

Developmental Response to Brain Inflammation

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TABLE OF CONTENTS

Publications and presentations	vii
List of Figures and Table	ix
Abbreviations	xiv
Abstract.....	xvi
CHAPTER 1	1
General Introduction	2
1.1 Molecular and cellular contributors in brain immune response.....	3
1.1.1 Immune system overview	3
1.1.2 Cytokines and adhesion molecules	6
1.1.3 Neutrophils.....	12
1.1.4 Microglia and perivascular macrophages	16
1.2 Neurodevelopmental disorders and inflammation	20
1.2.1 General aspects of neurodevelopmental disorders.....	20
1.2.1.1 Autism and schizophrenia.....	21
1.2.1.2 Cerebral Palsy (and preterm damage).....	22
1.2.2 The role of inflammation in neurodevelopmental disorders.....	24
1.2.2.1 Clinical evidence.....	24
1.2.2.2 Animal models for studying inflammation during development.....	28
1.2.3 "Windows of susceptibility" during development	37
1.3 The blood-brain barrier: interface between brain and periphery	39
1.3.1 Endothelial cells.....	39
1.3.2 Barrier mechanisms in the developing brain	43
1.3.3 The role of the adult blood-brain barrier in inflammation.....	45
1.3.4 The blood-brain barrier and inflammation during development.....	48

1.4 Other brain barriers and possible signalling routes from the central nervous system to the periphery	49
1.5 Acute phase response to brain injury	51
1.4.1 Acute phase response in the adult liver.....	52
1.4.2 Acute phase response to brain injury in adults	54
1.4.3 The role of the hepatic acute phase response during development.....	55
1.6 Aims.....	58
CHAPTER 2	59
Methods.....	60
2.1 Animals	60
2.2 Administration of substances.....	60
2.2.1 Intracerebral injection of IL-1 β	60
2.2.2 Macrophage depletion.....	61
2.2.2.1 Anti-colony stimulating factor 1 receptor.....	61
2.2.2.2 Clodronate liposomes.....	61
2.2.3 Intravenous injection of IL-1 β	62
2.3 Tissue preparation	62
2.4 Light-microscopy immunohistochemistry	63
2.4.1 Immunohistochemistry	63
2.4.2 Immunohistochemistry analysis.....	64
2.5 mRNA extraction	66
2.6 cDNA conversion and Real-Time polymerase chain reaction	66
2.7 Statistics	68

CHAPTER 3	73
An age specific perivascular cell population is found during “window of susceptibility” to brain inflammation	74
3.1 Introduction and aims	74
3.2 Results	75
3.2.1 Blood-brain barrier disruption and neutrophil recruitment were only observed at P14 after focal inflammation	75
3.2.2 The expression of key inflammatory mediators at basal level and after inflammation in the brain were age-dependent.....	80
3.2.3 Microglia responded to inflammation from P14 onwards	86
3.2.4 A specific cell population was identified at P14.....	86
3.3 Discussion	87
3.4 Conclusion	97
CHAPTER 4	98
Targeting perivascular cells to resolve the "window of susceptibility"	99
4.1 Introduction and aims	99
4.2 Results	100
4.2.1 Anti-CSF1R treatment did not deplete peripheral macrophages but affected microglia population	100
4.2.2 Clodronate liposomes depleted peripheral macrophages and significantly decrease the number of Iba ⁺ perivascular cells in the brain.....	103
4.3 Discussion	106
4.4 Conclusion	111

CHAPTER 5	113
Experimental activation of the acute phase response after focal brain injury inverts the "window of susceptibility"	114
5.1 Introduction and aims	114
5.2 Results	115
5.2.1 Basal expression of key inflammatory mediators and the number of neutrophils in the liver were highest in younger mice.....	115
5.2.2 Expression of key inflammatory mediators and the number of leukocytes in the liver increased after injection of a high dose of IL-1 β in the brain and were highest at P7 and P14.....	116
5.2.3 The "window of susceptibility" in the brain disappeared after 100ng IL- 1 β injection in the brain	127
5.2.4 Blood-brain barrier permeability and neutrophil recruitment to the brain decreased while brain expression of inflammatory mediators increased with the concentration of IL-1 β injected in the brain	132
5.2.5 The inverse relationship between the severity of the brain cellular injury and the magnitude of the hepatic acute phase response.....	134
5.2.6 Peripheral injection of IL-1 β after injection of a low dose of IL-1 β induced the hepatic acute phase response.....	139
5.2.7 Experimental activation of the acute phase response after intracerebral injection of a low dose of IL-1 β protected the brain from neutrophil recruitment and increased BBB permeability	143
5.3 Discussion	147
5.4 Conclusion	155

CHAPTER 6	157
General discussion	158
Final conclusion.....	170
References	171

PUBLICATIONS AND PRESENTATIONS

Publications

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- Sá-Pereira I., Anthony D.C., Stolp H.B. (November 22nd, 2013). Neurodevelopmental response to intracerebral inflammation. 3rd UK and Ireland Early Career Symposium. Cambridge, United Kingdom.
- Sá-Pereira I., Couch Y., Anthony D.C., Stolp H.B. (November 9th-13th, 2014). Age-specific susceptibility to neuroinflammation during postnatal development.

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- Sá-Pereira I., Roodseelaar J., Anthony D.C., Stolp H.B. (July 14th-18th, 2015). An age-specific intravascular macrophage population is associated with the murine "window of susceptibility" to CNS inflammation. The XII European Meeting on Glial Cell Function in Health and Disease (GLIA). Bilbao, Spain.
- Sá-Pereira I., Roodseelaar J., Stolp H.B., Anthony D.C. (April 19th-22nd, 2016). An inverse relationship between the severity of a brain injury and the magnitude of the APR in the early post-natal period. 9th International Symposium on Neuroprotection and Neurorepair. Leipzig, Germany.

LIST OF FIGURES AND TABLE

Chapter 1

Figure 1.1 Initiation of the innate immune response.	4
Figure 1.2 Neurodevelopmental disorders and their multifactorial origin.	24
Figure 1.3 Neurovascular Unit.	42
Figure 1.4 Brain barriers of the brain.	51
Figure 1.5 The acute phase response to brain injury in adult rodents.	57
Table 1.1 Immune activation impairs prepulse inhibition.	31
Table 1.2 Effects of immune activation on the inflammatory response, brain and/or behaviour.	36

Chapter 2

Figure 2.1 GeNorm analysis to select the best candidate reference gene(s) that are not affected by the experimental conditions and constitutively expressed in all brain samples.	71
Figure 2.2 GeNorm analysis to select the best candidate reference gene(s) that are not affected by the experimental conditions and constitutively expressed in all liver samples.	72
Table 2.1 Stereotaxic coordinates for injection of IL-1 β in the striatum of C57Bl/6 mice.	61
Table 2.2 Protocol for RNA conversion to cDNA.	69
Table 2.3 Protocol for RT-qPCR.	69
Table 2.4 Primers sequences.	70

Table 2.5 Mouse geNormPLUS kit gene list.	70
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Chapter 3

Figure 3.1 BBB permeability after focal inflammation during postnatal development. 77	
Figure 3.2 mRNA expression of transporters and tight junction proteins in the brain after focal inflammation at different time points during postnatal development.	78
Figure 3.3 Recruitment of neutrophils to brain parenchyma after focal inflammation during postnatal period.	79
Figure 3.4 mRNA expression of pro- and anti- inflammatory mediators in the brain during postnatal development.	83
Figure 3.5 mRNA expression of pro- and anti- inflammatory cytokines in the brain after focal inflammation during postnatal period.	84
Figure 3.6 mRNA expression of IL-1 β receptors, adhesion molecules and chemokines in the brain after focal inflammation during postnatal period.	85
Figure 3.7 Microglia population in naïve mice and after focal inflammation during postnatal development.	88
Figure 3.8 Perivascular macrophage population in the brain during postnatal development.	89

Chapter 4

Figure 4.1 Impact of anti-CSF1R treatment on macrophage population in the liver. ..	101
Figure 4.2 Microglia population after anti-CSF1R treatment.	102
Figure 4.3 Macrophages in the liver after injection of clodronate-filled liposomes. ...	104
Figure 4.4 Microglia and Iba-1 ⁺ perivascular cell (IPCs) populations after injection of clodronate-filled liposomes.	105

Chapter 5

Figure 5.1 mRNA expression of pro- and anti- inflammatory cytokines in the liver during postnatal development.	117
Figure 5.2 mRNA expression of CD11b and the number of neutrophils in the liver during postnatal development.	118
Figure 5.3 Effect of low and high doses of IL-1 β in the brain on mRNA expression of pro- and anti- inflammatory cytokines in the liver.	122
Figure 5.4 Effect of low and high doses of IL-1 β in the brain on mRNA expression of adhesion molecules and chemokines in the liver.	123
Figure 5.5 mRNA expression of CD11b and the number of neutrophils in the liver after injection of 100ng IL-1 β in the brain.	125
Figure 5.6 Neutrophil recruitment to the liver after injection of 100ng IL-1 β in the brain.	126
Figure 5.7 Spatial maps of neutrophil distribution after injection of 1ng or 100ng IL-1 β in the brain.	129
Figure 5.8 Recruitment of neutrophils to the brain after injection of 1ng or 100ng IL-1 β in the brain.	130
Figure 5.9 Effects of 1ng or 100ng IL-1 β in the brain on BBB permeability.	131
Figure 5.10 Effects of increasing doses of IL-1 β on BBB permeability during susceptible period.	132
Figure 5.11 Neutrophil recruitment after injection of 1ng, 10ng or 100ng IL-1 β in the brain during susceptible period.	135
Figure 5.12 Effect of increasing focal inflammation on the expression of IL-1 β and ICAM-1 in the brain.	136

Figure 5.13 Neutrophil recruitment to the liver after injection of 1ng, 10ng or 100ng IL-1 β in the brain during susceptible period.	137
Figure 5.14 The expression of inflammatory mediators in the liver after i.c. injection of 1ng, 10ng or 100ng IL-1 β during susceptible period.	138
Figure 5.15 Effect of experimental activation of the APR on the number of neutrophils recruited to the liver after injection of 1ng IL-1 β in the brain.	140
Figure 5.16 Effect of experimental activation of the APR on the hepatic expression of inflammatory mediators after injection of 1ng IL-1 β in the brain.	142
Figure 5.17 Effect of experimental activation of the APR on the number of neutrophils recruited to the brain and BBB permeability after injection of 1ng IL-1 β in the brain.	145
Figure 5.18 Effect of experimental activation of the APR on the expression of inflammatory mediators in the striatum after injection of 1ng IL-1 β in the brain.	146
Table 5.1 Hepatic mRNA expression levels of inflammatory mediators after injection of 100ng IL-1 β in the brain at different time points during postnatal period.	124

Chapter 6

Figure 6.1 Developmental response to a low dose of IL-1 β in the brain.	166
Figure 6.2 The effects of a high dose of IL-1 β in the brain on the inflammatory responses in the brain and liver.	167
Figure 6.3 The inflammatory response of P14 mice to increasing severity of inflammation in the brain.	168

Figure 6.4 Experimental activation of the acute phase response after injection of a low dose of IL-1 β in the brain.	169
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ABBREVIATIONS

APPs	Acute Phase Proteins
APR	Acute Phase Response
BBB	Blood-Brain Barrier
BCRP	Breast Cancer Resistance Protein
CCL	C-C chemokines
CD	Cluster of Differentiation
CNS	Central Nervous System
CSF	Colony-Stimulating Factors
csf	Cerebrospinal fluid
CSF1R	CSF1 Receptor
CXCL	C-X-C chemokines
CXCR	CXC Receptor
CX3CL	CX3C chemokines
CX3CR	CX3C Receptor
DAMPs	Damaged-Associated Molecular Patterns
E	Embryonic day
EAE	Experimental Autoimmune Encephalomyelitis
GLUT1	Glucose Transporter 1
HSCs	Hematopoietic Stem Cells
Iba-1	Ionized calcium-binding adapter molecule 1
i.c.	Intracerebral
ICAM	Intercellular Adhesion Molecule
IFN	Interferons

IgSF-CAMs	Immunoglobulin Superfamily of Cell Adhesion Molecules
IL	Interleukin
IL-1RA	IL-1 Receptor Antagonist
IL-1R1	IL-1 type 1 Receptor
IL-1R2	IL-1 type 2 Receptor
i.p.	Intraperitoneal
i.v.	Intravenous
IPCs	Large flattened irregular perivascular cells
LPS	Lipopolysaccharide
MBS	Anti-neutrophil serum HB199
MMP	Metalloproteinase
mRNA	Messenger Ribonucleic Acid
NMDA	N-Methyl-D-aspartic Acid
P	Postnatal day
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate-Buffered Saline
P-gp	P-glycoprotein
poly(I:C)	Polyinosinic:polycytidylic acid
PRRs	Pattern Recognition Receptors
ROS	Reactive Oxygen Species
RT-qPCR	Real-Time Polymerase Chain Reaction
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
ZO	Zonula Occludens

ABSTRACT

Perinatal inflammation contributes to neurodevelopmental diseases, and animal models have revealed that the inflammatory response within the central nervous system is age dependent. It remains unclear what intrinsic and/or extrinsic factors are responsible for this variation. Here, my aim was to discover the mechanisms responsible for the age-dependent changes in the inflammatory response of the brain by injecting interleukin-1 β (IL-1 β) into the brain of mice at postnatal day (P)7, P14, P21 or into adult mice.

A “window of susceptibility” was found at P14, which was associated with marked neutrophil recruitment and blood-brain barrier (BBB) breakdown, in response to a low dose of IL-1 β . Evaluation of cytokine, chemokine, and adhesion molecule mRNA transcripts failed to reveal any specific increases in basal or reactive expression following the injection of IL-1 β at P14. The extrinsic hepatic acute phase response (APR) was evaluated, but, once again, there was no evidence that an altered APR might account for the enhanced inflammatory response at P14. Indeed, an inverse relationship between the magnitude of the leukocyte recruitment to the brain and the APR was discovered. Enhancement of the APR with intravenous IL-1 β after injection of a low dose of IL-1 β into the brain was found to reduce the number of neutrophils and BBB permeability in the brain. While no molecular changes seem to account for the presence of the “window of susceptibility”, a population of Iba-1⁺ large, flattened and irregular perivascular cells was discovered within the P14 brain, that may contribute to the increased leukocyte recruitment at P14.

Although variations in the brain inflammatory response with development were not fully account for, my results highlight the importance of the systemic inflammatory response on the outcome of acute brain injury and suggest that the systemic APR might be manipulated therapeutically to protect the brain in the perinatal period.

CHAPTER 1

GENERAL INTRODUCTION

For a long time the brain was considered to be an immune-privileged site due, in part, to the presence of the brain barriers as well as an attenuated inflammatory response compared to other organs (Byrnes et al., 1996; Medawar, 1948; Stevenson et al., 1997). However, it is now evident that this concept is not absolute and describing brain as an “immunologically specialized” site may be more accurate (Galea et al., 2007). In the last few years, many studies have shown that inflammation and immune response in the brain have an important role in many neurological disorders, including multiple sclerosis, Alzheimer’s disease and Parkinson’s disease (Amor et al., 2010; Fung et al., 2012; Galea et al., 2007). Neuroinflammation has also been strongly implicated in the aetiology of neurodevelopmental disorders, such as autism, schizophrenia and cerebral palsy (Bayer et al., 1999; van Berckel et al., 2008; Kadhim et al., 2003; Li et al., 2009; Radewicz et al., 2000; Suzuki et al., 2013; Tetreault et al., 2012) and, therefore, suggests that the interplay between inflammation and neural development is key to understand the establishment and diversity of neurodevelopmental disorders. The impact that inflammation may have during development needs to be explored in the light of this hypothesis. The majority of our understanding of the inflammatory system in the brain comes from adult diseases, where a number of cellular and molecular mediators have been identified to modulate the immune response in the brain. However, there are further specializations of the developing brain and therefore, not all of the features observed in the adult may be exactly recapitulated in this context. In addition, the impact of inflammation on development is likely to be highly dependent on the timing of the challenge as brain development occurs within a very well controlled environment and includes a range of well-timed events (Bilbo and Schwarz, 2009, 2012, Hagberg et al., 2012, 2015). It is then important to specifically study immune modulation in the

developing brain in order to find concrete evidence of its contribution to neurodevelopmental disorders. Many studies using different adult rodent models of brain injury (reviewed in Anthony and Couch, 2014 and Anthony et al., 2012) and human data of adult brain pathologies (Di Napoli et al., 2005; Smith et al., 2006; Villapol, 2016) have also shown a correlation between the magnitude of the hepatic acute inflammatory response (acute phase response (APR)) and brain injury. However, very few studies have focused on the contribution and response of the peripheral immune response in the liver to brain injury in the fetus or neonates. It is likely that, as in adult, the hepatic APR may impact the magnitude of brain immune response and injury. This introduction seeks to provide an overview of the cellular and molecular mechanisms involved in brain and peripheral inflammatory responses and how these are known to be modulated during development.

1.1 Molecular and cellular contributors in brain immune response

1.1.1 Immune system overview

The major role of the immune system is to eliminate pathogenic bacteria, virus and any other toxic substances without an excessive inflammatory response that might cause further tissue damage. This is achieved by the collaborative work between the innate and adaptive immune systems. The innate immune system provides a rapid and non-specific reaction while the adaptive immune system specifically respond to different antigens, which allows the development of an immune memory that contributes to a faster and effective response to specific antigens when they are encountered a second time (reviewed in Chaplin, 2010; Delves and Roitt, 2000 and Parkin and Cohen, 2001). The innate immune response is mainly initiated after the interaction between pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs)

or/and damaged-associated molecular patterns (DAMPs) leading to the expression of many inflammatory cytokines (Figure 1.1).

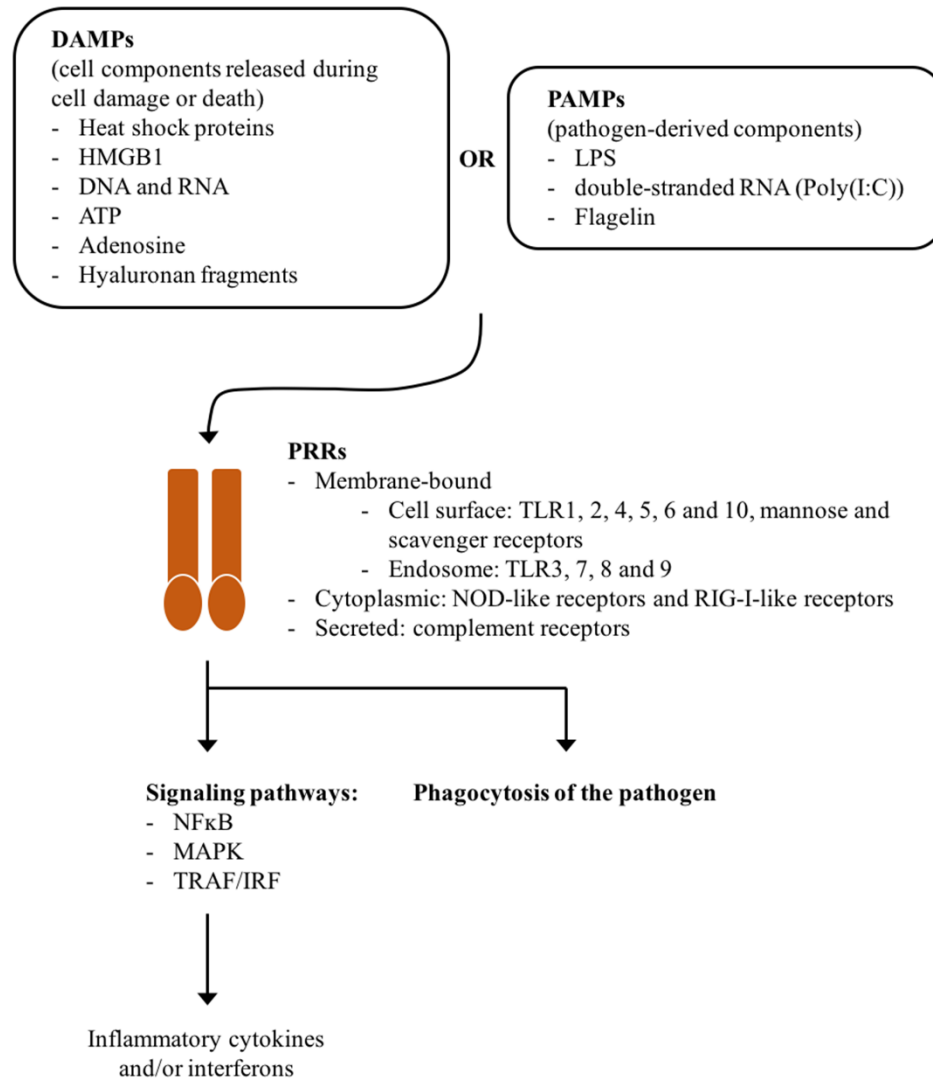


Figure 1.1 Initiation of the innate immune response. Damaged-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) bind to pattern recognition receptors (PRRs) on/in innate immune cells and, consequently, activate signalling pathways, which lead to the expression of inflammatory cytokines and/or interferons, or promote phagocytosis of pathogens. HMGB1: high mobility group box 1 protein, DNA: deoxyribonucleic acid, RNA: ribonucleic acid, ATP: adenosine triphosphate, LPS: lipopolysaccharide, Poly(I:C): polyinosinic:polycytidylic acid, TLR: toll-like receptor, NOD: nucleotide-binding oligomerization domain, NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells, MAPK: mitogen-activated protein kinase, TRAF: Tumor necrosis factor receptor associated factor, IRF: Interferon regulatory factor.

