arsenault *et al.*, 2014, showed that pups born to poly(I:C)-injected mothers gain weight less rapidly between P3 to P10 than control pups. Likewise, Murray *et al.*, 2018, found decreased body weight in male, but not female MIA rat pups at P21. Moreover, one study reported no differences in the body weight between MIA and control pups during peri-adolescence and adulthood, while showing increased visceral and subcutaneous fat in adult offspring (Pacheco-López et al. 2013). However their sample size was reduced to n=7/group and the lack of significant effects might be due to limited power. These conflicting data could possibly be explained by differences in the amount of poly(I:C) injected, the time and the route used for poly(I:C) injection or the species. Thus, while we injected C57Bl/6N pregnant dams with 5 mg/kg of poly(I:C) intraperitoneally at E12.5, Arsenault *et al.*, 2014 injected in C57Bl/6J pregnant dams with 5 mg/kg of poly(I:C) intravenously daily between E15 to E17, Pacheco-López *et al.*, 2013 injected C57Bl/6J pregnant dams with 5 mg/kg of poly(I:C) intravenously on day E9, and Murray *et al.*, 2018 injected Wistar rats with 10 mg/kg of poly(I:C) intraperitoneally on E15. Whatever the reasons for the discrepancies between our results and those reported by others, at least two mechanisms could explain why pups born to poly(I:C)-injected mothers gained weight more rapidly than control pups in our experiments. First, this phenomenon may be an indirect consequence of the impact of poly(I:C) on the nursing behaviour or milk quality of the mothers, the behaviour of their pups, or both. Indeed, poly(I:C) induces anxiety-related behavioural changes and pups born to poly(I:C)-injected females exhibit delays in growth and sensorimotor development (Arsenault et al. 2014). Alternatively, the injection of poly(I:C) to pregnant dams may directly impact the gastrointestinal tract of the mother and/or its pups, and therefore their ability to assimilate food nutrients. In support to this hypothesis, both changes in the composition of the intestinal microbiota and increased gut permeability have been reported in the progeny of mouse and rat pregnant mothers injected with poly(I:C) (Hsiao et al. 2013; S. M. Clark et al. 2019; Codagnone et al. 2019; Pacheco-López et al. 2013).

7. Why do pups born to poly(I:C)-injected mothers exhibit communication impairment?

Mice communicate by means of ultrasounds, in many behavioural contexts. They start producing USVs soon after birth as a response to accidental isolation in the cage, which calls for mother pup retrieval, as well as to hunger onset and thermal changes in their environment, all of which require maternal care. USV production is a time-dependent process, with mice gaining increased autonomy with the age and therefore changing their vocalisation pattern according to their newfound needs (Hofer, Shair, and Brunelli 2002; Nobuko, n.d.). Pup USVs start around P2 in most mouse strains and peak at the end of the first week of life, after which their number starts declining until pups reach the second week of life, when they stop being produced (Wiaderkiewicz et al. 2013). In adulthood, the context eliciting mouse vocalisation changes and often involves social interaction between same-sex individuals, mating behaviour or aggressive interactions (Hofer, Shair, and Brunelli 2002; Ferhat et al. 2016). Examination of rodent USVs can yield great insights into the pathogenesis and progression of diseases over time. Alterations of ultrasound patterns have been found in genetic and environmental mouse models of neurodevelopmental disorders, such as autism and schizophrenia (Scattoni, Crawley, and Ricceri 2009b). We have found that pups born to poly(I:C)-injected mothers produced fewer vocalisations (USV call rate) and exhibited a lower total USV duration, as compared to control pups born to PBS-injected mothers. Moreoever, we observed a significant alteration in the overall quality of USV patterns emitted between MIA and control pups, even though differences in individual syllable frequencies did not reach statistical significance between the MIA and control groups. Most neonatal USV studies on the MIA model so far have focused on the USV call rate and/or USV total duration, in which they found differences between MIA and control offspring (Malkova et al. 2012; Hsiao et al. 2013; Choi et al. 2016; Kim et al. 2017; Shin Yim et al. 2017). Most of these studies show that pups born from poly(I:C)-injected mothers displayed a decreased number of USVs in young pups, in the maternal isolation test (Malkova et al. 2012) and during social encounter in adults (Hsiao et al. 2013). However, studies from one group report increased numbers of USVs in MIA pups at P9 (Kim et al. 2017; Shin Yim et al. 2017; Choi et al. 2016a). Another study reported no differences between MIA and control pups at P9 (Morais et al. 2018). These inconsistencies are likely due to differences between the protocols used, in relation to the timing and concentration

of the injected Poly(I:C), the animal supplier and the presence or lack of animal habituation prior to the USV test. Also, in some studies, small sample size could explain the lack of significant effects due to limited power.