Downstream, these cytokines induce the production of other proteins and molecules, including reactive oxygen species (ROS), antimicrobial peptides, complement proteins and more cytokines by many cells. The activation of PRRs may also induce other non-transcriptional responses, such as phagocytosis and cell death (reviewed in Brubaker et al., 2015; Kumar et al., 2011 and Riera Romo et al., 2016). The main cellular components of the innate immune system are the neutrophils, monocytes, macrophages, eosinophils and basophils. They recognise senescent cells and microbes by PRRs and can act as antigen-presenting cells in order to activate the adaptive immune response. These cells can engulf pathogens and cell debris, and secrete many substances including cytokines, antimicrobial proteins, histamine, ROS and degradative enzymes (reviewed in Riera Romo et al., 2016). The adaptive immune response, which normally follows the innate immune system, is antigen-specific and is mainly mediated by lymphocytes. T lymphocytes are involved in cell-mediated immune response while B lymphocytes are involved in the humoral immune response (reviewed in Chaplin, 2010).

All immune cells arise from hematopoietic stem cells (HSCs). The development of the hematopoietic system starts very early in the embryonic development and occurs in a range of sites. The first hematopoietic cells start to be formed in the extraembryonic yolk sac at around embryonic day (E) 7.5 in mice, and later in allantois and placenta. The *novo* HSCs, which present characteristics of adult HSCs, are then produced at around E10.5 in an area surrounding the dorsal aorta named aorta-gonad mesonephros region and in the placenta. After being produced in the aorta-gonad mesonephros region, HSCs migrate to the placenta and foetal liver where they expand. Finally, cells start to migrate to the spleen at around E14 and to the bone marrow around birth (reviewed in Rieger and Schroeder, 2012). In adults, HSCs are located mainly in the bone marrow and are able to self-renew and differentiate into all blood cell types. It is thought that during hematopoiesis HSCs

and subsequent progenitors progressively lose their multilineage capacity in order to commit to specific cell lineages (Orkin and Zon, 2008). Within the leukocytes, T cells, natural killer cells and B cells constitute the lymphocytes while neutrophils, eosinophils, basophils, monocytes, mast cells and dendritic cells make part of the myeloid lineage (Rieger and Schroeder, 2012). Once in circulation, monocytes can migrate to different tissues and give rise to macrophages that can be divided in alveolar (pulmonary alveolus), osteoclast (bone), histiocyte (connective tissue), Kupffer cell (liver), intestinal macrophages and microglia (central nervous system (CNS)) (reviewed in Galli et al., 2011).

The brain represents a distinct immunological organ from the peripheral immune system. Microglia are considered to be the only innate immune cells present in the brain parenchyma in normal physiological conditions and the blood-brain barrier (BBB), which comprise the unique properties of the CNS parenchymal microvasculature, is an important player in the communication between central and peripheral immune systems. These important cellular components of the brain immune response will be discussed in the following sections.

1.1.2 Cytokines and adhesion molecules

Cytokines are small proteins that constitute one of the most diverse families of signalling molecules. They include tumour necrosis factors (now tumour necrosis factor (TNF) and lymphotoxin), interferons (IFN), interleukins (IL), transforming growth factors (TGF), colony-stimulating factors (CSF), platelet-derived growth factor, epidermal growth factor, fibroblast growth factors, macrophage migration inhibitory factors, insulin-like growth factors and chemokines (Bilbo and Schwarz, 2012). Chemokines are divided into four families according to the spacing of two conserved cysteine residues in the N-terminal position and include the C-C chemokines (CCL), the C-X-C chemokines

(CXCL), the C chemokines, and one CX3C chemokine (CX3CL) (Jaerve and Müller, 2012; Rostène et al., 2011). The inflammatory response is characterised by a highly dynamic balance between pro- and anti- inflammatory cytokines that can act synergistically and/or antagonistically. Usually, cytokines may act as anti- and/or pro-inflammatory mediators; generally pro-inflammatory cytokines include IL-1 α , IL-1 β , TNF and CCL-2, anti-inflammatory cytokines include IL-1 receptor antagonist (IL-1RA), IL-4, IL-10 and TGF- β and IL-6 is considered to be anti- and pro-inflammatory. Any disturbance to the dynamic balance of pro- and anti- inflammatory cytokines may contribute to pathology (reviewed in Garay and McAllister, 2010).

IL-1 family comprises 11 members that are divided into those with agonist activity (IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β , IL-36 γ), receptors antagonists (IL-1RA, IL-36RA and IL-38) and anti-inflammatory (IL-37) (Garlanda et al., 2013). IL-1 family members, as many other cytokines, are recognised by specific receptors expressed on the cell surface. For example, IL-1, which include IL-1 α and IL-1 β , signals through IL-1 type 1 receptor (IL-1R1) and, consequently, activates nuclear factor- κ B and mitogen-activated protein kinase pathways (O'Neill, 2008; Sims and Smith, 2010). IL-1 can also bind to IL-1 type 2 receptor (IL-1R2), which acts as a decoy receptor by inhibiting IL-1 signal transduction. In addition, IL-1RA is produced in parallel with IL-1 and inhibits IL-1 signalling by binding to IL-1R1 (Garlanda et al., 2013; Peters et al., 2013). This balance between activation and dampening of the inflammatory response is a recurring theme in inflammatory regulation, and illustrates processes of a carefully controlled inflammatory response. IL-1 α and IL-1 β are encoded by distinct genes but have similar biological effects. The major difference between these cytokines is that IL-1 β is secreted by cells with hematopoietic origin and circulates systemically, and IL-1 α is most present in epithelial cells, endothelial cells and astrocytes and only released upon necrosis.

Moreover, IL-1 α is fully active when released and IL-1 β needs be cleaved by caspase-1 in order to become active (Dinarello, 2011; Garlanda et al., 2013). In the brain, IL-1 can be expressed by microglia (Guadagno et al., 2015; Pearson et al., 1999; Pinteaux et al., 2002; Yao et al., 1992), endothelial cells (Corsini et al., 1996; Fabry et al., 1993) and astrocytes (Corsini et al., 1996; Lau and Yu, 2001; Pearson et al., 1999) upon stimulation. Once produced, IL-1 can act on many cells in the brain. IL-1, mainly IL-1 β , can induce the expression of chemokines, growth factors, adhesion molecules and cytokines in astrocytes (Ballestas and Benveniste, 1995; John et al., 2005; Moynagh et al., 1994; Shrikant et al., 1994), stimulate microglia to produce cytokines and chemokines (Hanisch, 2002; Lee et al., 1993), promote differentiation, maturation and survival of oligodendrocytes (Vela, 2002), and promote the recruitment and migration of leukocytes by inducing chemokine and adhesion molecules expression by the brain endothelial cells (Allen et al., 2012; Bernardes-Silva et al., 2001; Proescholdt et al., 2002). IL-1 α and IL-1 β have been implicated in many neurological disorders, including Alzheimer's disease, traumatic brain injury, epilepsy, Parkinson's disease and stroke (reviewed in Allan et al., 2005). Finally, an example of an anti-inflammatory cytokine is IL-10 that is recognized by its specific receptor and is able to activate Janus kinase signal transducer and activator of transcription pathway (Murray, 2006). IL-10 is known to decrease gene expression of cytokines, such as TNF, IL-1 β , CCL-2, CCL-5, CXCL-1 and CXCL-2, increase expression of anti-inflammatory mediators, including IL-1RA and TGF- β , and downregulate major histocompatibility complex class II antigens (reviewed in Lobo-Silva et al., 2016 and Moore et al., 2001). IL-10 was shown to ameliorate local inflammation during permanent ischemia in mice (Pérez-de Puig et al., 2013) and to decrease neurodegeneration in a mouse model of Parkinson's disease (Joniec-Maciejak et al., 2014). However, administration of IL-10 in the brains of two different amyloid

precursor protein transgenic mice increased amyloid- β accumulation and impaired memory (Chakrabarty et al., 2015). These studies suggest that any change, either increase or decrease, to the balanced inflammatory response may lead to negative effects depending on the injury/disease type.

In addition to their role in the immune response, cytokines have been shown to be important for CNS development. For example, TNF and IL-1 β were detected early in embryonic neocortical development in sheep (Dziegielewska et al., 2000), leading to the hypothesis, based on the timing of the expression, that these molecules contributed to synaptogenesis. An *in vitro* study using cells from the cerebral hemisphere of E18 rats has shown that incubation with cytokines from the IL-6 family of ligands increased the number of astrocytes and oligodendrocytes (Adachi et al., 2005). Finally, IL-6 knockout mice (12 weeks-old) were shown to have fewer proliferating neural progenitors cells in the hippocampal dentate gyrus and subventricular zone than their wild-type littermates (Bowen et al., 2011).

Chemokines are important chemotactic ligands for cell migration during development, homeostasis and inflammation by binding to their corresponding cell-surface receptors, which are members of the G protein-coupled receptor family. There are also other chemokine receptors that do not transduce the chemokine signalling as they lack an intracellular motif essential for signalling through Gi-type G protein. These receptors appear to have an important role in controlling chemokine gradients by scavenging and inducing transcytosis of chemokines (reviewed in Griffith et al., 2014 and Sokol and Luster, 2015). Chemokines are known to control the development and migration of immune cells during adult haematopoiesis. For example, CXCL-12 and its receptor CXCR4 are thought to be involved in the retention of neutrophils in the marrow and their release to circulation. The current hypothesis is that the retention of

immature neutrophils in the bone marrow is dependent on CXCR-4 signalling and, as these cells mature the expression of CXCR-4 on their surface decreased allowing them to leave the bone marrow (Suratt et al., 2004). In addition, it seems that the expression of CXCR-2, receptor for CXCL-1 to 8, on neutrophils is important for neutrophil mobilization from the bone marrow to the circulation (Eash et al., 2010). In the brain, chemokines have been implicated in many CNS processes and development (reviewed in Bilbo and Schwarz, 2012 and Rostène et al., 2011). For example, mice deficient for CXCL-12 or CXCR-4 at E18.5 were shown to have a disorganised cerebellum with a distorted architecture (Ma et al., 1998). Another study has demonstrated that CXCR-4 is expressed in interneuron precursors migrating from the basal forebrain to the neocortex during rat embryonic development and that CXCL-12 acted as chemoattractant for isolated striatal precursors from mice at E14.5 (Stumm et al., 2003). In addition, CXCL-9, CCL-2 and CCL-21 were shown to promote neuronal differentiation while CXCL-1, CXCL-9 and CCL-2 induced oligodendrocyte differentiation in a neurosphere cell culture generated from cells isolated from the subventricular zone of 6-8 week old C57BL/6 mice (Turbic et al., 2011). These results highlight the importance of chemokines in developmental processes and, therefore, any disturbance in chemokine levels from the induction of an inflammatory response may impact development and, consequently, lead to brain dysfunction. In adulthood, excessive expression of many chemokines and their receptors has been associated with the pathogenesis of neurological diseases, such as multiple sclerosis, Alzheimer's disease and stroke (reviewed in Cartier et al., 2005 and Veenstra and Ransohoff, 2012). The main role of chemokines during inflammation is to recruit immune cells. In the brain, it seems that CXCL-1 to 8 are important for the recruitment of neutrophils, which express the receptor CXCR-2. Direct administration of CXCL-1 or CXCL-2 in the striatum of mice at postnatal day (P) 21 was shown to promote

neutrophil recruitment 12 hours after the injection of the chemokines (Anthony et al., 1998). Injection of recombinant human adenovirus with rat CXCL-2 gene insertion in the dorsal hippocampus of adult mice was also shown to induced an extensive recruitment of polymorphonuclear leukocytes after 2 days. These cells were still present around the injury site after 4 and 7 days (Bell et al., 1996). Moreover, the recruitment of neutrophils to the brain was reduced in a CXCR-2 deficient mice 7 days after closed head injury when compared to wild-type animals. The decreased number of neutrophils in the injury site was correlated with reduce tissue damage and neuronal loss (Semple et al., 2010).

Another group of molecules that have been found to be very important in the immune response in the brain to injury is the group of cell adhesion molecules. They are proteins located on the cell surface and promote cell-cell or cell-extracellular matrix interactions. They comprise immunoglobulin superfamily of cell adhesion molecules (IgSF-CAMs), cadherins, integrins and selectins. Cell adhesion molecules are important for tissue formation and regeneration, and for immune response (reviewed in Horwitz, 2012). In the brain, integrins, IgSF-CAMs, such as neuronal adhesion molecule, and cadherins are important for brain development and function (reviewed in Sakisaka and Takai, 2005; Stoeckli, 2004 and Togashi et al., 2009), and the interaction between IgSF-CAMs, including intracellular adhesion molecule (ICAM)-1 and ICAM-2, integrins and selectins is essential for leukocyte recruitment during inflammation. The important role of adhesion molecules during the immune response in the brain will be discussed throughout this introduction.

As described above, cytokines, chemokines and cell adhesion molecules have been implicated in many developmental processes in the brain, which support their essential role in maintaining the homeostasis of the cerebral environment. Here it is hypothesized that any changes in the levels of cytokines, chemokines and cell adhesion molecules

during development may affect brain functioning and, consequently, lead to pathology. Therefore, it is important to characterize the brain immune environment regarding these important molecular mediators and to find how inflammation may change them, and consequently, lead to brain pathology during development. In addition, it is necessary to discover the effects of individual cytokines and cell adhesion molecules on the brain during development in order to find possible target-specific therapies for neurodevelopmental disorders.

1.1.3 Neutrophils

Neutrophils are considered important contributors to the pathogenesis of acute inflammation. These cells are polymorphonuclear cells with a short half-life (8-12 hours in mice) in circulation (Basu et al., 2002; Lord et al., 1991; Pillay et al., 2010) and migrate to the injury site in order to eliminate pathogens and resolve inflammation. Kolaczowska and Kubes 2013 have published a thorough review focused on neutrophil recruitment to different tissues. In the brain, from where neutrophils are normally excluded, many of the molecules involved in their recruitment are still unknown. This process involves a complex interaction between neutrophils and vessels' endothelial cells from the BBB. After being stimulated by different inflammatory mediators from inside the tissue, such as cytokines, or by direct interaction with pathogens, the endothelial cells increase expression of E-selectin and P-selectin on their surface (Petri et al., 2008). Consequently, these adhesion molecules bind to their glycosylated ligands present on neutrophils and lead to their tethering/capture and, consequently, to their rolling along the vessel (Zarbock et al., 2011). After, neutrophils need to be activated in order to completely adhere to the endothelium and this can be achieved by direct activation by pro-inflammatory cytokines, including IL-1 β and TNF, or by contact with the activated endothelial cells (Summers et al., 2010). Chemokines CXCL-1, CXCL-2 and CXCL-5

are also very important in promoting neutrophil activation through CXCR-2. The activation of G-protein-coupled chemokine receptors on neutrophils alters the conformation of integrins on the cell surface, which results in an increased affinity of these molecules to their cell adhesion molecule ligands on the endothelium surface. Thus, a firm adhesion occurs by interaction between integrins to adhesion molecules ICAM-1 and ICAM-2 on endothelial cells and, consequently, neutrophils start to crawl on the endothelium in order to find a permissive site for their migration. This last process is mediated by integrins on neutrophils that bind to ICAM-1 on endothelial cells (Phillipson et al., 2006). Neutrophil transmigration usually occurs at cell-to-cell junctions (paracellular) and involves integrins on neutrophils and, adhesion molecules and junctional proteins on endothelial cells (Petri et al., 2011; Phillipson and Kubes, 2011; Reymond et al., 2004). Neutrophils may also transmigrate through endothelial cells (transcellular) (Carman and Springer, 2004). Reorganization of the endothelial actin-cytoskeleton and rearrangement of endothelial cells attachment to basement membrane occurs during transmigration (Parsons et al., 2012). After migrating through the endothelial cells, neutrophils need to cross the basement membrane and pericytes. Once inside the tissue, neutrophils may follow different chemoattractants and release many inflammatory mediators, including cytokines, chemokines, vascular endothelial growth factors, proteases (mainly matrix metalloproteinase (MMP)-9) and oxidants (ROS) in order to resolve the injury (reviewed in Kolaczowska and Kubes, 2013).

In many situations, neutrophils may accumulate in the tissue and, consequently, induce damage through excessive and prolonged production of the mediators described above. Neutrophil recruitment to the brain and subsequent exacerbation of injury have been found in rodent models of CNS pathologies, such as traumatic brain injury (Kenne et al., 2012; Semple et al., 2010), Alzheimer's disease (Zenaro et al., 2015) and ischemic stroke

(Jin et al., 2010). Blond et al. (2002) have shown that direct injection of IL-1 β or TNF in the striatum of adult rats promoted marked neutrophil recruitment to the brain 24 hours after the injections of the cytokines. The same authors observed that excitotoxic lesion in the brain, induced by intracerebral (i.c.) injection of N-Methyl-D-aspartic acid (NMDA), was also able to cause neutrophil recruitment, which was observed together with neuronal death after 24 hours (Blond et al., 2002). In neonate rats (P7), neutrophil depletion before hypoxic-ischemic brain injury was shown to reduce brain swelling at 42 hours of recovery (Palmer et al., 2004). Injection of IL-1 β in the striatum of P21 rats was also shown to induce (4 hours after) neutrophil recruitment to the brain and increase vessel permeability, which was proven to be neutrophil-dependent (Anthony et al., 1997). This injection was also able to increase the expression of CXCL-1 and CXCL-2 in the brain. The injection of CXCL-1 in the striatum induced similar results, and the co-injection of IL-1 β and anti-CXCL-1 in the brain was able to inhibit vessel/BBB permeability and neutrophil recruitment, showing that IL-1 β -induced phenotype in the brain results from upregulation of chemokines (Anthony et al., 1998). Anthony et al. (1997) have shown that neutrophils are responsible for the increased BBB permeability observed after inflammation in the brain and this may be, in part, due to the fact that neutrophils can produce ROS. ROS can affect BBB integrity by activation of MMPs, oxidative damage to cellular molecules, modulation of tight junction proteins, cytoskeletal reorganization, and induction of inflammatory mediators. In addition, ROS may also increase neutrophil recruitment by promoting the expression of adhesion molecules on endothelial cells of the BBB (reviewed in Kunsch and Medford, 1999 and Pun et al., 2009). Neutrophils can release MMP-9 that can digest the basement membrane and/or extracellular matrix and contribute to the increased vessel permeability and disruption (Justicia et al., 2003; Rosell et al., 2008; Turner and Sharp, 2016). For example, Bauer et al. (2010) have shown that

inhibition of MMP-9 on an adult mouse model of hypoxia-induced vascular leakage reduced disorganization of tight junction proteins and vascular permeability. In human cases of fatal ischemic stroke with haemorrhagic complications, an excessive accumulation of MMP-9⁺ neutrophils surrounding brain microvessels associated with degradation of basement membrane (type IV collagen) and blood extravasation was observed (Rosell et al., 2008). Moreover, Moxon-Emre and Schlichter (2011) have shown that the majority of MMP-9⁺ cells in rat brains after i.c. haemorrhage were neutrophils and that depletion of neutrophils before i.c. haemorrhage decreased the expression of MMP-9 and blood-brain barrier permeability to endogenous immunoglobulin G (IgG) and, consequently, axonal injury. These results indicate that MMP-9⁺ neutrophils are major contributors to blood-brain barrier disruption. However, it is important to take into account the existence of many neutrophil subtypes that may contribute to injury in various ways (reviewed in Beyrau et al., 2012; de Oliveira et al., 2016 and Silvestre-Roig et al., 2016). For example, a subset of neutrophils expressing MMP-9, CXCR4 and vascular endothelial growth factor receptor 1 were shown to be recruited to hypoxic areas upon ischemia and, consequently, to promote vessel formation (Christoffersson et al., 2012; Massena et al., 2016). In cancer, two subsets of neutrophils have been found: pro-tumorigenic and immunosuppressive that express CXCR4, vascular endothelial growth factor and MMP-9 and, anti-tumorigenic and pro-inflammatory that express pro-inflammatory cytokines and chemokines and, are able to kill cancer cells (Fridlender et al., 2009). Finally, two subtypes of neutrophils were observed in a mouse model of methicillin-resistant *Staphylococcus aureus* infection when compared to non-infected mice: the first subtype isolated from methicillin-resistant *Staphylococcus aureus*-resistant mice and the second subtype isolated from methicillin-resistant *Staphylococcus aureus*-sensitive mice were shown to activate macrophages

toward a pro-inflammatory or anti-inflammatory phenotypes, respectively (Tsuda et al., 2004).

It is evident from published data that neutrophils play an important role in the acute response in the brain to a range of injuries in adults and neonates. Furthermore, the interplay between cytokines, chemokines, adhesion molecules and neutrophils seem to be an important step in the immune response in the brain. Thus, it is important to further investigate the role of these molecular and cellular mediators in inflammation during development.

1.1.4 Microglia and perivascular macrophages

The brain hosts a number of different myeloid populations, including parenchymal microglia, and perivascular, meningeal and choroid plexus macrophages. The function of myeloid cells in the brain is essential for both the regulation of the normal CNS milieu, as well as the response to injury (reviewed in Prinz and Priller, 2014; Prinz et al., 2011; Ransohoff and Cardona, 2010).