However, we wish to stress that our data regarding reduced USV emission in MIA pups during early development also agree with a number of studies on ASD genetic models, such as the *Tsc2*$^{+/-}$ tuberous sclerosis model (Young et al. 2010), the *Shank1*$^{-/-}$ mouse (Sungur, Schwarting, and Wöhr 2016) or *Oprm*$^{-/-}$ mice deficient for the μ-opioid receptor (*Oprm*$^{-/-}$) (Moles, Kieffer, and D'Amato 2004) and the BTBR mouse model of idiopathic ASD (Scattoni et al. 2008). Given that decreased neonatal USV can reflect a deficit in pup attachment behaviour and communication (Scattoni, Crawley, and Ricceri 2009a), we believe that our results can be interpreted as autistic-like symptoms in the context of the MIA model.

8. Why do pups born to poly(I:C)-injected mothers exhibit decreased locomotor activity?

We also assessed the impact of maternal poly(I:C) treatment on the locomotor activity and exploratory behaviour of young pups. Due to behavioural limitations imposed by the targeted age of our mice, we decided to record the pup distance travelled and time spent mobile, in order to assess their level of activity and based on this information, calculated their level of exploration of the environment. While pups born to poly(I:C)-injected mothers exhibited decreased distance travelled and time mobile compared to control pups, their exploratory behaviour was not impacted. This result points to a link between maternal poly(I:C) exposure and pup hypoactivity, which could be due to increased pup anxiety or level of fear of novel environments. Anxiety-induced changes in locomotor activity are commonly assessed by performing the openfield test for adult mice. In the MIA model, the openfield test was only assessed in adult mice, with the exception of one study, where the mice were tested at adolescence, at 5 weeks of age (Hsueh et al. 2018). In the MIA offspring subjected to the openfield test, the majority of studies report a decrease in the total distance travelled, the time spent in the center and the number of center entries (Shi 2003; Chow, Yan, and Wu 2016; Wu et al. 2015; Shin Yim et al. 2017; Kim et al. 2017; Hsiao and Patterson 2011; Hsueh et al. 2018). To our knowledge, our study is the first to report hypolocomotion in MIA offspring during the early postnatal period. This suggests that behavioural impairments appear very early in development in MIA offspring and persist into adulthood.

9. Why did we analyse our data using a multivariable statistical approach, and which conclusions could we draw?

Having validated the MIA model using standard univariate analysis, we then decided to include MIA confounders into a more sophisticated, multivariable analysis based on penalised regression. Our goal was to understand which factors had the largest influence on the MIA class. We have chosen to use this alternative approach based on recent discussions on the difficulties to generate a robust and reproducible MIA model both inter- and intra-institutionally (Roderick and Kentner 2018; Kentner et al. 2018; Careaga, Murai, and Bauman 2017), which created a lack of consensus on the MIA model among research groups. As you might have noticed in the previous chapter, when comparing our data with results obtained by other researchers, there were clear differences in the behavioural and immunological response of MIA offspring exposed to similar MIA induction protocols. Particularly, two recent studies (Mueller et al. 2019, 2018) have revealed crucial information on factors that influence the efficacy of the MIA model. These represent the housing system used for the MIA mice – whether open or ventilated cages (Mueller et al. 2018) - and the precise lot of Poly(I:C) used, which can contain lower or higher molecular weight Poly(I:C), each having different levels of immunogenicity, and therefore different degrees of MIA induction (Mueller et al. 2019). We therefore considered it essential to experimentally control for as many parameters as we could. We therefore followed the recently recommended guidelines to optimize the MIA model in our laboratory (Kentner et al. 2018; Mueller et al. 2019). As such, we used C57Bl/6N mice from Taconic, housed mice in open cages, focused on a single batch of poly(I:C) and analyzed only one sex (male). Also, mice were individually traced for all the parameters assessed in the course of the experiments (magnitude of MIA induction with maternal temperature and weight loss, parental age, litter size, pup weight, behavioural parameters), allowing us to take them into consideration using a multivariable modelling strategy. All other studies on the MIA model, as well as most published mouse work, have only been analysed using a classical class-comparison approach comparing each variable between classes, limiting data understanding and interpretation. The multivariable approach enabled to distinguish between protective and detrimental factors associated with MIA, of which some cytokines were appeared important players. Univariate analysis on cytokines of pups at P15 revealed no differences between MIA and control pups and proved to be limited