The origins of microglia have been studied and discussed for a long time. Early studies showed that all myeloid cells derive from the same progenitors during embryonic development and from bone marrow postnatally (Bertrand et al., 2005; Cumano and Godin, 2007). Monocytes are commonly transported in the peripheral circulation from the bone marrow to tissues, where they become tissue specific macrophages. However, recent studies now suggest that microglia, in contrast to other tissue-resident macrophages, derive from HSCs present in the yolk sac at E7.5 (Ginhoux et al., 2010; Kierdorf et al., 2013; Schulz et al., 2012). After entering the brain during embryonic development, these microglia proliferate to sustain and expand the microglial population throughout life (Ajami et al., 2007; Alliot et al., 1999) rather than by recruitment of peripheral myeloid cells (Ginhoux et al., 2010; Schulz et al., 2012). Microglia have also

been shown to support CNS development and function (Bilimoria and Stevens, 2014; Gomez-Nicola and Perry, 2015; Harry, 2013), which discriminate them from other tissue macrophages. As part of their many roles, microglia have been shown to ingest excess neuronal debris (Hosmane et al., 2012; Nielsen et al., 2009; Sierra et al., 2010), control neuron survival and proliferation (Chamak et al., 1994; Morgan et al., 2004; Nagata et al., 1993; Ueno et al., 2013), regulate synapse number through synapse pruning (Paolicelli et al., 2011; Schafer et al., 2012) and modulate synaptic function and maturation (Paolicelli et al., 2011; Zhan et al., 2014). Finally, microglia can also be distinguished from other tissue macrophages by their unique pattern of gene expression, which has been recently described in transcriptomic studies. Gautier et al., 2012, Hickman et al., 2013 and Butovsky et al., 2014 have shown that microglia in mice specifically express genes such as CX3CR1, integrin beta-5, solute carrier (Slc) family 2 member 5 and G protein-coupled receptor 34 when compared to macrophages from the peritoneum and spleen, highlighting the specialist role of microglia in the brain, above and beyond their macrophage-like functions.

Microglia are the resident innate immune cells in the CNS and, as other macrophages, they may perform phagocytosis, produce inflammatory mediators and act as antigen presenting cells (reviewed in Almolda et al., 2011; Greter et al., 2015; Hanisch and Kettenmann, 2007). They use their highly motile processes to monitor the CNS environment (Davalos et al., 2005; Nimmerjahn et al., 2005). Once they sense any disturbance, microglia rapidly migrate to the injury site in order to acutely respond and, consequently, resolve the injury by removing debris and restoring damaged tissue; however, if microglia activation is not properly controlled brain injury may expand and lead to long-term neuronal pathology (reviewed in Hu et al., 2014). This is achieved because microglia can produce ROS (Colton and Gilbert, 1987) and many pro-

inflammatory cytokines (Lee et al., 1993). Therefore, microglia have been found to have both beneficial and harmful effects in response to a stimulus. In order to minimise the extremes of these responses, microglial activity has to be very well controlled, which appears to be achieved by a tightly regulated balance between a range of microglial receptors, including receptors from the immunoglobulin superfamily, chemokine receptors, purinergic receptors, PRRs and, phosphatidylserine receptors and bridging proteins, that sense different environmental cues (reviewed in Garden and Möller, 2006; Hanisch, 2002; Harry, 2013 and Hu et al., 2014). These receptors can be characterised as "Off", which maintain microglia in their quiescence state and/or "On", which activate microglia during pathological conditions, and recognise ligands that are secreted or are present on the surface of other CNS cells. Cluster of differentiation (CD) 200 receptor or OX2 receptor, which belong to the immunoglobulin superfamily, is an example of an "Off" receptor. It is specifically expressed in macrophages/microglia and recognises CD200 on the surface of other cells and, consequently, maintains microglia in their "resting" state (Preston et al., 1997; Wright et al., 2000). In brains of CD200 deficient mice, microglia spontaneously present a more activated state, characterised by a more amoeboid morphology, when compared to wild-type mice, where microglia have many long processes. Furthermore, induction of experimental autoimmune encephalomyelitis (EAE), which is an animal model of multiple sclerosis in humans, in CD200^{-/-} mice resulted in an earlier disease onset when compared to CD200^{+/+} mice (Hoek et al., 2000). In addition, administration of an antibody against CD200 receptor after the induction of EAE in rats resulted in a more severe EAE with higher clinical disease score than control animals (Wright et al., 2000). It is evident from these studies that increased activation of microglia resulting from unbalanced expression of receptors exacerbates neurological injury. Another receptor specifically expressed in microglia is the CX3C receptor

(CX3CR)-1 or fractalkine receptor that recognises the chemokine CX3CL-1 that can be secreted or recognised at cell surface level. CX3CL-1 is mainly expressed by neurons and, therefore, plays an important role in neuron-microglial crosstalk (Harrison et al., 1998; Meucci et al., 1998; Nishiyori et al., 1998) and argued to maintain the microglia phenotype. A previous study have shown that in a toxic model of Parkinson's disease CX3CR^{-/-} mice presented more neuronal loss in the substance nigra pars compacta when compared to wild-type mice (Cardona et al., 2006). Cho et al., (2011) have also shown that CX3CL-1 levels in the brain were lower in Alzheimer's disease patients when compared to age-matched controls and that deletion of CX3CR in a mice model of Alzheimer's disease exacerbated microglial activation, increased Tau phosphorylation and memory deficits. However, in a model of focal cerebral ischemia, brains from CX3CR^{-/-} adult mice were shown to have smaller infarcts, less BBB permeability, and fewer apoptotic cells and leukocytes infiltrates than brains from wild-type animals (Dénes et al., 2008). All these previous studies suggest that CX3CR role in CNS injury may vary with the type of disorder. Many other chemokine receptors, such as CCR2, CCR5 and CXCR4, are expressed by microglia and are responsible for microglial activation, including cell migration to the injury site and production of pro-inflammatory cytokines (reviewed in Hu et al., 2014). Regardless their receptors, the activation of microglia have been correlated and/or implicated in many neurological disorders, including Alzheimer's disease, Amyotrophic Lateral Sclerosis, Parkinson's disease and Huntington's disease (reviewed in Block et al., 2007; Gomez-Nicola and Perry, 2015; Prinz et al., 2011). Microglia also seem to play a role in disease progression in neurological disorders with a developmental origin. Published data supporting this hypothesis, along with the contribution of other immune mediators to the development

of disorders such as autism, schizophrenia and cerebral palsy, is discussed in the next section.

Perivascular cells or macrophages are located in the perivascular space in the CNS and they perform immune surveillance mainly by acting as antigen presenting cells (Graeber et al., 1992; Hickey and Kimura, 1988). These cells are also able to sense neuronal injury and death, ingest CNS antigens and sense alterations in the periphery, such as cytokines and endotoxin levels. The ability to receive signals from both sides, periphery and CNS, make these cells an important player in the interaction between the CNS and peripheral immune system (reviewed in Williams et al., 2001). Perivascular macrophages are bone marrow derived and were shown to be continuously replaced in adult rats by blood-borne monocytes in physiological conditions (Bechmann et al., 2001). Perivascular macrophages were shown to decrease amyloid burden in models of Alzheimer's disease (Hawkes and McLaurin, 2009; Mildner et al., 2011). However, more perivascular macrophages were found in brains from multiple sclerosis patients when compared to control brains (Zhang et al., 2011). These contradictory results suggest that the role of perivascular macrophages may vary with the type of injury. Evidence of the presence of perivascular macrophages and their contribution to brain function and injury during development are lacking.

1.2 Neurodevelopmental disorders and inflammation

1.2.1 General aspects of neurodevelopmental disorders

Neurodevelopmental disorders are a group of conditions that result from disturbances during CNS development and are characterized by impairments in motor function, learning, social behaviour, cognition or/and language.

1.2.1.1 Autism and schizophrenia

Autism spectrum disorder, commonly named autism, is an early onset (<3 years, (Owen et al., 2016)) neuropsychiatric disorder that is characterised by deficits in social interaction and communication, and repetitive and restrictive behaviour, interests or activities that can be accompanied by defects in language and intellectual impairment. This disorder has a worldwide prevalence of 7:1000 (Lai et al., 2014). There is no unique cause for autism, but a multifactorial aetiology is proposed (Figure 1.2). Genetic background and environmental factors, such as sex and infections during development, may play a role (reviewed in Bourgeron, 2015; Constantino and Charman, 2015 and Watts, 2008). Such factors are thought to disrupt normal brain development and, consequently, generate region-specific functional changes. However, despite this widely held view, it remains unclear what specific brain regions and neural mechanisms are involved in autism. Brain dysfunction may arise from disturbed neural connectivity and growth, changes in neural migration, unbalanced excitatory-inhibitory networks, abnormal dendritic morphology, neuroimmune changes, calcium signalling defects and neuronal loss (reviewed in Bourgeron, 2015; Donovan and Basson, 2016; Waterhouse and Gillberg, 2014 and Watts, 2008). However, once again there is remarkably little evidence for many of these claims.

Schizophrenia is a heterogeneous neuropsychiatric disorder with an age of onset of 16-30 years (Owen et al., 2016) and a prevalence of 5:1000 (McGrath et al., 2008). It manifests as one or more behavioural abnormalities, including delusions, hallucinations, impaired motivation, reduction in spontaneous speech and social withdrawal. This can be accompanied by cognitive impairment (Owen et al., 2016). Schizophrenia, as autism, has a multifactorial aetiology (Figure 1.2) where genetic background and environmental factors during development are responsible for the different phenotypes found in

schizophrenia patients (reviewed in Siever and Davis, 2004). Anatomical or functional abnormalities in the brain can include changes in neurotransmission, decreases in inhibitory neurons and reductions in grey matter (reviewed in Bakhshi and Chance, 2015; Haijma et al., 2013; Lewis, 2012 and Owen et al., 2016).

Autism and schizophrenia have been shown to overlap at many levels. Both disorders share similar impairments in neuro- and social-cognition functioning (Eack et al., 2013) while intellectual deficits are more common in autism (Christensen et al., 2016; Cooper, 1997; Morgan et al., 2008). These two neurodevelopmental disorders also share many genetic mechanisms and environments risks, including prenatal infection, gestational diabetes, paternal age and mother nutrition (Chisholm et al., 2015; Meyer et al., 2011). Regarding brain anatomy and function, both disorders show volume loss of grey matter (Cheung et al., 2010) and changes in neurotransmission, such as glutamatergic and dopaminergic dysfunction (reviewed in Bourgeron, 2015; Chisholm et al., 2015 and Owen et al., 2016).

1.2.1.2 Cerebral Palsy (and preterm damage)

Cerebral palsy is a neurodevelopmental disorder very distinct from autism and schizophrenia that has an prevalence of 2:1000 live births (Oskoui et al., 2013) and an age of onset of around 18 months (Rosenbaum et al., 2007). It is characterised by a range of phenotypes of motor impairment that may be accompanied by cognitive and visual deficits (Pakula et al., 2009). There are subtypes of cerebral palsy, which have different levels of disability, that are normally classified according to topographical distribution. They may result from diverse insults in different areas of the CNS during embryonic development, at birth or postnatal period (Figure 1.2). Risk factors include preterm birth, genetic background, infections/inflammation, asphyxia/hypoxia (mainly during birth), toxic factors, maternal factors and sex. Regarding brain anatomy and function, cerebral

palsy patient can present different lesions, such as infarcts and white matter injuries (including periventricular leukomalacia: injury to the white matter surrounding the ventricles of the brain). These may then lead to cell death/loss and neuronal impairment, which in turn can lead to movement dysfunction when they occur in different areas involved in motor function (including cortex, cerebellum, basal ganglia and thalamus) (reviewed in Marret et al., 2013).

As stated above, preterm birth is one of the risk factors of cerebral palsy; however, it is also considered a multifactorial syndrome leading to many clinical complications. It is estimated that approximately 15 million babies (more than 1 in 10 babies) are born preterm (before 37 completed weeks of gestation; 37 to <42 weeks = term), representing 11.1% of all livebirths worldwide, and that this number is increasing. In addition, preterm complications are considered the leading cause of death in children under 5 years old, being responsible for almost 1 million deaths each year (Blencowe et al., 2012; World Health Organization: fact sheet n°363, November 2015). Furthermore, a large percentage of the survivors of preterm birth may suffer long-term neurological impairments (Marlow et al., 2005). Preterm birth can be classified into those that happen spontaneous/naturally or those that result from medical order (caesarean section or labour induction). Approximately 70% of preterm births in high-income countries are spontaneous preterm and represent the syndrome that is responsible for diverse pathologies. Spontaneous preterm results from interaction of many factors that induce the activation of maternal and foetal tissues, which promotes the production of mediators, including prostaglandins and cytokines and metalloproteinases. Consequently, these mediators induce myometrial contractions and/or rupture of foetal membranes. Risk factors include inflammation/infection, haemorrhage, uterine distension, immune dysregulation, stress and cervical insufficiency and many other genetic, biological, behavioural, social, clinical

and environmental events (reviewed in Harrison and Goldenberg, 2016 and Rubens et al., 2014).

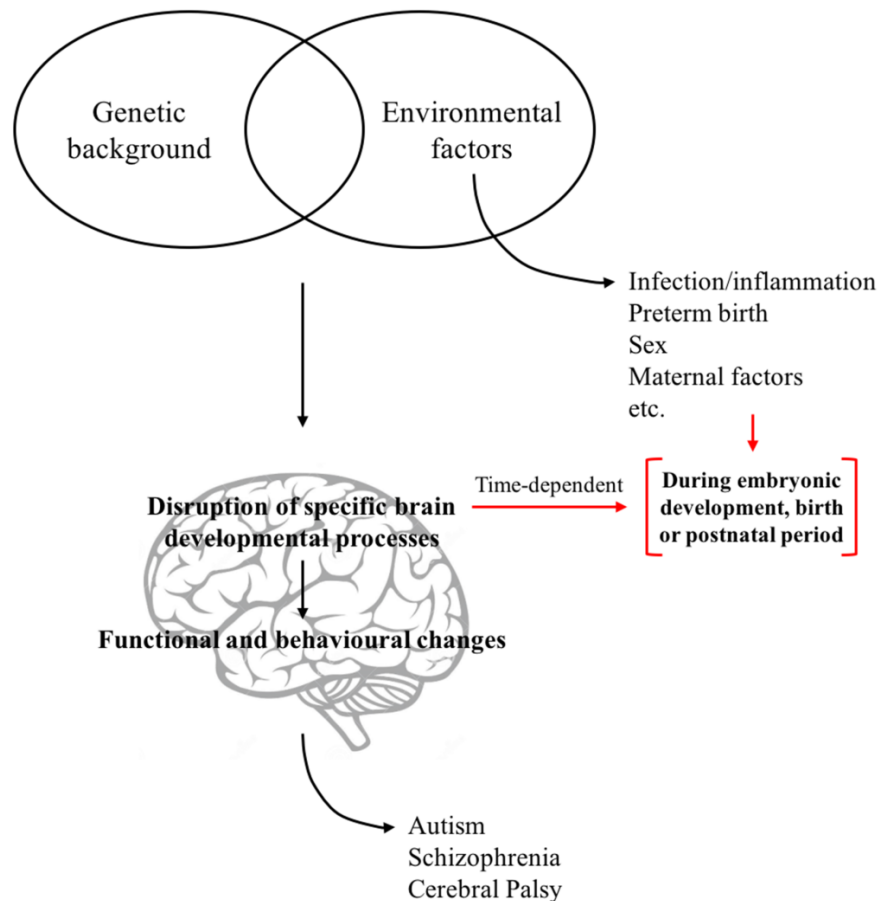


Figure 1.2 Neurodevelopmental disorders and their multifactorial origin. Neurodevelopmental disorders, such as autism, schizophrenia and cerebral palsy, are complex conditions that result from the interaction of a genetic predisposition and environmental factors. These may occur at different time points during development and, consequently, influence specific brain developmental processes that may lead to deficits in function and behaviour.

1.2.2 The role of inflammation in neurodevelopmental disorders

1.2.2.1 Clinical evidence

Many clinical studies have correlated inflammatory processes with neurodevelopmental disorders. Li et al. (2009) have found increased levels of TNF, IL-6 and CXCL-8 in post-mortem frontal cortex of autistic patients when compared to controls. Vargas et al. (2005)

have also observed elevated levels of CCL-2 and TGF- β 1 in the cerebellum and cortex, and of many other inflammatory mediators, such as IL-6, IL-10, CCL-7, in the cortex of post-mortem brains from autistic patients when compared to controls. Astrogliosis and microglial activation was also observed in both regions analysed, but it was more prominent in the cerebellum. The same authors found that levels of CCL-2, CCL-4, CXCL-7, CXCL-10, IL-6 and IFN- γ in the cerebrospinal fluid were higher in autistic than in control patients (Vargas et al., 2005). A later study by Suzuki et al. (2013) have shown a prominent microglial activation in the cerebellum of autistic subjects when compared to controls by using positron emission tomography (PET) and a radiotracer for microglia ([¹¹C](R)-PK11195). It seems that the cerebellum in brains of autistic subjects is one of the main regions where neuroinflammation occurs. This is in concordance with previous observations showing that the cerebellum is one of the foci of pathological abnormalities in autism (Bailey et al., 1998; Courchesne, 1991; Courchesne et al., 1988; Kemper and Bauman, 2002). It is known that cerebellum processes inputs from many areas of the brain and it is thought to play a major role in motor/movement control. However, evidence has suggested that cerebellum may also be important for cognitive function, such executive, visual spatial, and linguistic impairments, and affective dysregulation (Schmahmann and Sherman, 1998; Stoodley and Schmahmann, 2010) and that this may be, in part, due to the connectivity loop existing between cerebellum and prefrontal cortex, which is essential for cognitive function (Watson et al., 2014). Indeed, deficits in cognitive tasks, such as executive control, attention, memory functions and language, in autism have been correlated with cerebellar impairments (reviewed in Fatemi et al., 2012). Thus, immune dysfunction found either in cerebellum and prefrontal cortex (Li et al., 2009; Suzuki et al., 2013; Vargas et al., 2005) may contribute to many anatomical and functional changes on these same brain regions (Donovan and Basson,

2016) and, consequently, to behavioural deficits in autism. In fact, Vargas et al. (2005) not only observed glial activation but also neuron loss in the cerebellum of 9 out of 10 autistic brains. Peripheral immune activation have also been correlated with autism as many cytokines and chemokines, including CCL-2, CCL-5, CCL-11, CXCL-8, IL-1 β and IL-6 were found to be elevated in plasma from patients suffering from autism when compared to control subjects (Ashwood et al., 2011a, 2011b). Many studies have also discovered immune activation in schizophrenia. For example, elevated levels of inflammatory mediators, such as IL-2, IL-6 and CXCL-8, were found in serum of schizophrenic patients when compared to controls (Zhang et al., 2004). Bayer et al. (1999) have shown microglia activation in frontal cortex and hippocampus and Radewicz et al. (2000) have observed microglia activation in temporal and frontal cortex in post-mortem brains of schizophrenic patients when compared to controls. All these studies clearly support that inflammation plays an important role in autism and schizophrenia as high levels cytokines and reactive glia were found in brain areas that are responsible for many cognitive tasks that are normally impaired in these disorders. However, it is important to study and correlate inflammatory mediators with specific developmental processes in the brain in order to discover target-specific therapies and clear connections between brain dysfunction and behavioural impairments in autism and schizophrenia.

Evidence of inflammation has also been found in cerebral palsy cases. Yoon et al. (2000) have studied the relationship between the occurrence of cerebral palsy in 3 years-old children and the presence of funisitis (inflammation of the connective tissue of the umbilical cord), amniotic fluid concentrations of IL-6 and CXCL-8, and amniotic fluid white blood cell count. The authors found that newborns, which were born with ≤ 35 weeks, that later developed cerebral palsy had higher rate of funisitis and were born to mothers with higher levels of IL-6 and CXCL-8 and higher white blood cell counts in the

amniotic fluid than newborns that did not developed cerebral palsy (Yoon et al., 2000). An earlier study observed that preterm newborns (26 to 35 weeks-old) that later developed periventricular white matter (periventricular leukomalacia) lesions were born to mothers with higher concentrations of IL-6, IL-1 β and TNF and IL-1RA in the amniotic fluid than those without white matter lesions. In addition, the authors found that newborns that developed periventricular leukomalacia were delivered at a lower mean gestational age and birth weight, and had a higher rate of significant complications, such as infection-related complications, intraventricular haemorrhage and cerebral palsy, when compared to those that did not have white matter lesion. In fact, all neonates that were diagnosed with cerebral palsy had periventricular white matter lesions (Yoon et al., 1997), which is characteristic of cerebral palsy (Folkerth, 2005). Kadhim et al. (2001) have also shown higher immunoreactivity for IL-1 β and TNF in and around periventricular leukomalacia lesions in brains of neonates that died shortly after birth when compared to brains without any lesion (Kadhim et al., 2001). Later, Kadhim et al. (2003) observed higher immunoreactivity of IL-1 β and TNF in neurons from neocortex and deep grey matter structures, such as thalamus, putamen and hippocampus, of neonates diagnosed with periventricular leukomalacia when compared with neonates without the lesion (Kadhim et al., 2003). This suggest that deficits in cortical and other grey matter regions, which may result from immune activation, may be involved in cerebral palsy as periventricular leukomalacia is the main neuropathology associated to this disorder. In fact, cortical and deep gray structures impairments have been observed in periventricular leukomalacia and cerebral palsy cases (Hoon A.H. et al., 1997; Inder et al., 1999; Marín-Padilla, 1997; Menkes and Curran, 1994). Lesions in cortex and other grey matter regions may explain not only motor (cortex and putamen) but also the

cognitive and visual (cortex, thalamus and hippocampus) impairments observed in many cerebral palsy subjects.

As described above, Yoon et al. (1997) have found that newborns with periventricular white matter lesions were delivered at a lower mean gestational age and had higher rates of significant complications, such as infection-related complications, intraventricular hemorrhage and cerebral palsy, when compared to those that did not have white matter lesions. This data suggests that newborns with low gestational age are more susceptible to many postnatal complications and, consequently, brain injury than newborns with a higher gestational age. Many other studies have correlated postnatal bacterial infections in preterm infants and neurodevelopmental impairments and/or brain injury (reviewed in Strunk et al., 2014). Furthermore, Kuban et al. (2015) have demonstrated that increased levels and number of inflammatory proteins in the blood of preterm infants (<28 weeks) during the first two weeks after birth were positively associated with increasing risk of disorders of brain function and structure at 2 years of age. Later, Kuban et al. (2017) positively correlated increased levels of inflammatory mediators, including CXCL-8 and ICAM-1, in the blood of preterm children (<28 weeks) during the first postnatal month with cognitive deficits at 10 years of age. It is evident that preterm babies are at higher risk of brain injury, which may result from immune activation. It is therefore important to study the effects of inflammation during the intensive care period in order to specifically discover how and when the brain of preterm infants is susceptible to brain injury.