in explaining how serum markers relate to the MIA class. However, multivariate analysis showed that cytokines, such as TNF and IL-15, were positively associated with the MIA class and could therefore be considered detrimental to pup development. In contrast, cytokines, such as CXCL10 and IL-5, were shown to be positively associated to the control group and were therefore considered as being protective.

10. What is known about the role of TNF, CXCL10, IL-5 and IL-15 in neurodevelopment and brain function?

Our data suggest that at least four cytokines, i.e. TNF, CXCL10, IL-5 and IL-15, are associated with being born from poly(I:C)-injected mothers. While it remains to be established whether these cytokines contribute to the neurodevelopment deficits that are observed in the MIA model, it is noteworthy that all these cytokines have been proposed to play a role in neurodevelopment or brain function as described in the brief overview below.

TNF: As previously mentioned in the introduction section, TNF is expressed early in brain development and plays an important role in synaptogenesis and neurogenesis (Dziegielewska et al. 2000; Garay et al. 2013). TNF constitutive expression in the brain is maintained in homeostatic conditions (Vitkovic, Bockaert, and Jacque 2000; Stellwagen and Malenka 2006), and it appears necessary for optimal brain development and function. However, during immune activation, TNF promotes neuroinflammation, which has been associated with deleterious consequences on brain function and behaviour, in particular in the context of sickness behaviour (Dantzer et al. 2008). In the MIA model of ASD, TNF was also found to be increased in the serum and placenta of pregnant dams immediately after Poly(I:C) injection (Garay et al. 2013; U. Meyer 2006; Gilmore, Jarskog, and Vadlamudi 2005), as well as in the amniotic fluid (Gilmore, Jarskog, and Vadlamudi 2005). However, no change was reported in the brains of offspring from Poly(I:C) – injected mothers immediately after Poly(I:C) injection (U. Meyer 2006; Gilmore, Jarskog, and Vadlamudi 2005), but decreased TNF levels were reported in the neonatal brain at P1 (Gilmore, Jarskog, and Vadlamudi 2005). Nonetheless, human studies on ASD reported elevated levels of TNF in the serum and cerebrospinal fluid in children diagnosed with ASD, as compared with healthy children (Ashwood et al. 2011b; Molloy et al. 2006; Vargas et al. 2005; Abdallah et al. 2013; Chez et al. 2007), as well as increased TNF levels in *post mortem* brain tissue of ASD patients (Li et al. 2009). Furthermore, the levels of TNF were found to be associated with the severity of autism (Chez et al. 2007). Last, but not least, increased TNF has also been positively associated with gastrointestinal (GI) dysfunction in LPS-stimulated PBMCs from children with autism (Rose et al. 2018).