1.2.2.2 Animal models for studying inflammation during development

Many rodent models have been developed in order to study the effects of infection/inflammation on brain development and behaviour. It is widely accepted that the third trimester in humans are roughly equivalent to P0-P10 in rats, which have a total

gestation time of 21 days, and mice, which have a total gestation time of 18.5 days, and that the entire gestation period in rats and mouse is approximately equivalent to the first two trimesters in humans (Clancy et al., 2007; Workman et al., 2013). Moreover, it is described that there are specific "windows of susceptibility" to the development of autism, schizophrenia and cerebral palsy. A first epidemiological study has shown an positive association between schizophrenia and influenza virus infection during the second trimester during the 1957 influenza A2 epidemic in Finland (Mednick et al., 1988). Later, Brown et al. (2004) reported an increased risk of schizophrenia in the offspring of mothers infected with influenza virus during the first trimester but not during the second or third trimester. The differences between these two studies may rely on the fact that the first one defined timing of exposure to the virus by the dates of the peak periods of influenza epidemics and the second used quantification of influenza antibody levels in the serum of each pregnancy, which is more accurate. In another epidemiologic study, admission to hospital due to viral or bacterial infection in the first or second trimester, respectively, was positively associated with autism spectrum disorder in the offspring (Atladóttir et al., 2010). While schizophrenia and autism were both correlated with infection during the first and second trimester, cerebral palsy was shown to result from different types of infection/inflammation occurring during late gestation, birth and neonatal period (reviewed in Dubowitz et al., 1985; Hermansen and Hermansen, 2006; Nelson, 2008; Nelson and Willoughby, 2000 and Reddihough and Collins, 2003). Many authors have taken this information into account in order to study the effects of infection/inflammation on neuropathology and behaviour in rodents at specific time points relevant for the different developmental disorders.

Different immunogens have been used in animal models for immune activation during development. Lipopolysaccharide (LPS) (component of Gram negative bacteria wall) has

been used to mimic bacterial infection while polyinosinic:polycytidylic acid (poly(I:C)) has been used to mimic viral infection. Both induce a signalling cascade, through toll-like receptors, that induce the transcription of inflammatory mediators, including anti- and pro- inflammatory cytokines, chemokines and complement proteins. Virus, such as influenza, are also used for prenatal immune activation. In addition, direct administration of cytokines during embryonic or postnatal periods have also been used in order to study the effects of specific component of the immune system on neurodevelopment (reviewed in Boksa, 2010 and Harvey and Boksa, 2012). Behaviour and brain pathology, including molecular, neurochemical and morphologic changes are the main outcomes measured in these models.

At behavioural level, prepulse inhibition is one of the most frequently measured outcomes in animal models as it is a core characteristic of schizophrenia patients (Kumari et al., 2000) and it is also observed in autism (Perry et al., 2007). Moreover, it can be assessed in rodents and human using similar paradigms (Harvey and Boksa, 2012). Prepulse inhibition of the startle reflex response is the capacity of a weak prestimulus to inhibit the response to a subsequent strong sensory stimulus and it is used to study deficits in attention and information processing (sensomotor gating) (Kumari et al., 2000; Perry et al., 2007). Deficits in prepulse inhibition have been observed in rodent models of maternal immune activation by prenatal administration of poly(I:C) (Howland et al., 2012; Meyer et al., 2008; Wolff and Bilkey, 2008), LPS (Fortier et al., 2007; Romero et al., 2010; Wischhof et al., 2014) and IL-6 (Smith et al., 2007) (Table 1.1). Delivery of influenza virus by intra-nasal infusion in pregnant mice at around E9.5 not only caused deficits in prepulse inhibition in the adult offspring but also deficits in social interaction and exploratory behaviour, which can be observed in autism and schizophrenia patients (Shi et al., 2003). Prenatal administration of poly(I:C) in mice at E17 (Meyer et al., 2006)

or from E12 to 17 (Ozawa et al., 2006), LPS in mice at E8 (Coyle et al., 2009) and IL-6 in rats at pregnancy days 16, 18 and 20 (Samuelsson et al., 2006) has also been shown to alter diverse learning and memory tasks that indicated decreased spatial exploration and perseverative behaviour (Meyer et al., 2006) and, deficits in recognition memory (Coyle et al., 2009; Ozawa et al., 2006) and spatial memory (Samuelsson et al., 2006) in the adult offspring. Another study has shown that intraperitoneal (i.p.) injection of poly(I:C) at E10.5, 12.5 and 14.5 decreased social interaction and increased repetitive/stereotyped behaviour in adult mice, which are the two principal symptoms of autism spectrum disorder (Malkova et al., 2012). Also, i.p. injections of LPS from E17 until the end of gestation induced motor dysfunction, which is characteristic of cerebral palsy disorder, in adult rat offspring (Girard et al., 2009).

Table 1.1 Immune activation impairs prepulse inhibition.

Immunogen	Species	Injection route	Time of administration	Reference
Poly(I:C)	Rat	i.v.	E15	Wolff and Bilkey 2008
	Mouse	i.v.	E9	Meyer et al. 2008
	Rat	i.v.	E15	Howland et al. 2012
LPS	Rat	i.p.	E15 and 18	Fortier et al. 2007
	Rat	s.c.	From E1 to 21	Romero et al. 2010
	Rat	i.p.	E15 and 16	Wischhof et al. 2014
IL-6	Mouse	i.p.	E12.5	Smith et al. 2007
Influenza virus	Mice	intra-nasal	E9.5	Shi et al. 2003

Details of published studies showing that immune activation during embryonic development decreases prepulse inhibition in the offspring are shown in this table. i.v.: intravenous, i.p.: intraperitoneal, s.c.: subcutaneous, E: embryonic day.

Much work has been done to identify the morphological, molecular and neurochemical impairments in the brain that underlie the behavioural deficits characteristic of

schizophrenia, autism and cerebral palsy. Animal models of immune activation, either during embryonic development or postnatally, have shown many neuropathologies similar to those observed in autism, schizophrenia and cerebral palsy. These include cerebellar abnormalities (Shi et al., 2009), altered dopaminergic system (Fortier et al., 2004; Meyer et al., 2008), and demyelination and white matter injury (Bell and Hallenbeck, 2002; Paintlia et al., 2004; Pang et al., 2003). Most importantly, studies have shown the direct relationship between brain dysfunction and behavioural impairments in rodent models (Table 1.2). Wischhof et al. (2014) have shown that i.p. injection of LPS at E15 and 16 in rats reduced prepulse inhibition and myelination in the orbitofrontal cortex and basolateral amygdala (both areas important for making predictions to guide behaviour) (Sharpe and Schoenbaum, 2016)), and affected GABAergic system (decreased calcium-binding protein Parvalbumin) in the prefrontal cortex and hippocampus in adult offspring. Impairments in GABAergic system are involved in the neuropathology of schizophrenia and they are likely to contribute to cognitive deficits when affecting prefrontal cortex and hippocampus (Wischhof et al., 2014). Meyer et al. (2008) have shown that intravenous (i.v.) injection of poly(I:C) in mice at E9 impaired prepulse inhibition and the dopaminergic system (reduced prefrontal dopamine D1 receptors) in adult offspring, which is common in autism and schizophrenia. Injections (i.p.) of poly(I:C) in mice from E12 to E17 were also shown to induce deficits in cognitive impairment (recognition memory), decrease prepulse inhibition and alter the dopaminergic system (increased dopamine turnover and decreased receptor binding of D₂-like receptors in the striatum, which is important for motor learning/modulation and reward-guided behaviours (Cerovic et al., 2013)) in adult offspring (Ozawa et al., 2006). It is clear from these studies that immune activation early in development induces brain pathology in specific areas of the brain that may explain certain behaviour deficits in

rodents. However, it is essential to discover the pathways that link immune activation and these impairments. As discussed above, human data have suggested that inflammation may contribute for brain dysfunction and, consequently, to behaviour changes observed in neurodevelopmental disorders. Many studies using rodent models have been developed in order to mimic the human condition regarding inflammation and brain/behaviour in neurodevelopmental disorders, and to discover possible links between inflammatory mediators and specific brain/behaviour deficits. As in humans, increased levels of inflammatory mediators have been found in rodent models of immune activation during development. Most of these studies have focused on embryonic development in rodents, which are clinically relevant for autism and schizophrenia. Mice that received i.p. injection of LPS at E15 were shown to have increased levels of IL-1 α , TNF and IL-6 in serum and of IL-1 α and IL-6 in the amniotic fluid a few hours after injection (Fidel et al., 1994). LPS (2.5mg/kg) injection (i.p.) at E16 also increased levels of TNF in the amniotic fluid, and of IL-6 and TNF in the placenta but decreased TNF in the rat foetal whole brain 2 hours after LPS administration (Urakubo et al., 2001). Cai et al. (2000) have studied the expression of TNF and IL-1 β in the foetal rat brain after i.p. injection of LPS (4mg/kg) at E18 and observed that these cytokines were upregulated in whole brain 1 hour after LPS administration when compared to saline-controls. In contrast to Cai et al. (2000), Gayle et al. (2004) have demonstrated that i.p. injection of LPS (0.1mg/kg) at E18 increased levels of cytokines in maternal serum (TNF, IL-1 β , IL-6 and IL-10) and amniotic fluid (TNF, IL-1 β and IL-6) within 6 hours, but did not induce the expression of the same cytokines in rat foetal whole brain. A later study supported the observation by Gayle et al. (2004), as i.p. injection of LPS (0.05mg/kg) at E18 increased levels of TNF, IL-1 β and IL-6 in maternal plasma and placenta within 8 hours, induced levels of IL-1 β in rat foetal plasma 4 hours after LPS administration but did not change protein

levels of any cytokine in the foetal whole brain at any time point studied (Ashdown et al., 2006). The differences between these three studies may rely on the fact that Cai et al. (2000) used higher concentrations of LPS than the two later studies. Many other studies have shown that poly(I:C) i.p or i.v. injections at different embryonic days (E9, E16 or E18) were shown to increase cytokines and chemokines in the foetal brain of mice and rats (reviewed in Arrode-Brusés et al., 2012 and Patterson, 2009). Although most of the published data are focused on embryonic development, and are, therefore, correlated with autism and schizophrenia, there are also few other studies where immune activation is induced in newborn rodents, being clinically relevant for cerebral palsy and preterm newborns, which are very susceptible to infections/inflammation. Arrode-Brusés et al. (2012) have observed that i.p. injection of Poly(I:C) in P4 mice increased many inflammatory mediators, including IL-1 β , CCL-11, CCL-2, CCL-3, CCL-4, CCL-5, CXCL-10, CXCL-1, CXCL-2, CXCL-5 and CXCL-9 while other cytokines, such as IL-6 and IL-10 decreased in whole brain 24 hours after poly(I:C) exposure. Also, direct injections of LPS in both brain hemispheres of P2 mice promoted increases in the expression of many inflammatory mediators, such as IL-1 β , IL-6, TNF, CCL-2, CXCL-1, CXCL-10 and ICAM-1, in the brain 12 hours after LPS administration (Christensen et al., 2014). All these studies clearly show that immune activation by bacterial or viral components during embryonic development or in newborn rodents changes the levels of pro- or/and anti- inflammatory in maternal and/or foetal/newborn tissues. However, in order to create clinically relevant rodent models to study the role of immune activation in neurodevelopmental disorders it is important to correlate the increased/decreased levels of inflammatory mediators and microglia activation with brain pathology and/or behaviour impairments similar to those observed in humans (Table 1.2). Ribeiro et al. (2013) have observed that daily i.p. injection of poly(I:C) from P5 to P7 in rats promoted

activation of microglia in the hippocampus and striatum, and this was accompanied by deficits in prepulse inhibition. Also, i.p. injection of LPS at E9 in mice promoted activation of microglia throughout the brain at E18 and at birth, impairments in social behaviour in P7 offspring and excessive repetitive grooming behaviour in adult offspring (Le Belle et al., 2014). Both social and repetitive behavioural deficits are core in autistic patients. Rousset et al. (2006) have also shown that LPS (0.3mg/kg) i.p. injection in rats at E19 and 20 was able to promote the expression of IL-1 β in the brain of P1 offspring and this was correlated with increased cell death in periventricular white matter (characteristic of cerebral palsy) and striatum that was still present in P7 rats. Finally, Favrais et al. (2011) have studied myelination and oligodendrogenesis, and behaviour in mice that received i.p. injections of IL-1 β from P1 to P5. The authors showed that IL-1 β injections increased blood levels of IL-1 β and TNF at P5, induced severe memory impairments at P29 to P30, decreased brain white matter fractional anisotropy at P35, reduced many myelin proteins and increased the number of nonmyelinated axons in the brain at P15 and P30 and, impaired maturation of oligodendrocytes from P5 to P30 when compared to control mice. Interestingly, this last study was able to correlate the three steps that are suggested to occur after immune activation during development: increased inflammatory mediators, brain pathology and behavioural deficits. Moreover, it demonstrated cytokine-specific effects as the authors have used IL-1 β for immune activation from P1 to P5 (equivalent to preterm newborns/third trimester of pregnancy in humans). More studies need to be performed in order to correlate specific mechanisms of brain damage with each cytokine that is known to be altered in neurodevelopmental pathologies in humans and rodents.

Table 1.2 Effects of immune activation on the inflammatory response, brain and/or behaviour

Immunogen	Species	Injection route	Time of administration	Immune response	Brain pathology	Behaviour deficits	Reference
Poly(I:C)	Mouse	i.v.	E9	-	Altered dopaminergic system (P183)	Decreased prepulse inhibition (P98-P112)	Meyer et al. 2008
	Rat	i.p.	From P5 to P7	Microglia activation (P74)	-	Decreased prepulse inhibition (P74)	Ribeiro et al. 2013
LPS	Rat	i.p.	E19 and 20	Increased IL-1 β in the brain (P1)	White matter injury including cell death and hypomyelination (P7)	-	Rousset et al. 2006
	Rat	i.p.	E15 and 16	-	Decreased myelination (P70 and P120)	Deficits in memory (P70) and prepulse inhibition (P90)	Wischhof et al. 2014
	Mouse	i.p.	E9	Microglia activation (E18 and P0)	-	Impaired social behaviour (P45-70)	Le Belle et al. 2014
IL-1 β	Mouse	i.p.	From P1 to P5	Increased IL-1 β in the blood (P5)	Impaired white matter (P35) and myelination (P15-30)	Memory impairment (P29-30)	Favrais et al. 2011

Details of published data showing association between immune activation during development and inflammatory response, brain pathology and/or behaviour deficits are described in this table. i.v.: intravenous, i.p.: intraperitoneal and E: embryonic day.

In summary, either studies using human tissue and/or animal models have shown that inflammation has an important role and a profound impact on the development of brain disorders. Many rodent models have been developed in order to mimic different aspects of neurodevelopmental disorders by using different type and doses of immunogens, and injection times. In general, all studies so far support the current hypothesis that immune activation during development leads to immune imbalance (altered levels of anti- and pro-inflammatory cytokines) and, consequently, neuropathology and behaviour deficits. It is important now to explore the contribution of specific inflammatory mediators to neurodevelopmental injury and study how brain capacity to respond to inflammation vary during development and, consequently, contribute to final pathological outcome.

1.2.3 "Windows of susceptibility" during development

It is clear from many published studies focused on infection/inflammation and neurodevelopment disorders that the deficits observed vary with the time of immune activation. Both human and rodent data have shown specific "windows of susceptibility" to different types of infection/inflammation during development. In response to this, researchers studied the effects of infection/inflammation on the immune response, neuropathology and behavioural outcome at different time points during development in rodent models. Meyer et al. (2008) have shown that i.v. injection of poly(I:C) at E9 induced deficits in prepulse inhibition and in the dopaminergic system (reduced prefrontal dopamine D1 receptors), while poly(I:C) injection at E17 impaired working memory and reduced expression of hippocampal NMDA-receptor subunit 1 in adult mouse offspring. The authors were able to mimic two core features of autism and schizophrenia (deficits in prepulse inhibition and dopaminergic system) by inducing viral infection at E9 and, therefore, supported the current idea that infection/inflammation occurring early in gestation is correlated with the development of these two disorders.

Moreover, immune activation later in embryonic development was shown to promote deficits in hippocampus that may explain the observed memory deficiency and, therefore, demonstrate that long-term brain and cognitive abnormalities are modulated by the timing of immune activation during pregnancy in mice (Meyer et al., 2008). Another study has shown that i.p. injection of LPS at E15-16 and E18-19 but not at E10-11 decreased prepulse inhibition in adult rat offspring (Fortier et al., 2007). Meyer et al. (2006) have also shown that maternal immune activation (i.v. injection of poly(I:C)) at different embryonic time points (E9 or E17) promoted differential cytokine (IL-1 β , IL-6, TNF and IL-10) production in foetal brain shortly after the injection (4h or 6h). Specifically, at 6 hours, poly(I:C) treatment at E17 increased gene expression of IL-6, TNF and IL-10 whereas the same treatment performed at E9 did not induce the expression of any cytokine but decreased expression of IL-10 instead. The same authors have observed that immune activation at E9 decreased open-field exploration while at E17 it promoted perseverative behaviour in the adult offspring (Meyer et al., 2006). Injection of LPS (i.p.) in rats at P1, P21 or P70 promoted an acute (2h) increase in the expression of many pro-inflammatory cytokines (IL-1 β , IL6 and TNF) and chemokines (CXCL-1, CCL2 and CCL4) in the brain at P21 and P70. At P1, increased expression of these inflammatory mediators was only observed 6 hours after LPS injection when compared to the other ages, indicating a delayed inflammatory response (Ortega et al., 2011). Finally, direct injection of LPS in both brain hemispheres promoted a cytokine (IL-1 α , IL-1 β , IL-2, IL-5, IL-6, TNF) and chemokine (CCL2, CCL3, CXCL9 and CXCL10) response 12 hours after injection at P2 but not at P21 in mice (Christensen et al., 2014). All these studies show that many immunogens affect behavioural, neurological and immune processes according to the time when they are administered. Most importantly, they have demonstrated that distinct behavioural, neurological and inflammatory

outcomes may be generated when immune activation occurs at different time points within each equivalent susceptible period for developmental disorders in humans. This finding suggests that the time when inflammation occurs it may, in part, explain why there are so many "subtypes" of deleterious outcomes at brain and behavioural level during human development. Thus, characterization of the immune environment in the brain before and after administration of inflammatory mediators at different time points during development needs to be further studied in order to discover the molecular pathways that may explain why brain susceptibility to injury changes throughout development.

1.3 The blood-brain barrier: interface between brain and periphery

It is well known that the brain develops and functions within a very strictly-controlled environment. The BBB represents the interface between the blood and the brain parenchyma, it regulates the movement of molecules, ions and cells between the two compartments, and protects the brain from toxins and pathogens. It ensures a well-controlled homeostasis and, consequently, allows normal neuronal function (Daneman and Prat, 2015). This barrier is composed by endothelial cells, basement membrane, pericytes and astrocytes endfeet. The interaction between these components and microglia and perivascular cells is also crucial for maintenance of the BBB properties and, together, they all form the neurovascular unit (Sá-Pereira et al., 2012) (Figure 1.3).

1.3.1 Endothelial cells

Endothelial cells from the BBB have unique and distinguishable morphological, biochemical and functional features, and are considered to be the anatomic basis of the BBB (Engelhardt and Sorokin, 2009; Hawkins and Eggleton, 2006). Endothelial cells have

a cytoplasm with a uniform thickness and no fenestrae (Fenstermacher et al., 1988), sparse pinocytic vesicular transport systems (Sedlakova et al., 1999) and a negative surface charge that repulses negatively charged compounds (de Boer and Gaillard, 2006; Ribeiro et al., 2011). They also have a large number of mitochondria that enhance the energy potential for enzymatic activity and for selective transport systems, which actively transport nutrients and other compounds into and out of the brain (de Boer and Gaillard, 2006; Oldendorf et al., 1977). In addition to these properties, the monolayer of endothelial cells in the brain capillaries has a selective and sealed phenotype. They create a cellular barrier by expressing junction complexes at their surface that bind to adjacent endothelial cells at the intercellular space (reviewed in Tietz and Engelhardt, 2015) (Figure 1.3). These complexes include tight and adherens junctions that prevent any free paracellular diffusion of substances from the blood to the brain and block the penetration of macromolecules (Abbott et al., 2010; Tietz and Engelhardt, 2015; Wolburg and Lippoldt, 2002). Tight junctions comprise a complex of transmembrane proteins, including claudins (Furuse and Tsukita, 2006), occludins (Furuse et al., 1993) and junctional adhesion molecules (Martin-Padura et al., 1998), and cytoplasmatic proteins, including zonula occludens (ZO) -1, 2 and 3, cingulin, AF-6 and 7H6 (Balda and Anderson, 1993; Citi et al., 1989; Haskins et al., 1998; Jesaitis and Goodenough, 1994; Stevenson et al., 1986; Zhong et al., 1994). Cytoplasmic proteins are responsible for binding the tight junctions to the actin cytoskeleton in the endothelial cells (Tietz and Engelhardt, 2015). Adherens junctions contribute to cell-cell adhesion through VE-cadherin and catenins, which act as an anchor to the actin filaments in the cells (Dejana et al., 2008, 2009). Besides their impermeable paracellular structure, endothelial cells also have receptors and specific transport systems that control transcellular transport (reviewed in Saunders et al., 2013 and Strazielle and Ghersi-Egea, 2015). The solute

carrier family of proteins ensures the transport of water-soluble molecules such as glucose, which is a major source of energy in the brain, amino acids, which are important for synthesis of many neurotransmitters, and ions, which are involved in neurotransmission, from the blood to the brain. Some of these carriers have high substrate specificity and, therefore, are only able to transport compounds that are very similar to endogenous substrates (e.g. GLUT1, transporter for glucose). Many others are more permissive and they may transport a broader range of substrates, which make them a possible entry route for exogenous/drugs (reviewed in Saunders et al., 2013, 2016). ATP-binding cassette transporters or ABC transporters, including P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins, are responsible for the efflux of harmful and toxic compounds from the brain to the blood (Cooray et al., 2002; Leslie et al., 2005; Schinkel and Jonker, 2003; Schinkel et al., 1996; Strazielle and Ghersi-Egea, 2005). These efflux transporters have a broad substrate specificity and are able to export many drugs, such as anti-inflammatory, immunosuppressive, anti-infectious and psychotropic drugs, and this contributes to the fact that these transporters represent an obstacle for the delivery of drugs to the brain (reviewed in Strazielle and Ghersi-Egea, 2015). Finally, vesicular trafficking through caveolae can also occur in the endothelial cells. Caveolae are small invaginations of plasma membrane that are involved in endocytosis, signal transduction and transcytosis. Caveolae are very rich in proteins, such as caveolin-1, and lipids, including cholesterol and sphingolipids (reviewed in Cardoso et al., 2010; Parton et al., 1994; Simionescu et al., 2002 and Stan, 2002).

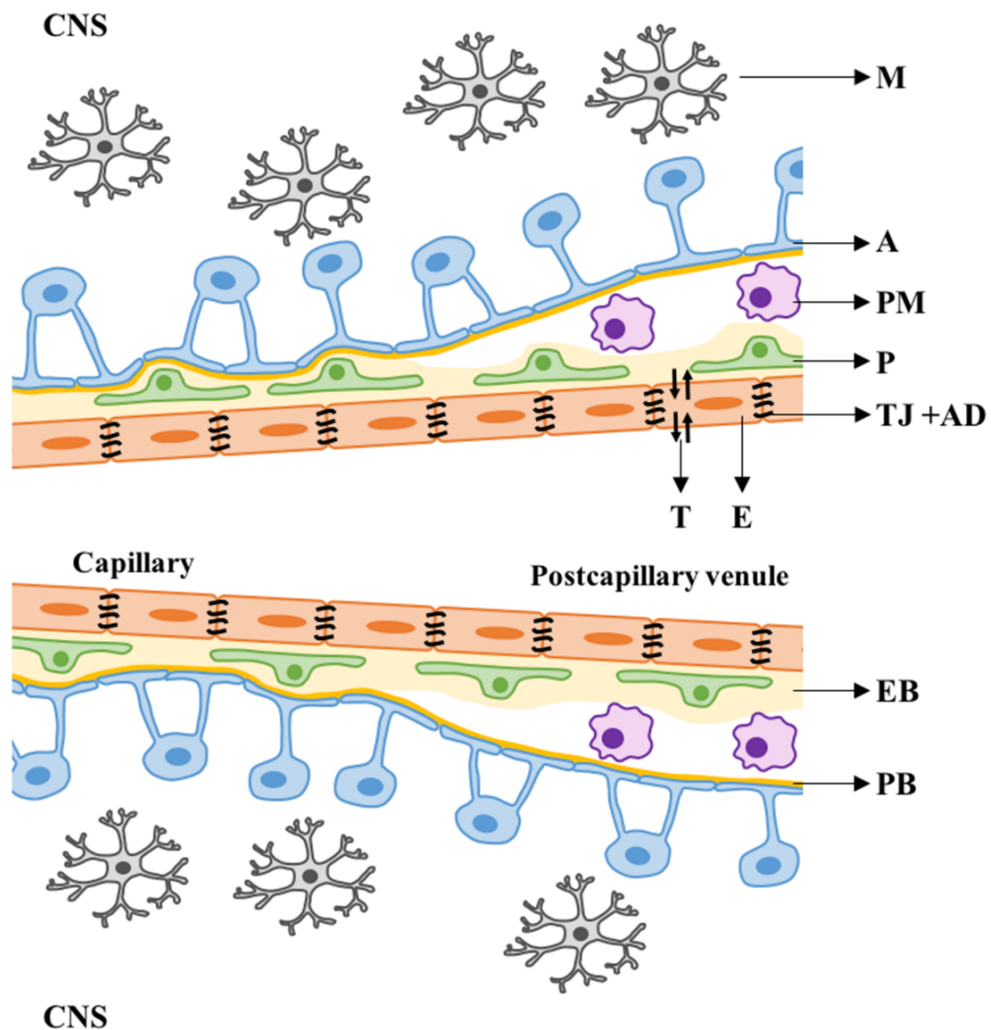


Figure 1.3 Neurovascular Unit. Neuroanatomy of the neurovascular unit. The endothelial cells (E) are the anatomical basis of the BBB and constitute a selective barrier by their low pinocytic vesicular transport, negative surface, tight (TJ) and adherens (AJ) junction complexes and, influx and efflux transport systems (T). These cells are surrounded by the endothelial basement membrane (EB) in which many pericytes (P) are embedded. Another basement membrane can be found on top of the EB, which is named parenchymal basement (PB). Astrocytes (A) sit on top of the PB and together they form the glia limitans perivascularis. At capillary level, both EB and PB merge and cannot be distinguished. In post-capillary venules, both basement membranes are separated and delimit the cerebrospinal fluid-drained perivascular space where antigen presenting cells, such as perivascular macrophages (PM), can be found. Microglia (M) are present in the CNS parenchyma and they make part of the neurovascular unit as they are important for BBB function. Adapted from Engelhardt and Ransohoff 2012.

1.3.2 Barrier mechanisms in the developing brain

It is known that tight junctions and many of the transport mechanisms, including solute-linked carrier and efflux transporters, are present in the BBB very early in the development and that this translates into functional effectiveness of the blood–brain barrier (reviewed in Ek et al., 2012 and Saunders et al., 2012).

Early studies using transmission electron and/or freeze fracture have shown that tight junctions were present and well formed from early vascularization in the brain of humans (10 and 13 weeks of gestation) and sheep (18 weeks of gestation; total gestation time: 21 weeks) (Mollgård and Saunders, 1975), baboons (mid-gestation) (Bass et al., 1992) and mice (E10) (Bauer et al., 1993). These results supported even earlier studies showing that after injecting dye systemically, the brain of many rodent animals and humans was free from dye early in the embryonic development as well as in adults animals (reviewed in Saunders et al., 2014). Moreover, Ek et al. (2006) have shown that the BBB was functionally restrictive to small water-soluble molecules very early in brain vascularization (when first vessels penetrate into the neocortex: P5 marsupial opossum) and this was correlated with the presence of claudin-5 in vessels (Ek et al., 2006).

The GLUT1 transporter has been observed in brain vessels early in development both in mice (E11; (Bauer et al., 1995a)) and rats (E14.5; (Dermietzel et al., 1992)). Vannucci et al. (1994) also studied GLUT1 expression in cortical microvessels from E19 to P30 in rats and determined rate constants for the transport of glucose into and out of the brain in 7, 14 and 21 day old rats. The authors observed that GLUT1 was present and glucose was transported from the earliest time point analysed and that both parameters increased with age (Vannucci et al., 1994). Braun et al. (1980) have demonstrated higher brain uptake rates for amino acids, such as arginine, choline, and adenine, in newborn rabbits when compared to adult rabbits. In addition, Cornford et al. (1982) have studied the uptake of

amino acids and glucose to the rabbit brain at different postnatal ages and found that the transport rate of arginine, choline, adenine and tryptophan decreased while the transport of glucose was the same from newborn to adult animals. Lefauconnier and Trouvé (1983) have also shown that transport of amino acids, such as alanine, serine, cysteine and threonine, across the BBB decreased from P5 to P19 rats while of others, including leucine, tryptophan, did not change with age. On a later study, Daneman et al. (2010) have isolated endothelial cells from brains of mice neonates (P2-8) and adults (P60-70) and were able to produce a transcriptome and, consequently, compare the expression of many transporters between the developing and adult brain. Many transporters were found to be upregulated in the neonate, including influx transporters, such as Slc1a1 (glutamate), Slc2a1/Glut-1 (glucose), Slc7a1 and Slc7a3 (cationic amino acids), Slc7a5 and Slc38a5 (neutral amino acids), Slc12a6 (K^+/Cl^-) and Slco2b1 (prostaglandins, organic anions) and efflux transporters, such as Abcb1a (P-gp), Abcc4 (multidrug resistance associated protein 4) and Abcg2 (BCRP). Some transporters were also downregulated in the neonate. These included influx transporters, such as Slc7a8 and Slc7a7 (cationic amino acids), Slc39a8 (metal ion transporter), Slc1a4 (glutamate, neutral amino acids) and Slco2a1 (organic anions), and efflux transporters, such as Abcg1 and Abca1 (cholesterol efflux regulatory protein) (Daneman et al., 2010). Finally, another study has shown that multidrug resistance-associated proteins 1 and 4, P-gp and BCRP were expressed in the rat brains from E13 onwards (Ek et al., 2010).

Although they are some contradictory results, mainly regarding glucose transport, between all studies presented above, they all support the concept of a functional and tight BBB very early in the development. Variation in the BBB composition regarding influx transport of amino acids (for synthesis of neurotransmitters), ions (for synapses), glucose (major energy source) and neurotransmitters (glutamate), and efflux transport of

xenobiotics, drugs and other toxic substances supports the hypothesis that the function of the BBB may vary during development in order to protect and fulfil the metabolic demands of the developing brain. In fact, the brain microenvironment during development may need to be carefully controlled as or even more than in adults in order to make sure that cell positioning and connections are established in a way that will guarantee a healthy and functional brain later in life. Any alterations to this complex and controlled process has the potential to cause major deficits in the brain.

1.3.3 The role of the adult blood-brain barrier in inflammation

The BBB represents an effective barrier between the periphery and the CNS. This barrier may also mediate the cross-talk between the CNS and immune system in disease and physiologic states. For example, the adult BBB may (1) transport circulating cytokines into the CNS, (2) respond to circulating cytokines and other inflammatory mediators by producing more cytokines and other inflammatory mediators and, (3) interact with immune cells allowing their transport into the brain. Moreover, BBB dysfunction and disruption can also occur in response to inflammation and, consequently, contribute to brain injury (reviewed in Banks, 2015 and Erickson et al., 2012).

Different cytokines and chemokines, such as IL-1 β , IL-1 α , TNF and CCL-2 have been shown to cross the BBB from the blood to the brain in rodents. However, the transport of these inflammatory mediators from the brain to the blood through the BBB has not been reported (reviewed Banks and Erickson, 2010). Many more studies have demonstrated the importance of the endothelial cells in receiving and transmitting signals from and to the periphery. As discussed in section 1.1.2, cytokines are recognized by receptors expressed on different cells and induce different signalling pathways that contribute to the immune response. Endothelial cells of the BBB are known to express receptors for different cytokines and, consequently, induce the recruitment and migration of leukocytes

to the brain by producing chemokines and adhesion molecules (reviewed in Daneman and Prat, 2015 and Larochelle et al., 2011). An endothelial-specific IL-1R1 knockdown mice model injected with IL-1 β (intracerebroventricular) showed inhibition of leukocyte infiltration to the brain compared with wild-type mice (Ching et al., 2007). Besides IL-1 β , other cytokines have been reported to interact with their receptors at BBB endothelium. These include TNF and lymphotoxin that may act together to modulate chemokines, cytokines and cell adhesion molecules expression in microvessels, and IL-6, which can affect cell adhesion molecules expression in endothelial cells and leukocyte recruitment (reviewed in Larochelle et al., 2011 and Meager, 1999). Many *in vitro* studies have also shown that endothelial cells are able to produce different cytokines, such as IL-1 β , IL-1 α and IL-6, constitutively and/or when stimulated with inflammatory stimulus, including IL-1 β and LPS (*mice*: Fabry et al., 1993; *rhesus monkey*: Reyes et al., 1999). An important feature of the endothelial cells of the BBB is that they have an abluminal cell membrane in contact with the CNS and a luminal cell membrane connected with the periphery. This polarity allows them to receive signals from one side, and produce and secrete inflammatory mediators to the other side, representing one important mechanism of immune signalling between the CNS and the peripheral immune system (reviewed in Erickson et al., 2012). A good example of this process is the *in vitro* work by Verma et al. (2006). The authors used a monolayer of mouse brain endothelial cells on a Transwell system, and studied the production and release of cytokines after LPS incubation. The endothelial cells were able to release IL-6, IL-10 and TNF either to the abluminal (top chamber) and to the luminal (bottom chamber) side 24 hours after incubation of LPS (100 μ g/ml) in the abluminal side (Verma et al., 2006). Another study using the same Transwell system with rat brain endothelial cells has demonstrated that IL-1 β incubation for 5 hours on the luminal side upregulated ICAM-1 (quantified by immunogold electron

microscopy) on the luminal but also on the abluminal cell surface when compared to unstimulated cells (Staykova et al., 2000).

Disruption and/or dysfunction of the BBB may also happen during inflammation and, consequently, this barrier may no longer promote the ideal CNS environment for normal brain function. Adult BBB disruption/dysfunction may involve increased permeability, tight junction protein redistribution and/or decreased expression, deficits in influx or/and efflux transporters, disrupted basement membrane, pericytes detachment and loss of astrocyte endfeet. (reviewed in Obermeier et al., 2013; Strazielle and Gherzi-Egea, 2015 and Tietz and Engelhardt, 2015). BBB disruption/dysfunction has been observed in many CNS pathologies, such as multiple sclerosis, stroke and Alzheimer's disease (reviewed in Obermeier et al., 2013 and de Vries et al., 2012). Altered ZO-1 presence was observed in human brains of different multiple sclerosis cases and this was associated with fibrinogen leakage, which indicates increase BBB permeability (Kirk et al., 2003). Reduced expression of P-gp by the endothelial cells in a EAE rat model and in human brains of multiple sclerosis was also observed and coincided with the presence of perivascular infiltrates of lymphocytes (Kooij et al., 2010). Also, in a rat model of stroke the expression of ZO-1, claudin-5 and occludins was decreased and correlated with increases in BBB permeability (Jiao et al., 2011). Loss of claudin-5, occludin and ZO-1 associated with activated microglia was observed in human cortex capillaries where beta-amyloid accumulated (brains with capillary cerebral amyloid angiopathy, which is frequently observed in Alzheimer's patients) (Carrano et al., 2011). Moreover, Bolton et al. (1998) have shown that occludin and ZO-1 were absent from vessels presenting extensive neutrophil recruitment in the striatum of P21 rats that received i.c. injection of IL-1 β (Bolton et al., 1998). Finally, many *in vitro* studies have shown that different cytokines

decrease the expression of tight and adherens junctions in the endothelial cells (reviewed in Rochfort and Cummins, 2015).

1.3.4 The blood-brain barrier and inflammation during development

Daneman et al. (2010) have shown that mouse neonatal endothelial cells express many inflammatory transcripts needed for immune response. ICAM-1 and integrin $\alpha 6$, which are important for immune cell migration through the BBB, were shown to be expressed early in development. Transcripts of IL-1 β and one of its receptors (IL-1R2), IL-6, and IL-6RA and IL-6RB, which make up the cell-surface type I cytokine receptor complex, were also observed in the neonate endothelial cells. Finally, TNFR1, TNFR2 and several other TNF receptor family members transcripts were highly expressed in the neonate endothelial cells (Daneman et al., 2010). The expression of these inflammatory transcripts by the endothelial cells in neonates indicates that the BBB early in the development may be capable of responding to inflammation and, consequently, contribute to neurodevelopmental brain injuries. Thus, studies focused on the role of the BBB in inflammation during development are needed. BBB dysfunction/disruption may also happen during development. Many studies have shown BBB susceptibility to neuroinflammation or peripheral inflammation at early stages and this seems to vary with age, and the time and the location of the insult (Stolp et al., 2013), which is in concordance with the presence of "windows of susceptibility" in neurodevelopmental disorders. For instance, an IL-1 β injection in the striatum has been shown to produce a significant increase in vessel permeability to horseradish peroxidase and neutrophil recruitment around the injection site 4 hours after injection in P21 rats, but not at P0 or in adult animals. This increase in BBB permeability was found to be neutrophil-dependent as no BBB leakage was observed in P21 brains after neutrophil depletion (Anthony et al., 1997). The same authors have also shown that TNF injection in the

striatum did not induce BBB permeability or neutrophil recruitment to the injected site at any time point (Anthony et al., 1997), suggesting that BBB dysfunction varies according to the inflammation induced in the brain. Lawson and Perry (1995) have observed that LPS injection in the striatum of BALB/c mice promoted a rapid increase in leukocyte recruitment at P7 and not at P0 (Lawson and Perry, 1995). Furthermore, immune activation by i.p. injection of LPS transiently increased BBB permeability in the periventricular white matter tract during the first postnatal week in rats (Stolp et al., 2005) and i.p. injection of LPS followed by hypoxia-ischemia increased neutrophils recruitment and BBB permeability at P12 and not at P1 in Lewis rats (Brochu et al., 2011). The observed developmental differences in the response of the BBB to inflammation are likely to be dependent on developmental stage of the CNS, BBB immune signalling mechanisms, and developmental stage of the peripheral immune system at the time of injury (reviewed in Stolp et al., 2013). Future studies should examine all these aspects in order to find specific molecular and/or cellular mechanisms that can explain susceptibility to inflammation during development.

1.4 Other brain barriers and possible signalling routes from the central nervous system to the periphery

Besides the BBB, other barriers can be found in the CNS: the blood-cerebrospinal fluid (csf), which is formed by junctional proteins between epithelial cells of the choroid plexus within the cerebral ventricles; the outer csf-brain barrier at the level of the pia arachnoid, formed by junctional proteins between endothelial cells of the arachnoid vessels; the inner csf-brain barrier, present only in early embryonic development, formed by strap junctions between the neuroependymal cells covering the ventricular surfaces (Stolp et al., 2013) (Figure 1.4). Blood-csf barrier, like the BBB, expresses tight junctions

and, influx and efflux transporters, which are important for controlling the composition of the csf. It is also currently accepted that this barrier is functional from early in development (reviewed in Liddelow, 2015) and that choroid plexus can produce many neurotrophic molecules important for brain development (reviewed in Johansson, 2014). This barrier, together with the outer csf-brain barrier and BBB, is thought to allow the entrance of macrophages, dendritic cells and T-cells to the csf in physiologic conditions, where these cells perform immunosurveillance (reviewed in Ousman and Kubes, 2012). Moreover, the blood-csf barrier may also act as an entry site for immune cells during CNS injury. For example, neutrophils were shown to be transported from the periphery through blood-csf barrier in traumatic brain injury in adult rodents (Szmydynger-Chodobska et al., 2009). Once in the csf, these cells may migrate to brain parenchyma at the level of the BBB, outer csf-brain barrier or through neuroependymal cells lining the ventricular surfaces. Antigens from CNS may also be transported into the csf at the level of the BBB, outer csf-brain and neuroependymal cells covering the ventricular surfaces and, consequently, to circulation through vessels present in all three barriers. Finally, choroid plexus can also express many inflammatory mediators, such as CXCL-1, IL-1 β , ICAM-1, upon immune activation and, therefore, contribute to the inflammatory response in the CNS (Marques et al., 2009).

Recently, functional lymphatic vessels were found lining the dural venous sinuses, which are able to carry fluid and immune cell from the csf and are associated to the deep cervical lymph nodes (Louveau et al., 2015). This may then be a route for diverse signals and immune cells from the CNS and to the periphery either in physiological and/or pathological conditions and, consequently, important for surveillance and injury resolution in the CNS.

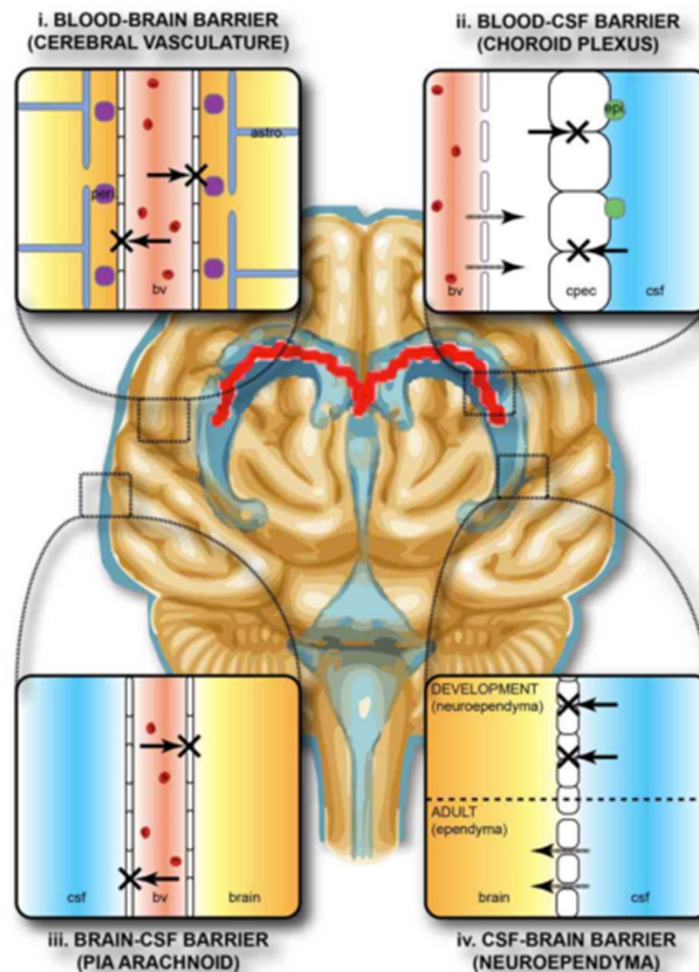


Figure 1.4 Brain barriers of the brain. Choroid plexuses are shown in red. csf: cerebrospinal fluid (blue), astro, astrocyte; bv, blood vessel; cpec, choroid plexus epithelial cell; Csf, cerebrospinal fluid; peri, pericytes. This figure was provided by Stolp et al. (2013).