IL-15: IL-15 is a pleiotropic cytokine with important roles in promoting the survival, proliferation and activation of natural killer (NK) and CD8+ T cells. IL-15 and its receptor IL-15R are known to be expressed by glial cells and neurons in the mouse (Hanisch et al. 1997) and human brain (Kurowska et al. 2002), having regional- and development- dependent expression. *Il15r*$^{-/-}$ mice deficient for IL-15 receptors exhibit depressive-like behaviour (X. Wu et al. 2011). Moreover, IL-15 has been shown to have roles in memory formation, regulation of the circadian rhythm and metabolism in mice, and appears therefore to be required for proper brain functioning (He et al. 2010; Pan et al. 2013). Interestingly, the expression of IL-15 receptors at the blood-brain-barrier is modulated by TNF (Pan et al. 2009). In the MIA mouse model, IL-15 is decreased in multiple areas of the brain in MIA offspring between P7 to P30 (Garay et al. 2013). In human studies, the role of IL-15 has been shown to be different in neuropsychiatric pathologies compared to neurodegenerative disorders: Rentzos *et al.*, 2006 have found increased levels of IL-15 in Alzheimer's disease (AD) patients, compared to patients with noninflammatory neurological disease. Moreover, other studies considered IL-15 to be a possible biomarker of AD and pathologies involving cognitive impairment (Hall 2012; Bishnoi, Palmer, and Royall 2015). However, IL-15 in female schizophrenia patients was shown to be negatively associated with Positive And Negative Symptoms Scale score for schizophrenia (PANSS scores) (Ramsey et al. 2013). This suggests that IL-15 plays complex roles and that further work is required to understand its involvement in the early postnatal neurodevelopment.

IL-5: IL-5 is a cytokine with important roles in mucosal immunity and is known to be extensively implicated in the development of allergies and asthma. It has recently also been positively associated with ASD in several human studies. Unstimulated PBMCs from ASD patients were shown to have elevated levels of T_H2-produced cytokines, among which IL-5 (Molloy et al. 2006). Moreover, IL-5 and IL-4 were found to be elevated in the serum of mothers with a child with ASD at mid-gestation, and are associated with a 50% increase in the risk of developing ASD in the child (Goines et al. 2011). Furthermore, a recent meta-analysis has shown that IL-5 levels were positively associated with ASD diagnosis (Saghazadeh et al. 2019). Increased IL-5 levels in children with autism have also been associated with GI dysfunction, as compared with ASD and healthy children with no GI dysfunction. Although ASD patients displayed increased levels of T_H1-produced cytokines

IFN-γ and IL-8 in the brain, compared to control, no difference was observed in the brain levels of T_H2-derived cytokines IL-5, IL-4 and IL-10 between ASD patients and healthy individuals (Li et al. 2009). Our results of a negative association between IL-5 levels and belonging to the MIA class are therefore *a priori* not in line with the reported deleterious roles of IL-5. However, it is possible that IL-5 levels might fluctuate transiently in the MIA offspring, during a specific developmental window.

CXCL10: CXCL10 is a cytokine with chemotactic roles produced by monocytes and endothelial cells in the periphery in response to IFN-γ. CXCL10 also plays roles in cerebral function, as can be produced by human neural precursor cells upon TNF-α stimulation (Sheng 2005). Moreover, it was found to be increased in several neurodegenerative diseases, such as AD (Xia et al. 2000), multiple sclerosis (T. L. Sørensen et al. 2001) and HIV-associated dementia (Cinque et al. 2005). A few studies also looked at CXCL10 expression in children with ASD and found contradictory results: one study showed decreased levels of several chemokines, among which CXCL10 in autistic patients, which were associated with social behaviours (Shen et al. 2016), while another study showed no differences in the levels of CXCL10 between ASD and healthy children (Ashwood, *et al.*, 2011b)(Ashwood et al. 2011a). The differences could be due to the studies being conducted in two distinct populations, with the first being a Chinese Han population, while the second being a Caucasian population. It remains that, further studies are warranted to precisely understand the possible roles of CXCL10 in neurodevelopment.

Take home message

While many authors have investigated the role of cytokines on neurodevelopment, very little is known on the impact of cytokines on the behaviour of the mouse during the first two weeks after its birth. Here we have identified four cytokines i.e. TNF, IL-5, IL-15 and CXCL10, that were associated with altered odds of being born to a mother in which the immune system has been activated during pregnancy. We further demonstrated that TNF could accelerate the acquisition of developmental milestones and promote exploratory behaviour in infant mice. While we have only obtained preliminary insights into underlying mechanisms, the protocols that we have developed and more specifically the use of an innovative multivariable analysis for analysing behavioural and biological data in the MIA model provide a framework for further studies.

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