1.5 Acute phase response to brain injury

Peripheral infection/inflammation has been strongly implicated in neurodevelopmental disorders. As discussed above, many *in vivo* studies have shown that immune activation in the periphery may increase inflammatory mediators and injury in the brain. However, it is also known that the peripheral immune system can respond to injury to the brain in adults and, as a consequence, contribute to further brain damage. Thus, it is important to understand the interaction between the peripheral and central immune responses and

study the systemic inflammatory response to brain inflammation during development in order to discover its contribution to neurodevelopmental disorders.

1.4.1 Acute phase response in the adult liver

The liver is an important immunological organ that contains a range of immune cells, including Kupffer cells, monocytes, neutrophils, dendritic cells, natural killer cells, and natural killer T cells (Robinson et al., 2016). PRRs, such as toll-like receptors, are expressed on many of these cells and others, such as endothelial cells and hepatocytes (Broering et al., 2008; Preiss et al., 2008; Wu et al., 2007, 2010; Xia et al., 2008; Zhang et al., 2009). These receptors are able to recognize exogenous PAMPs or endogenous signals from damaged cells and, consequently, induce the production of cytokines (Broering et al., 2011). Within the immune cell population, Kupffer cells and neutrophils are the most prominent cells in the acute immune response in the liver (Bieggs and Trautwein, 2013). Kupffer cells sit on the surface of a fenestrated layer of liver sinusoidal endothelial cells, which constitute a network of sinusoids capillaries. Therefore, the endothelial cells and the Kupffer cells have easy access to blood content and, consequently, are key to the transduction of immune responses in the liver (Ishibashi et al., 2009; Si-tayeb et al., 2010). Kupffer cells are the first cells to detect exogenous PAMPs and endogenous DAMPs through their surface-expressed PRRs. These cells are able to act as phagocytes and secrete pro-inflammatory cytokines, such as TNF, IL-1 β and IL-6, and chemokines that contribute to inflammation and initiate the acute-phase response (reviewed in Bieggs and Trautwein, 2013). In addition, cytokines induce the expression of adhesion molecules, such as ICAM-1, by the liver sinusoidal endothelial cells, which can participate in the recruitment of immune cells, including neutrophils (Ando et al., 2007; Ohira et al., 1994, 2003; Sakamoto et al., 1997). As discussed above, neutrophils are important innate immune cells that quickly migrate to the injury site and,

consequently, produce cytotoxic signals, including cytokines, chemokines and oxidants, and recruit more neutrophils and monocytes.

The APR is a rapid systemic reaction to any homeostasis changes that result from many types of injury and its mainly coordinated by the liver that produces a range of plasma proteins (acute phase proteins (APPs)) as a consequence (Gabay and Kushner, 1999; Gruys et al., 2005). At the site of injury, many pro-inflammatory cytokines are secreted and, consequently, diffuse into extracellular fluid and blood. These inflammatory mediators activate receptors on hepatocytes, mediating the production of many APPs (reviewed in O'Reilly and Eckersall, 2014). TNF, IL-1 β and IL-6 are main mediators in the activation of the APR, being IL-6 the cytokine that upregulates most of the APPs (Castell et al., 1989; Heinrich et al., 1990; Le and Vilcek, 1989). The APPs produced by hepatocytes have a range of functions that promote pathogen/injury clearance. APPs that are secreted include (1) complement proteins that may increase phagocytosis, cell lysis/degranulation and vascular permeability; (2) iron-binding proteins that reduce free iron; (3) antimicrobial proteins; (3) clotting proteins; (4) inflammatory proteins, such as IL-6 that increase other pro-inflammatory mediators and amplify the APR; (5) protease inhibitors that act as anti-inflammatory mediators as they inhibit coagulation and neutrophils, and (6) other proteins, such as c-reactive protein and serum amyloid P that activate complement system and increase phagocytosis, and serum amyloid A that recruits myeloid-derived suppressor cells (reviewed in Robinson et al., 2016). Other APPs are downregulated during the APR and they include albumin and transferrin. This last may contribute to decreasing levels of iron, which are important for microbial growth (Gruys et al., 2005). In summary, the APR in the liver to diverse injuries in the organism is a very controlled and balanced mechanism. Hepatic secretion of many pro- or anti-inflammatory APPs allows the resolution of the damage and contribute to tissue repair.

1.4.2 Acute phase response to brain injury in adults

It is known that inflammation in the CNS is followed by an APR in the liver in adult rodents in order to eliminate the inflammatory stimuli, attenuate local inflammation, and promote tissue repair and regeneration (reviewed in Anthony and Couch, 2014 and Anthony et al., 2012). Many published studies support the concept that inflammatory brain injury is followed by an APR in the liver, which is characterized by the production of cytokines, chemokines and other APPs. These are then able to circulate in the blood and induce the mobilization of leukocytes to the circulation, and, consequently, to the brain in order to resolve the initial injury or contribute to further damage, if an excessive inflammatory response occurs (Figure 1.5). Injection of IL-1 β in the striatum of adult rats increased CXCL-1 levels in the liver and serum after 2 hours, and this was accompanied by increased numbers of neutrophils in the peripheral blood, liver and brain. This pathway was inhibited by an antibody against CXCL-1 when administered systemically 10 minutes before i.c. injection of IL-1 β (Campbell et al., 2003), highlighting the role of CXCL-1 as a strong chemoattractant for neutrophil recruitment to the liver and brain. Another study has also shown that i.c. injection of TNF induced a rapid APR in the liver, including upregulation of CXCL-1 and CCL-2, and increased the recruitment of leukocytes to the brain (Campbell et al., 2005). An earlier study has observed a rapid upregulation of APPs, including serum amyloid A2 and serum amyloid P, in the liver of adult mice after injection of IL-1 β in the striatum (Wilcockson et al., 2002). Increases in CXCL-1 and IL-6 levels in plasma and liver were also observed 4 hours after focal cerebral ischemia in adult mice (Chapman et al., 2009). Traumatic brain injury in adult mice has also been shown to induce the APR in the liver (increased serum amyloid A1, recruitment of neutrophils and macrophages and, expression of CXCL-1 and CXCL-10) (Villapol et al., 2015). Finally, human studies have observed that circulating levels of c-

reactive protein and IL-6 of ischemic stroke patients were positively correlated with brain infarct volume, stroke severity and clinical outcome (Acalovschi et al., 2003; Smith et al., 2004, 2006; Vila et al., 2000). All these studies have shown that APR in the liver is activated after different types of injury in the adult brain and suggest that production of inflammatory mediators by the liver may contribute to the recruitment of leukocytes to the injury site. In addition, it appears that there is a correlation between the magnitude of the acute hepatic inflammatory response and the extent of brain injury. As stated above, APR in the liver have been positively correlated with the magnitude of brain injury in ischemic stroke patients. Furthermore, Campbell et al. (2003) have observed that i.c. injection of IL-1 β induced the production and release of CXCL-1 by the liver and the recruitment of neutrophils to the liver, blood and brain in a dose-dependent manner (Campbell et al., 2003).

1.4.3 The role of the hepatic acute phase response during development

Whilst it is common to assess how peripheral inflammation affects brain injury in development, there has been little study of the reverse situation to date. Most of rodent studies have shown that immune activation in the periphery during either embryonic development or in neonates contribute to increased levels of inflammatory mediators in the blood and/or in the brain, brain dysfunction and behavioral deficits. Clinical studies have also shown a positive correlation between the levels of many cytokines and chemokines (either in circulation or in the brain) and the development of brain injury in cases of autism, schizophrenia, cerebral palsy and in preterm newborns. In these situations, increased levels of inflammatory cytokines/chemokines in the periphery may: (1) be the cause of brain injury or/and (2) be induced after brain injury and further aggravate brain damage. Due to the difficulty of studying this dual hypothesis in humans, rodent studies are needed to discover the role of the peripheral immune response to brain

injury during development. Very little is known about the hepatic APR during development. One study by Aly et al. (2011) have measured the concentration of serum amyloid A in circulation of full-term or near-term newborns (gestational age: 39 ± 2.1 weeks) diagnosed with asphyxia and admitted to the neonate intensive care unit, and age-matched controls in the first 24 hours and at P7. The authors have demonstrated that levels of serum amyloid A were higher in newborns with asphyxia than controls at P1 and P7. In addition, the concentration of serum amyloid A was positively correlated with the severity of hypoxic ischemic encephalopathy at both time points (Aly et al., 2011). Only one animal study so far has studied the hepatic APR to brain injury. The authors have shown that hypoxia-ischemia in P7 rats decreased TNF, IL-1 β and CCL-2 but increased CXCL-1 expression in the liver 3 hours after injury and this was followed by the recruitment of neutrophils to the affected brain hemisphere (Bonestroo et al., 2013). While this seems to be different from adults, it still demonstrates that the APR may play a role in the development of brain injury at early ages. However, more studies should be performed in order to validate this hypothesis.

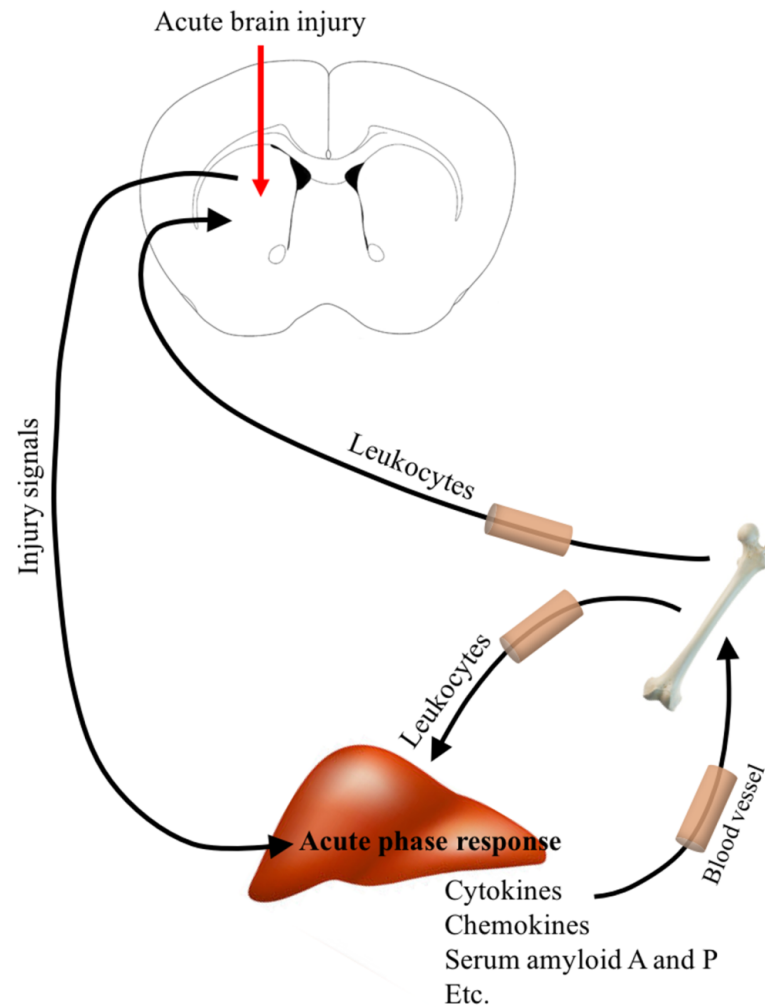


Figure 1.5 The acute phase response to brain injury in adult rodents. Proposed mechanism for the interaction between acute brain injury and APR in the liver. After acute brain injury, injury signals, which may include cytokines, from the brain are transmitted to the periphery and, consequently, induce an APR in the liver characterized by the production of inflammatory cytokines, chemokines and other APPs, such as serum amyloid A and P. These inflammatory mediators are then released to circulation and induce the recruitment of neutrophils from their main production site (bone marrow) to circulation, liver and brain, where their main goal is to resolve the initial injury. However, excessive inflammatory response and neutrophil persistence in the brain may contribute to further damage. Adapted from Anthony and Couch (2014).

1.6 Aims

The principal aim of this thesis is to study the brain and hepatic acute responses to focal inflammation induced by the injection of IL-1 β into the brain at different postnatal ages in mice. It is clear from human and rodent data that inflammation is implicated in different types of brain injury that occur during development. However, it remains unclear which factors might be responsible for the age-specific susceptibility of the brain to inflammation. It is my hypothesis that the developmental stage of the brain, specifically its capacity to respond to inflammation, and the APR in the liver modulate CNS immune cell recruitment after focal inflammation. Thus, work in this thesis was conducted in order to test the following hypotheses:

- That the cytokine expression profile accounts for the susceptibility of the brain to focal inflammation during postnatal development.
- That brain macrophage populations modulate the response of the developing brain to focal inflammation during the postnatal period.
- That the hepatic APR is related to the severity of the brain inflammatory response and controls/influences the recruitment of leukocytes to the CNS after focal inflammation during postnatal development.

CHAPTER 2

METHODS

2.1 Animals

C57Bl/6 mice were used at P7, 12, 13, 14 and 21 days. Eight-week-old mice were also used. Mice were obtained from Harlan, UK, at least 3 days before the experiments, to allow animals to acclimatise to the local environment prior to experimentation. These animals were housed in specific pathogen-free facilities under a standard light/dark cycle with food and water ad libitum. All animal procedures were conducted in accordance with institutional ethical guidelines and with UK Home Office approval.

2.2 Administration of substances

2.2.1 Intracerebral injection of IL-1 β

Wild-type animals were anaesthetised with 2-3% isoflurane in oxygen. All surgical procedures were performed under an operating microscope. Stereotaxic surgery was performed as previously described (Couch et al., 2014) between 9 and 10am. The head of the anaesthetised animal was shaved and held in a stereotaxic frame. The skull was exposed with a midline sagittal incision and dental burr drill was used to make a small hole for injection into the left striatum. Stereotaxic coordinates were used to locate this region (Table 2.1). A pulled glass microcapillary needle was used to inject 0.5 μ l containing 1, 10 or 100ng of rat recombinant IL-1 β (rrIL-1 β , 501-RL/CF; 2ng/ μ l, 20ng/ μ l or 200ng/ μ l; R&D Systems, UK) or vehicle (0.9% saline). The cytokine was delivered over a period of 5 minutes to allow the diffusion through the parenchyma. The wound was closed using surgical clips. The animals were allowed to recover in a heated chamber. Mice were anaesthetized with pentobarbital 4 hours after stereotaxic injection for tissue collection. rrIL-1 β shares approximately 90% amino acid sequence identity with mouse

(obtained by Blast analysis) and it is thought to signal through IL-1R1 in mice (Zetterström et al., 1998). It is possible that the extent of the response may vary between rrIL-1 β and mouse recombinant IL-1 β and, therefore, comparison studies should be performed in the future.

Table 2.1 Stereotaxic coordinates for injection of IL-1 β in the striatum of C57Bl/6 mice.

Age	Stereotaxic coordinates
P7	Bregma: AP +0.5mm, ML +1mm, DV -1.7mm
P14	Bregma: AP +0.5mm, ML +1mm, DV -2mm
P21	Bregma: AP +0.5mm, ML +1.5mm, DV -2mm
8 weeks	Bregma: AP +0.5mm, ML +1.8mm, DV -2mm

2.2.2 Macrophage depletion

2.2.2.1 *Anti-colony stimulating factor 1 receptor*

Rat anti-mouse CSF1 receptor (CSF1R) (120 μ g/20 μ l; M279, Amgen, USA) or Rat IgG (120 μ g/20 μ l; Sigma I4131, UK) was administered by i.p. injection, twice weekly from P0 to P14. Mice were anaesthetized with pentobarbital at P14 for tissue collection.

2.2.2.2 *Clodronate liposomes*

Wild-type P12 and P13 mice were anaesthetized with 2-3% isoflurane in oxygen. All surgical procedures were performed under an operating microscope. Liposomes containing clodronate or PBS (0.3mg/60 μ l; ClodLip BV – ClodronateLiposomes.com, NL) were injected intravenously into the tail vein. The animals were allowed to recover in a heated chamber. Mice were anaesthetized with pentobarbital at P14 for tissue collection.

2.2.3 Intravenous injection of IL-1 β

Wild-type P14 mice were anaesthetised with 2-3% isoflurane in oxygen. All surgical procedures were performed under an operating microscope. Saline or 100ng of rrIL-1 β (50 μ l) were injected into the tail vein of naïve mice or immediately after i.c. injection of 0.5 μ l containing 1ng of rrIL-1 β or vehicle (0.9% saline). The animals were allowed to recover in a heated chamber. Mice were anaesthetized with pentobarbital 4 hours after i.v. injection for tissue collection.

2.3 Tissue preparation

Mice were anaesthetized with pentobarbital (20% w/v pentobarbital sodium, i.p.; J M Loveridge Ltd, UK) and then transcardially perfused with cold 0.9% saline with heparin (Sigma, UK). Liver and striatum were removed to dry ice and later to -80 for extraction of messenger ribonucleic acid (mRNA). The striatum was dissected into ipsilateral (injected) and contralateral side from saline and IL-1 β injected animals. The contralateral side from naïve animals was also removed for the same purpose. Alternately, mice were transcardially perfused fixed with 4% paraformaldehyde (in 0.1M phosphate buffer, pH 7.4) after 0.9% saline with heparin (Sigma, UK). Brain and liver were collected and immersed into 4% paraformaldehyde at 4°C. After 24 hours they were immersed in 30% sucrose at 4 °C until they sink and, subsequently, they were embedded in optimal cutting temperature (O.C.T.) compound (CellPath, UK). Ten-micrometer-thick sections were cut on a cryostat (Leica Microsystems, UK) and mounted on gelatine-coated slides (made in house).

2.4 Light-microscopy immunohistochemistry

2.4.1 Immunohistochemistry

Slides were dried at 37°C for 15 minutes and quenched for endogenous peroxidase activity in hydrogen peroxide solution (3% of a 0.03% hydrogen peroxide solution in methanol; Sigma, UK). Using Shandon Sequenza® slide racks (Thermo Scientific, UK), slides were blocked for non-specific Fc-dependent binding in 10% serum in phosphate-buffered saline (PBS). Endogenous biotin in the liver was reduced using an avidin-biotin blocking kit (Vector Laboratories, UK). Sections were incubated in primary antibody at 4°C overnight. These include ionized calcium-binding adapter molecule 1 (Iba-1) (Abcam, 1:500), CD68 (Bio-Rad, 1:200) and CD206 (Bio-Rad, 1:100) for macrophages, laminin1+2 (Abcam, 1:500) and Glut-1: (Calbiochem, 1:500) for vessels and an anti-neutrophil serum HB199 (MBS) (made in house, 1:1000) for neutrophils. MBS has been validated by the use of other antibodies, such as anti-neutrophil elastase (Friedrich et al., 2011) and has been widely used to identify neutrophils (Anthony et al., 1998, 1997; Blamire et al., 2000; Bolton and Perry, 1998; Campbell et al., 2002, 2003, 2007a, 2008, 2010; Couch et al., 2014; Ferrari et al., 2004; Hughes et al., 2003; Losey et al., 2015; Sibson et al., 2002). Sections were then incubated for 2 hours with the appropriate biotinylated secondary antibody (Vector Laboratories, UK) followed by an avidin-biotin-peroxidase solution (Vectastain Elite ABC, Vector Laboratories, UK) for 1 hour. Peroxidase was then exposed to the chromogen diaminobenzidine hydrochloride (0.5µg/ml, Sigma, UK) in 0.1M phosphate buffer with 0.05% hydrogen peroxide. Finally, slides were washed, counterstained with cresyl violet, dehydrated/xylene and mounted with DPX medium. BBB permeability was measured by the presence of endogenous IgG in the brain. For that, the slides were incubated with biotinylated antibody anti-mouse IgG (Vector Laboratories, UK). Double immunohistochemistry for Iba-1 and Glut-1 or

MBS and laminin1+2 was also performed by two sequential immunohistochemistry processes. These were separated by an avidin/biotin blocking (Avidin/Biotin blocking kit, Vector Laboratories, UK). More specifically, the slides were dry at 37°C for 15 minutes, quenched for endogenous peroxidase activity and blocked for non-specific Fc-dependent binding as described above. Sections were then incubated with the first primary antibody at 4°C overnight. Sections were incubated for 2 hours with the appropriate biotinylated secondary antibody followed by an avidin-biotin-peroxidase solution for 1 hour. Peroxidase was then exposed to the chromogen diaminobenzidine hydrochloride in 0.1M phosphate buffer with 0.05% hydrogen peroxide. Slides were washed in PBS and incubated with 1:20 avidin blocking solution for 15min followed by one PBS wash and 1:20 biotin blocking solution for 15min. Slides were then washed with PBS and blocked again for non-specific Fc-dependent binding. This was followed by the second primary antibody incubation at 4°C overnight. The sections were again incubated for 2 hours with the appropriate biotinylated secondary antibody followed by an avidin-biotin-peroxidase solution for 1 hour. Peroxidase was then exposed to the VECTOR VIP substrate (VECTOR VIP peroxidase substrate kit, Vector Laboratories, UK), which gives a purple colour. Finally, sections were washed in distilled water, dehydrated/xylene and mounted with DPS medium. A citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) antigen retrieval step was added to the laminin 1+2 staining protocol.

2.4.2 Immunohistochemistry analysis

Cell counts were performed by light microscopy (Leica, UK) under 40x magnification. A microscope graticule (10 by 10 squares) was used to randomly sample positive cells (microglia in the striatum, and neutrophils or macrophages in the liver) throughout the tissue area, and a micrometer slide with 1mm scale was used for calibration in order to obtain the final number of cells per mm². The total number of neutrophils, and Iba-1⁺,

CD68⁺ and CD206⁺ cells associated with vessels in the striatum were counted in the whole ipsilateral striatum of at least two brain coronal sections approximately 120µm apart. The area of the striatum was measured by tracing it under a Camera Lucida (4x magnification), which was attached to the microscope (Leitz Laborlux S, Leica Biosystems, GE). A 1mm scale was also drawn from the micrometer slide for calibration. All drawings were scanned and the area of the striatum was measured in ImageJ with appropriate calibration. The number of positive cells per mm² was then calculated by dividing the total number of cells by the area of the striatum. The total number of Iba-1⁺ cells in vessels (either in the lumen or attached to vessels) in each brain was represented as the average of the total number of positive cells in two entire brain coronal sections that were approximately 120µm apart and encompassed the striatum.

To schematically represent neutrophil recruitment patterns in the brain, a 10µm coronal section at approximately Bregma +0.5mm immunolabelled for neutrophils was selected from the injection site of mice in each relevant treatment group. The section was viewed at 1.6x magnification using a light microscope attached to a Camera Lucida. The total number of neutrophils were dotted with a black marker pen onto white paper forming a spatial map of the pattern of recruited neutrophils. The brain limit, corpus callosum and ventricles were also drawn with a pencil. Spatial maps were then scanned and converted onto a transparent background using Adobe Photoshop. Spatial maps were superimposed onto coronal figure 23 from *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin).

2.5 mRNA extraction

mRNA was isolated using Rneasy mini kit (Qiagen, UK) following manufacturer's guidelines. Tissue was disrupted and homogenized in Buffer RLT containing 1% of β -Mercaptoethanol (Sigma, UK) using a pellet pestle motor (Kontes, UK). Lysate was transferred directly into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2min at full speed. Supernatant was then carefully mixed with 1 volume of 75% ethanol, transferred to a Rneasy spin column placed in a 2ml collection tube and centrifuged for 15s at 10000 rpm. The flow-through was discarded. Buffer RW1 was added to the column and centrifugation for 15s at 10000 rpm was performed. The flow-through was discarded and the previous step was repeated. The same was done using buffer RPE. The Rneasy spin column was then transferred to a new 1.5ml collection tube. Finally, 25 μ l of nuclease free water was added to the column and the tube was centrifuged for 1min at 10000 rpm to elute the mRNA. The quantity and quality of mRNA were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, UK). A ratio of sample absorbance at 260 and 280 of $\sim$ 2.0 and ratio of sample absorbance at 260 and 230 of 1.8-2.2 were considered as "pure" for mRNA.

2.6 cDNA conversion and Real-Time polymerase chain reaction

mRNA was converted to cDNA using nanoScript2TM reverse transcript kit (Primerdesign, UK) (Table 2.2). Real-Time polymerase chain reaction (RT-qPCR) was performed using a LightCycler[®] 480 (Roche Diagnostics, UK) and SBYR green mastermix (Primerdesign, UK) (Table 2.3). ICAM-1 and E-selectin primers were obtained from InvitrogenTM and, BCRP, P-gp, Caveolin-1, Glut-1, Claudin-5, TNF and CD11b primers were obtained from Roche Life Science. The rest of the primers were obtained as custom designed real-

time PCR assays from Primerdesign (Table 2.4). House-keeping genes were selected by a GeNorm analysis for the same samples (geNormPLUS 12 gene kit, Primerdesign, UK) (Table 2.5). GeNorm is a system for selecting the most appropriate candidate reference gene(s); one or more that are not affected by the experimental conditions and constitutively expressed in all the samples. To perform geNorm the expression of 12 reference (house-keeping) genes was measured by RT-qPCR in a representative set of brain or liver samples, including naïve tissue and tissue from animals injected with saline or IL-1 β into the striatum. Then, the GeNorm software (qbase+) was used to provide a ranking of the reference genes in order of stability of expression throughout the samples and to select the best house-keeping genes for each experiment (Figure 2.1 and 2.2). In detail, the software creates two graphs: the first one (Figure 2.1A and 2.2A) represents the average expression stability value (geNorm M) of reference genes at each step during stepwise exclusion of the least stably expressed reference gene. Starting from the least stable gene at the left, the genes are ranked according to increasing expression stability, ending with the most stable gene on the right; the second graph (Figure 2.1B and 2.2B) determines the optimal number of reference genes by illustrating the variation in average stability of the reference genes (geNorm V). This graph shows sequential addition of each reference gene starting with the two most stably expressed genes on the left with the inclusion of a third, fourth and fifth gene etc. moving to the right. This measure is known as “pairwise variation, $V(n/n+1)$ ”. The Cq values obtained using these selected reference genes are then averaged to give the normalisation factor. The optimal number of reference genes n is found when $V(n/n+1)$ drops below 0.15. E.g. if a $V_{2/3}$ score of 0.25 is achieved using two reference genes but a $V_{3/4}$ score of 0.14 is achieved using a third reference gene then the ideal normalisation factor would be calculated using the top three reference genes in future experiments. In these experiments, the optimal number of reference

targets in this experimental situation is 2 (geNorm $V < 0.15$). As such, the optimal normalization factor can be calculated as the geometric mean of reference targets Zfp91 and Cdc40 for brain (Figure 2.1A and B) and Csnk2a2 and Pak1ip1 for liver (Figure 2.2C and D) (information obtained from qbase^{PLUS} software and geNorm SYBRgreen Handbook). All experimental brain samples were compared to Zfp91 and Cdc40 and liver samples to Csnk2a2 and Pak1ip1. A relative quantification was performed using $\Delta\Delta C_t$ analysis. The results were represented as fold change of naïve expression levels.

2.7 Statistics

Data are presented as mean $\pm$ standard error of the mean (SEM) and considered statistically significant with a $p < 0.05$. Data were analysed using one-way ANOVA with Tukey's multiple comparison test, Kruskal-Wallis with Dunn's multiple comparisons test or two-way ANOVA with Bonferroni–Dunn post-hoc test for multiple groups where appropriate. All details regarding statistical tests and the number of animals used in each group can be found in figure legends.

Table 2.2 Protocol for RNA conversion to cDNA.

Step	nanoScript2™ reverse transcript kit	
1	RNA template (up to 2µg)	x µl
	Oligo-dT primer and Random nonamer primer	1.0 µl
	RNAse/DNAse free water	X µl
	Final volume	10 µl
2	65°C	5 min
3	Ice	
4	nanoScript2 4X Buffer	5.0 µl
	dNTP mix 10mM	1.0 µl
	RNAse/DNAse free water	3.0 µl
	nanoScript2 enzyme	1.0 µl
	Final volume	10 µl
5	25°C	5 min
	42°C	20 min
	75°C	10 min
	-20°C	∞

Table 2.3 Protocol for RT-qPCR.

Step	Primerdesign Ltd - RT-qPCR protocol	
1	PrecisionPLUS-SYBRgreen mastermix	10 µl
	Primer	1.0 µl
	Template (25ng)	5.0 µl
	RNAse/DNAse free water	4.0 µl
	Final volume	20 µl
Enzyme Activation HotStart	95°C	2 min
Denaturation*	95°C	15s
Data collection*	60°C	60s
Melting Curve		

* Cycling x40

Table 2.4 Primers sequences.

Primers	Foward	Reverse
interleukin 1 beta (IL-1 β)	GCTATGGCAACTGTTCTGAA	ACAGCCCAGGTCAAAGGTT
interleukin 6 (IL-6)	AATTCCAGAAACCGCTATGAAGT	ATCCTCTGTGAAGTCTCCTCTC
interleukin 4 (IL-4)	AGCAACGAAGAACACCACAG	GCAGCTCCATGAGAACACTAG
interleukin 10 (IL-10)	GGGAAGAGAAACCAGGGAGAT	GCCACAGTTTCAGGGATGA
interleukin 1 receptor, type I (IL-1R1)	CCCCGAACCGTGAACAAC	AAACGATCTGATTTGGATATTCTGTA
interleukin 1 receptor, type II (IL-1R2)	GGCAAGAAGCAGCAAGGTA	TTACTAACACAAGCAAGATGAACA
interleukin 1 receptor antagonist (IL-1RA)	GACCTTTTACCTGAGAAACAAC	TAGGCACCATGTCTATCTTTTC
chemokine (C-X-C motif) ligand 1 (CXCL-1)	GCTGGGATTACCTCAAGAAC	TGTGGCTATGACTTCGGTTTG
chemokine (C-X-C motif) ligand 2 (CXCL-2)	CAGAAGTCATAGCCACTCTCAAG	AGCCTTGCCTTTGTTCAGTATC
chemokine (C-X-C motif) ligand 5 (CXCL-5)	AAAGATTTCTGAGGACTCTGACC	TTTTCTCATCAAAGCAGGGAGT
Intercellular Adhesion Molecule 1 (ICAM-1)	CCCACGCTACCTCTGCTC	GATGGATACCTGAGCATCACC
E-Selectin	TCCTCTGGAGAGTGGAGTGC	GGTGGGTCAAAGCTTCACAT
Breast Cancer Resistance Protein (BCRP)	GCCTTGGAGTACTTTGCATCA	AAATCCGCAGGGTTGTTGTA
P-glycoprotein (P-gp)	GGGCATTTACTTCAAACCTTGTC	TTTACAAGCTTCATTTCTTAATTCAA
Caveolin-1 (Cav-1)	CCAGGGAAACCTCCTCAGA	CCGGATGGGAACAGTGTAGA
Glucose transporter 1 (Glut-1)	ATGGATCCCAGCAGCAAG	CCAGTGTTATAGCCGAAGTGC
Tumor Necrosis Factor alpha (TNF)	TGCCTATGTCTCAGCCTCTTC	GAGGCCATTTGGGAACCTTCT
Cluster of differentiation molecule 11B (CD11b)	CAATAGCCAGCCTCAGTGC	GAGCCCAGGGGAGAAGTG

Table 2.5 Mouse geNormPLUS kit gene list.

Mouse geNormPLUS kit gene list (<i>Mus musculus</i>)
adaptor-related protein complex 3, delta 1 subunit (Ap3d1), mRNA
casein kinase 2, alpha prime polypeptide (Csnk2a2), mRNA
cell division cycle 40 homolog (yeast) (Cdc40), mRNA
F-box and WD-40 domain protein 2 (Fbxw2), mRNA
F-box protein 38 (Fbxo38), mRNA
HIV TAT specific factor 1 (Htatsf1), mRNA
MON2 homolog (yeast) (Mon2), transcript variant 1, mRNA
PAK1 interacting protein 1 (Pak1ip1), mRNA
zinc finger protein 91 (Zfp91), mRNA
18S rRNA gene
actin, beta, cytoplasmic (ACTB), mRNA
glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA

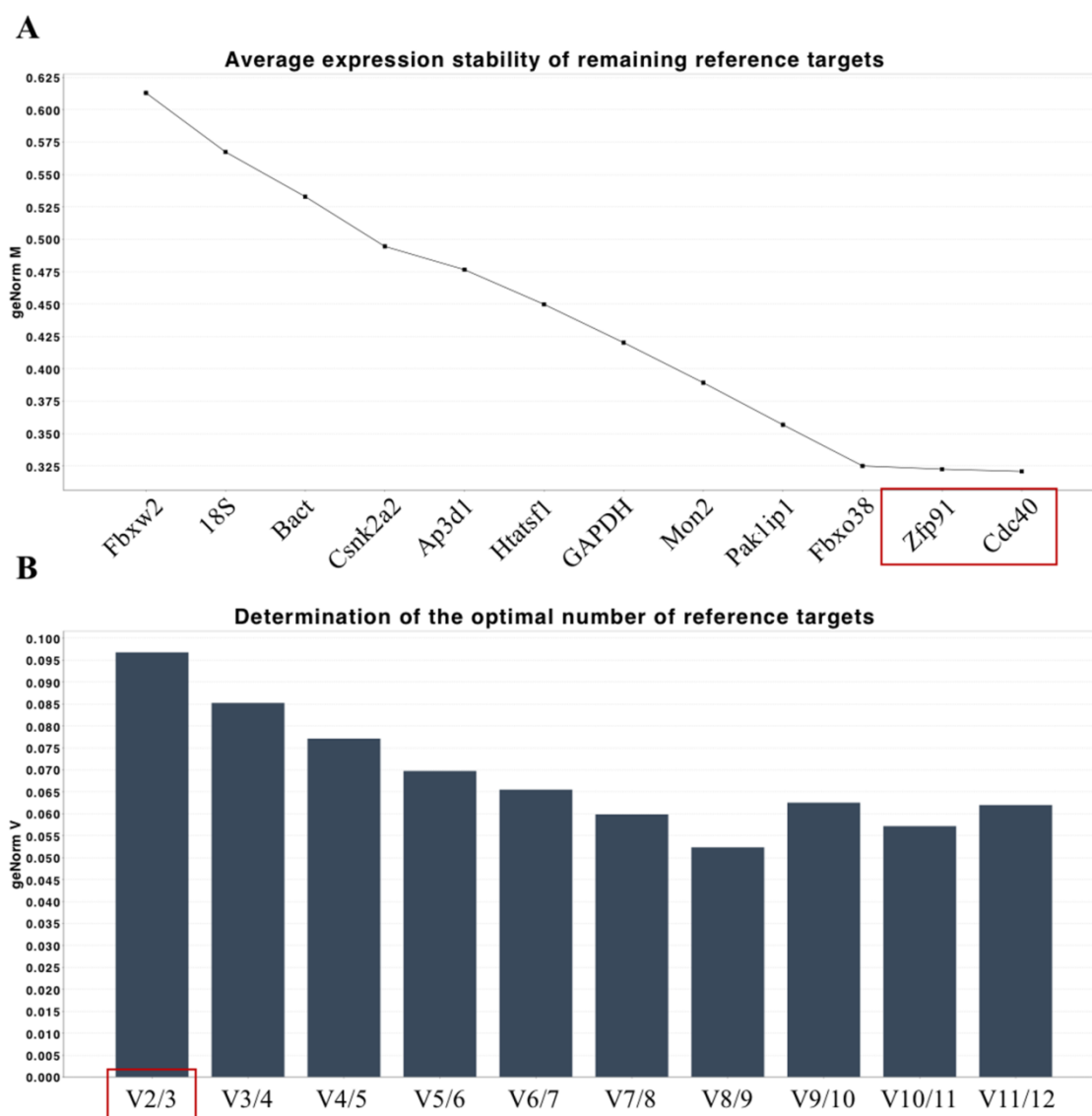


Figure 2.1 GeNorm analysis to select the best candidate reference gene(s) that are not affected by the experimental conditions and constitutively expressed in all brain samples. A) Average expression stability value (geNorm M) of reference genes at each step during stepwise exclusion of the least stably expressed reference gene. B) Determination of the optimal number of reference genes by illustrating the variation in average stability of the reference genes (geNorm V). The optimal normalization factor can be calculated as the geometric mean of reference targets Zfp91 and Cdc40. Information in this legend was obtained from qbasePLUS software and geNorm SYBRgreen Handbook.

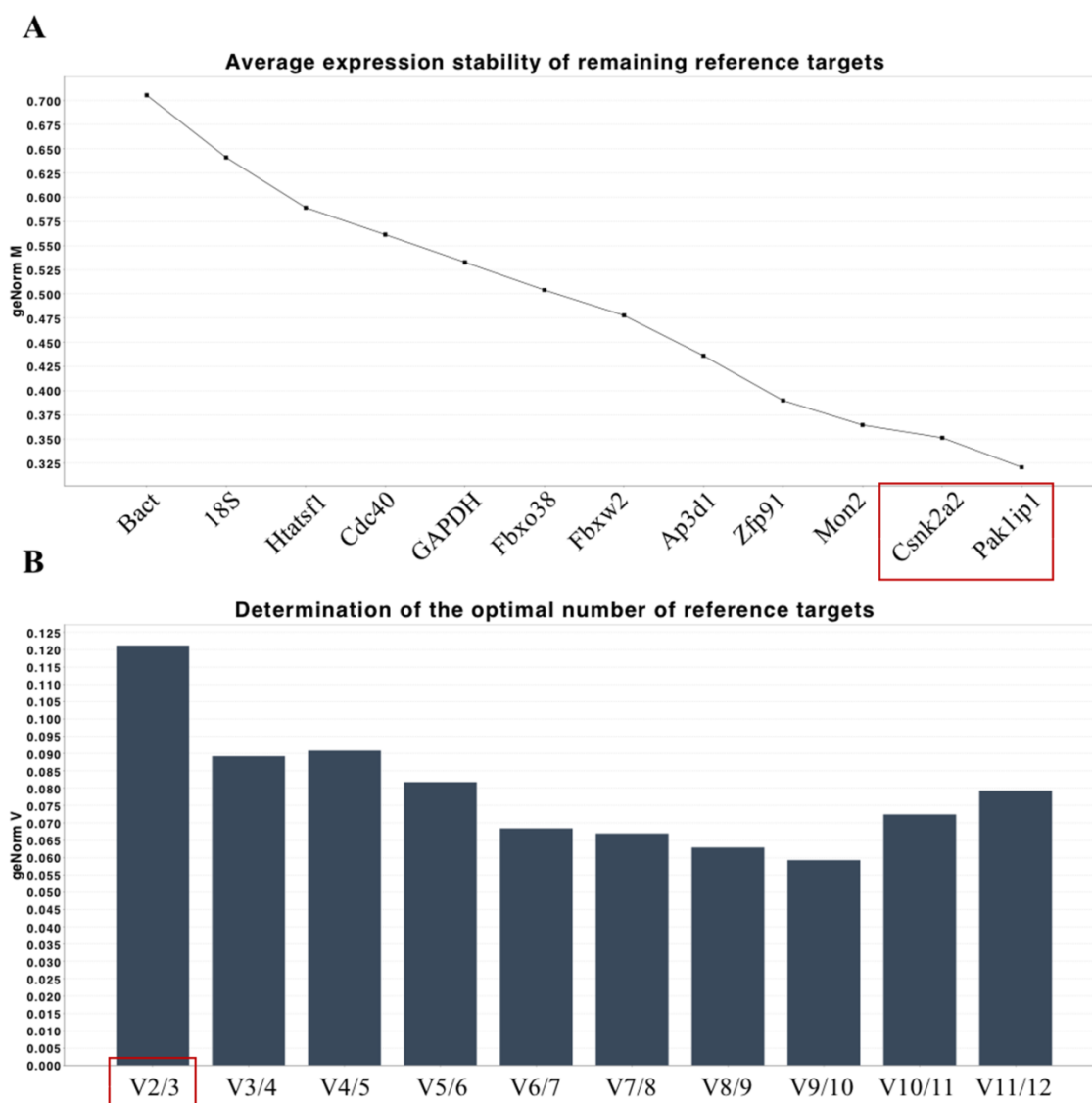


Figure 2.2 GeNorm analysis to select the best candidate reference gene(s) that are not affected by the experimental conditions and constitutively expressed in all liver samples. A) Average expression stability value (geNorm M) of reference genes at each step during stepwise exclusion of the least stably expressed reference gene. B) Determination of the optimal number of reference genes by illustrating the variation in average stability of the reference genes (geNorm V). The optimal normalization factor can be calculated as the geometric mean of reference targets Csnk2a2 and Pak1ip1. Information in this legend was obtained from *qbase^{PLUS}* software and *geNorm SYBRgreen Handbook*.

CHAPTER 3

AN AGE SPECIFIC PERIVASCULAR CELL POPULATION IS FOUND DURING “WINDOW OF SUSCEPTIBILITY” TO BRAIN INFLAMMATION

3.1 Introduction and aims

In this chapter, the response of the brain to focal inflammation was studied during postnatal development in mice. It is clear from previous studies that many immune activators affect neurological processes, often in a manner specific to the stage of brain development. However, the majority of these studies only focused on a specific time point of the brain development and did not consider the effects of immune activation throughout development. This is of particular importance as rodent data suggest the inflammatory response in the brain is increased during development (Campbell et al., 2002) and, therefore, may contribute to the dysfunction of important developmental neuronal processes. Anthony et al. (1997) previously showed that in response to an i.c. inflammation, there was an age-specific increase in BBB permeability which was associated with a substantial influx of neutrophils. While this work showed that the BBB permeability was neutrophil-dependent it did not establish the inflammatory signalling pathways involved in this response. Therefore, in this chapter the acute molecular and cellular responses in the brain to focal inflammation were investigated in order to characterize the molecular profile of the brain inflammatory response that may explain the brain susceptibility to inflammation during development. The principal aims of this chapter were (1) to study BBB integrity, (2) to evaluate leukocyte recruitment to the brain, 3) to measure the expression of various inflammatory mediators in the brain and

(4) to examine the response of microglia and perivascular macrophages, in each case, 4 hours after i.c. injection of 1ng IL-1 β in the striatum of P7, P14 or P21 C57Bl/6 mice.

3.2 Results

3.2.1 Blood-brain barrier disruption and neutrophil recruitment were only observed at P14 after focal inflammation

Focal inflammation in the brain was induced by 1ng IL-1 β injection into the left striatum of C57Bl/6 mice at different postnatal ages including P7, P14 and P21. Naïve or saline injected brains were used as controls. BBB permeability was revealed using an antibody against endogenous IgG, which does not cross the BBB in physiological conditions. In naïve and saline injected brains endogenous IgG could not be observed macroscopically. The injection of 1ng IL-1 β increased BBB permeability in the ipsilateral striatum only at P14 (Figure 3.1A). Increased BBB permeability was not detected in the contralateral striatum at P14, or in brains at P7 and P21 (Figure 3.1A). Microscopic examination of brain immunolabelled for endogenous IgG with cresyl violet counterstain confirmed this observation as IgG was only found in the ipsilateral parenchyma at P14. At P7 and P21 i.c. injection of IL-1 β did not promote IgG extravasation into the parenchyma as IgG was only observed in vessels (Figure 3.1B).

The mRNA expression of different BBB transporters, including GLUT-1, P-gp and BCRP, vesicular trafficking protein caveolin-1, and tight junction proteins claudin-5 and ZO-1 was measured in the ipsilateral striatum injected with saline or 1ng IL-1 β . Within each age group, the expression of all transport proteins, claudin-5 and ZO-1 did not change after 1ng IL-1 β injection in the brain, except claudin-5 at P7 that significantly increased from saline to IL-1 β injected brains (Figure 3.2). GLUT-1 expression

significantly increased from P7 to P14 and P21 ($p < 0.05$) in control brains and from P7 to P21 ($p < 0.001$) in the IL-1 β injected brains (Figure 3.2).

To determine if focal inflammation caused leukocyte migration to the brain parenchyma and if their presence was correlated with BBB permeability, double immunohistochemistry was performed for neutrophils (MBS) and vessels (laminin 1+2) on IL-1 β injected brains and in control brains (naïve and saline injected) (Figure 3.3A). Representative spatial maps of neutrophil distribution were generated using the immunolabelled slides. More neutrophils were recruited to the left hemisphere injected with IL-1 β at P14 compared to P7 and P21 brains (Figure 3.3B). Neutrophils were counted in the entire ipsilateral striatum on the basis of their presence in the brain parenchyma or vessels (either in the lumen or attached to vessels). No neutrophil recruitment was observed in control brains (Figure 3.3A). The number of neutrophils were $11 \pm 2/\text{mm}^2$ in vessels and $23 \pm 10/\text{mm}^2$ in the parenchyma at P7, $8 \pm 1/\text{mm}^2$ in vessels and $85 \pm 21/\text{mm}^2$ in the parenchyma at P14, and $8 \pm 2/\text{mm}^2$ in vessels and $13 \pm 4/\text{mm}^2$ in the parenchyma at P21 (Figure 3.3C). A two-way ANOVA analysis with Bonferroni–Dunn post-hoc test showed that the number of neutrophils in the parenchyma was only significantly higher than in vessels at P14 ($p < 0.001$) and that the number of neutrophils in the parenchyma was significantly higher at P14 than at P7 and P21 ($p < 0.01$) (Figure 3.3C).

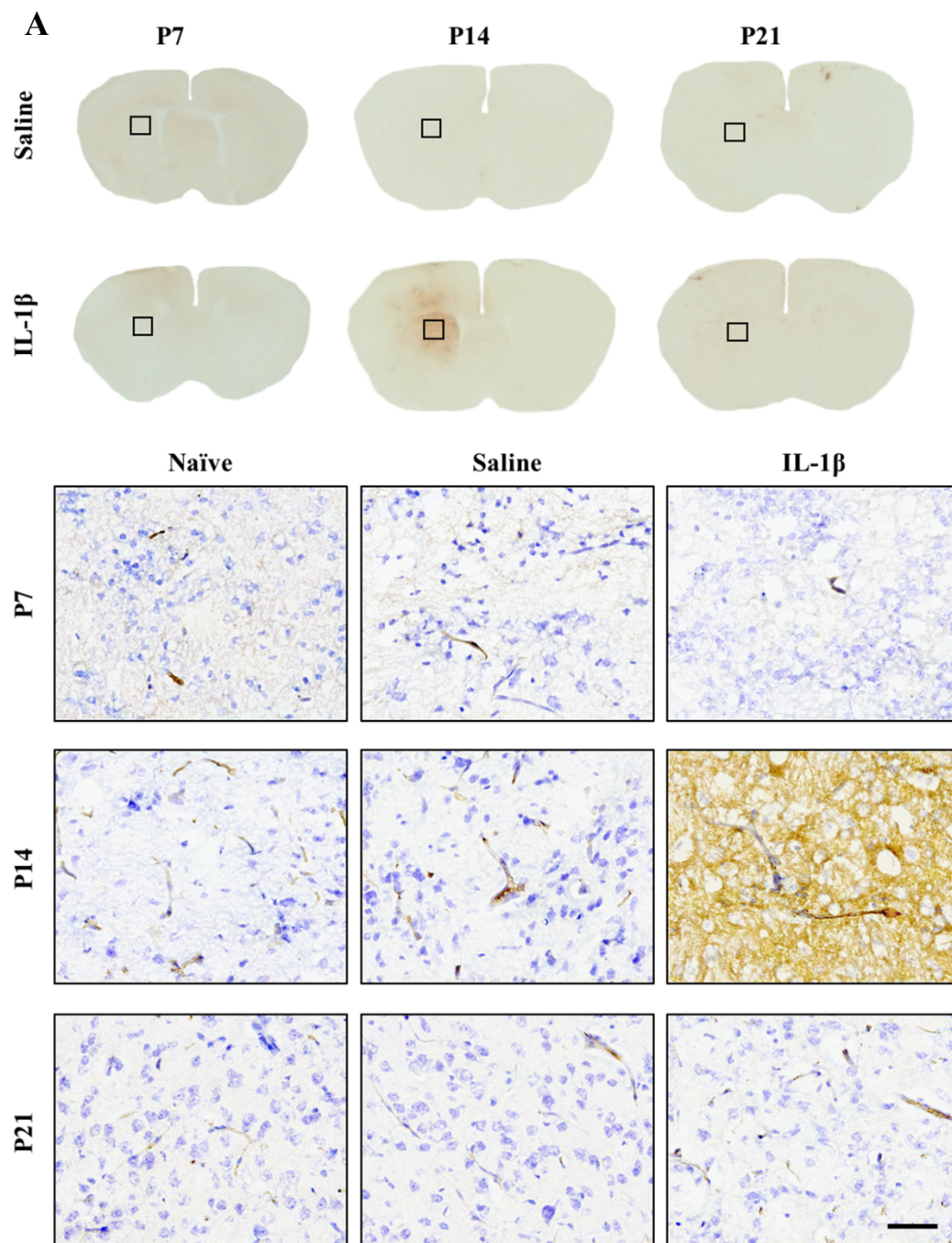


Figure 3.1 BBB permeability after focal inflammation during postnatal development. BBB permeability to endogenous IgG was examined in naïve brains and in brains 4 hours after the i.c. injection of saline or 1ng IL-1 β at P7, P14 or P21 by immunohistochemistry. A) Representative pictures of the whole brain injected with saline or IL-1 β at P7, P14 or P21. B) Representative pictures of IgG immunohistochemistry with cresyl violet counterstain in the ipsilateral striatum of naïve brains and of brains injected with saline or IL-1 β at P7, P14 or P21. Scale bar = 50 μ m. BBB permeability was highest in the ipsilateral striatum at P14. Squares boxes in A represent area from where pictures were taken in B and the injection site. Four brains of each age and condition group were used.

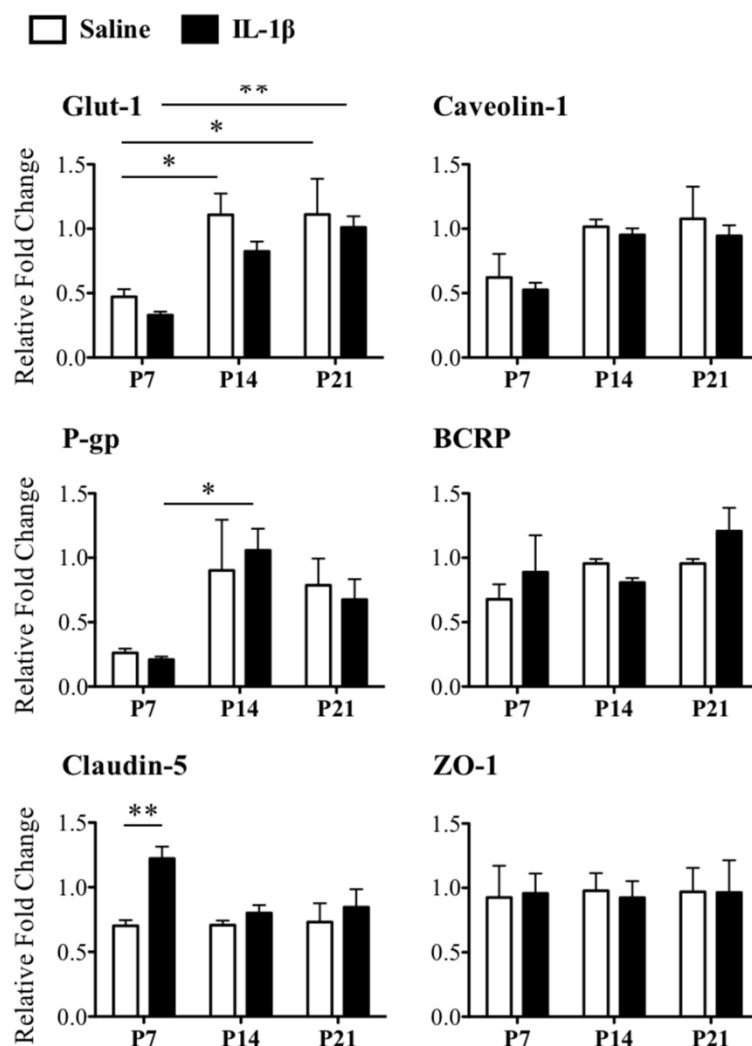


Figure 3.2 mRNA expression of transporters and tight junction proteins in the brain after focal inflammation at different time points during postnatal development. mRNA expression of GLUT-1, caveolin-1, P-gp, BCRP, claudin-5 and ZO-1 was measured by RT-qPCR in the ipsilateral striatum injected with saline or 1ng IL-1 β at P7, P14 or P21. There was no significant decrease in the expression of any of the transcripts measured 4 hours after focal inflammation, suggesting that changes in the expression of these molecules do not explain the increased BBB permeability at P14. Data are represented as relative fold change from contralateral expression levels of P21 naïve mice. Bars represent mean $\pm$ SEM. * p <0.05 and ** p <0.01. Statistics: two-way ANOVA with Bonferroni–Dunn post-hoc test. n =4 each group.

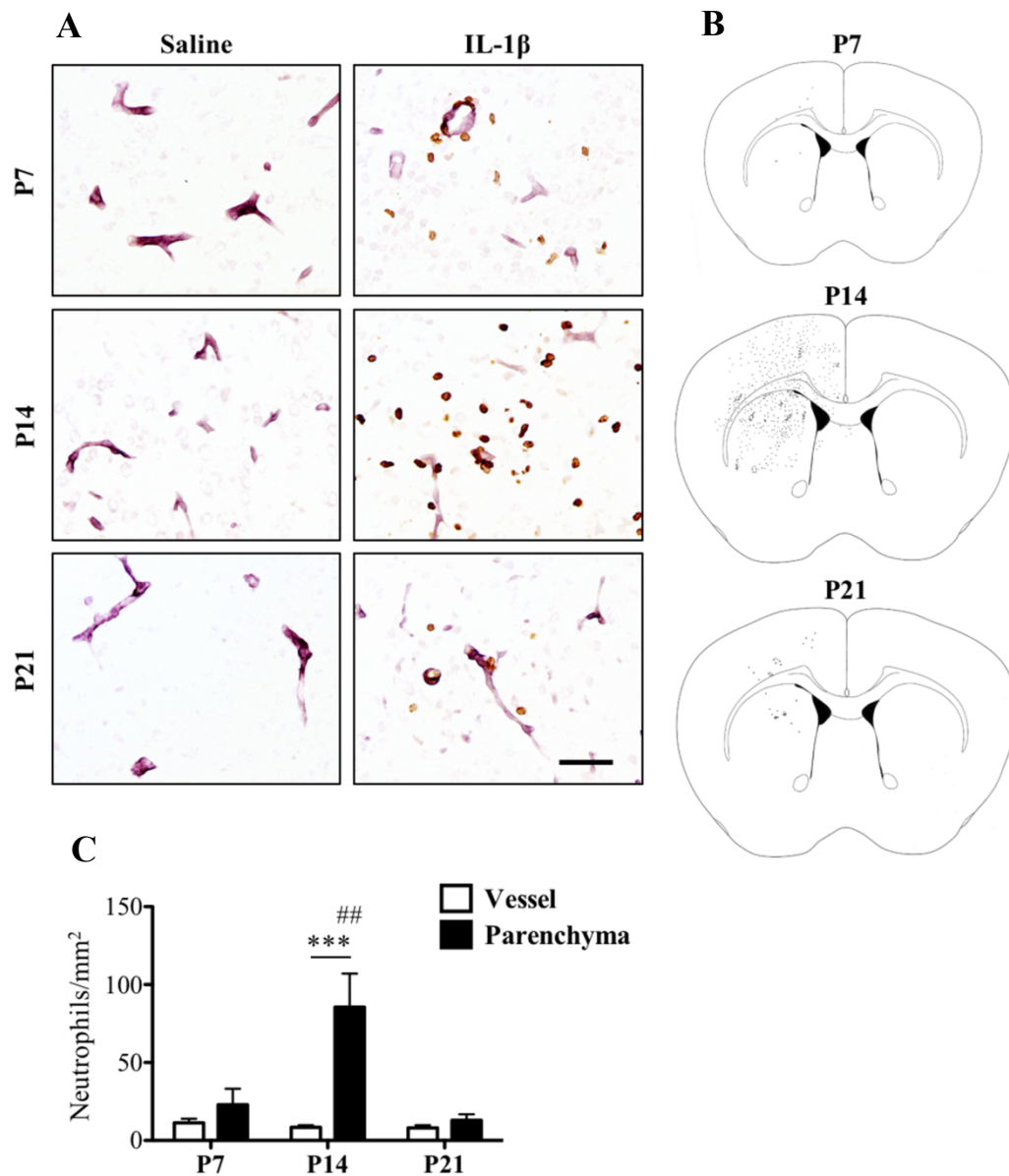


Figure 3.3 Recruitment of neutrophils to brain parenchyma after focal inflammation during postnatal period. Double immunohistochemistry for neutrophils (MBS, brown) and vessels (laminin 1+2, purple) on brains injected with saline or 1ng IL-1 β was performed (A). Scale bar = 50 μ m. Representative spatial maps of neutrophil distribution were obtained using a representative coronal immunolabelled section from each treatment group (B). Spatial maps of saline injected brains are not shown as no neutrophils were found in these brains. More neutrophils were present in the P14 ipsilateral hemisphere 4 hours after the injection of IL-1 β when compared to P7 and P21. Neutrophils were counted on the basis of their presence in the parenchyma or in vessels (C). More neutrophils were observed in the parenchyma at P14 than at P7 and P21 after focal inflammation. Data are represented as number of neutrophils per mm² of striatum. ***p<0.001, ##p<0.01 vs. P7, P21 parenchyma values. Statistics: two-way ANOVA with Bonferroni–Dunn post-hoc test. P7: n=4, P14: n=5 and P21: n=4.

3.2.2 The expression of key inflammatory mediators at basal level and after inflammation in the brain were age-dependent

To determine if age-dependent basal or post-injury levels of inflammatory mediators contributed to the observed increase in BBB permeability and neutrophil recruitment at P14, RT-qPCR for different inflammatory mediators was performed. The mRNA expression of both pro- and anti-inflammatory cytokines, IL-1 β receptors, adhesion molecules and chemokines was measured in naïve contralateral striatum, and in the ipsilateral and contralateral striatum 4 hours after saline or 1ng IL-1 β injection. mRNA expression levels were represented as relative fold change from contralateral expression levels of P21 naïve mice.

In naïve striatum, mRNA expression of pro-inflammatory cytokines, including IL-1 β and IL-6, was highest at P7 (Figure 3.4). IL-1 β mRNA was upregulated at P7 (2.34 ± 0.34) when compared to P14 (1.69 ± 0.30) and P21 (1.02 ± 0.13) though only P7 and P21 levels were significantly ($p < 0.05$) different. IL-6 expression was significantly higher at P7 (2.07 ± 0.26) than at P14 (1.18 ± 0.06) and P21 (1.03 ± 0.14). Anti-inflammatory cytokines, IL-4 and IL-10, presented different expression profiles from each other. IL-4 was highly expressed ($p < 0.05$) in P7 (2.35 ± 0.39) and P14 mice (2.45 ± 0.07) when compared to P21 animals (1.08 ± 0.21) and IL-10 was highest at P7 (2.09 ± 0.33) and significantly ($p < 0.05$) decreased from P7 to P14 (0.91 ± 0.14) and P21 (1.05 ± 0.17). IL-1RA was also highest in P7 mice ($p < 0.01$, P7: 3.34 ± 0.47 vs. P14: 1.21 ± 0.13 and P21: 1.05 ± 0.19). In contrast to the pattern for the previously mentioned transcripts, IL-1R1 expression significantly ($p < 0.05$) increased from P7 (0.46 ± 0.08) and P14 (0.46 ± 0.04) to P21 (1.08 ± 0.24). IL-1R2 was significantly ($p < 0.01$) higher in P7 (1.76 ± 0.19) animals compared to P14 (0.95 ± 0.03) and P21 (1.03 ± 0.15) mice. Expression of the adhesion molecule

ICAM-1 in the naïve brain did not change throughout postnatal development, but E-selectin expression was significantly ($p < 0.05$) higher at P7 (1.38 ± 0.05) than at P14 (1.04 ± 0.01) and P21 (1.01 ± 0.10). Finally, CXCL-1 exhibited a similar mRNA expression pattern to E-selectin. This chemokine significantly decreased from P7 (1.76 ± 0.27) to P14 (0.62 ± 0.05) and P21 (1.03 ± 0.14) in the naïve brain.

Four hours after i.c. injection of saline or 1ng IL-1 β , mRNA expression of the inflammatory mediators discussed above was differentially upregulated in the ipsilateral striatum (Figure 3.5 and 3.6). A two-way ANOVA with Bonferroni-Dunn post-hoc test was performed at the different ages in order to compare ipsilateral and contralateral (internal control) expression levels within each treatment group and to compare ipsilateral or contralateral expression levels between treatment groups. IL-1 β expression did not change at P7, but was significantly upregulated in the IL-1 β ipsilateral striatum at P14 (32.24 ± 12.82 vs. 7.27 ± 3.26 IL-1 β contralateral ($p < 0.05$) and 1.78 ± 0.54 ipsilateral saline ($p < 0.01$)) and P21 (224.60 ± 83.03 vs. 16.74 ± 10.22 IL-1 β contralateral ($p < 0.01$) and 1.00 ± 0.06 ipsilateral saline ($p < 0.01$)). IL-6 expression significantly ($p < 0.05$) increased from saline ipsilateral striatum (2.88 ± 0.86) to IL-1 β ipsilateral striatum (9.09 ± 2.92) at P7. IL-6 was also upregulated in the IL-1 β ipsilateral striatum of P21 animals (121.10 ± 45.44 vs. 10.13 ± 5.65 IL-1 β contralateral ($p < 0.01$) and 1.63 ± 0.42 saline ipsilateral ($p < 0.01$)). No changes in expression of anti-inflammatory cytokines (IL-4 and IL-10) were detected following IL-1 β injection at any point during postnatal development. IL-1RA mRNA expression increased in the IL-1 β ipsilateral striatum in all ages. At P7, IL-1RA expression was upregulated in the IL-1 β ipsilateral striatum (8.79 ± 1.63) though only significantly ($p < 0.05$) different from saline ipsilateral striatum (2.58 ± 0.35) and not from IL-1 β contralateral striatum (7.67 ± 2.80). IL-1RA

expression in the IL-1 β ipsilateral striatum significantly increased at P14 (8.79 ± 1.63 vs. 7.67 ± 2.80 IL-1 β contralateral ($p < 0.05$) and vs. 2.58 ± 0.35 saline ipsilateral ($p < 0.01$)) and at P21 (40.50 ± 20.63 vs. 3.73 ± 1.76 IL-1 β contralateral ($p < 0.05$) and 1.10 ± 0.17 saline ipsilateral ($p < 0.05$)) (Figure 3.5). IL-1R1 mRNA expression in the IL-1 β ipsilateral striatum was only upregulated in P21 mice (2.50 ± 0.30 vs. 1.60 ± 0.22 IL-1 β contralateral ($p < 0.5$) and 1.24 ± 0.20 saline ipsilateral ($p < 0.01$)). In comparison, the expression of the second IL-1 β receptor, IL-1R2, significantly ($p < 0.05$) increased in the IL-1 β ipsilateral striatum at P14 (1.46 ± 0.35) when compared to saline ipsilateral striatum (0.79 ± 0.09) and not to IL-1 β contralateral striatum (1.02 ± 0.11). ICAM-1 was upregulated in the IL-1 β ipsilateral striatum (8.72 ± 1.85) of P7 animals though only significantly ($p < 0.05$) higher than saline ipsilateral striatum (3.81 ± 0.95). The expression of this adhesion molecule in the IL-1 β ipsilateral striatum increased at P14 (9.17 ± 1.84 vs. 4.87 ± 0.74 IL-1 β contralateral ($p < 0.05$) and 3.33 ± 0.21 saline ipsilateral ($p < 0.01$)) and P21 (20.11 ± 3.31 vs. 4.17 ± 1.06 IL-1 β contralateral ($p < 0.001$) and 3.51 ± 0.32 saline ipsilateral ($p < 0.001$)). E-selectin mRNA expression was upregulated in the IL-1 β ipsilateral striatum (1.65 ± 0.44) at P14, but was only significantly ($p < 0.05$) different from saline ipsilateral striatum (0.48 ± 0.10) and not from IL-1 β contralateral striatum (1.21 ± 0.24). The expression of this adhesion molecule was also significantly upregulated at P21 (10.86 ± 2.95 vs. 1.23 ± 0.41 IL-1 β contralateral ($p < 0.001$) and 1.14 ± 0.66 saline ipsilateral ($p < 0.001$)). Finally, CXCL-1 significantly ($p < 0.05$) increased from saline ipsilateral striatum (1.92 ± 0.32) to IL-1 β ipsilateral striatum (56.32 ± 32.31) at P14 and was significantly ($p < 0.01$) upregulated in the IL-1 β ipsilateral striatum at P21 (456.00 ± 169.80 vs. 34.72 ± 21.88 IL-1 β contralateral and 1.34 ± 0.69 saline ipsilateral) (Figure 3.6). A two-way ANOVA analysis with Bonferroni–Dunn post-hoc test showed that the

expression of IL-1 β , IL-6, IL-1RA, IL-1R1, ICAM-1, E-selectin and CXCL-1 was highest in the IL-1 β ipsilateral striatum at P21 when compared to the other ages.

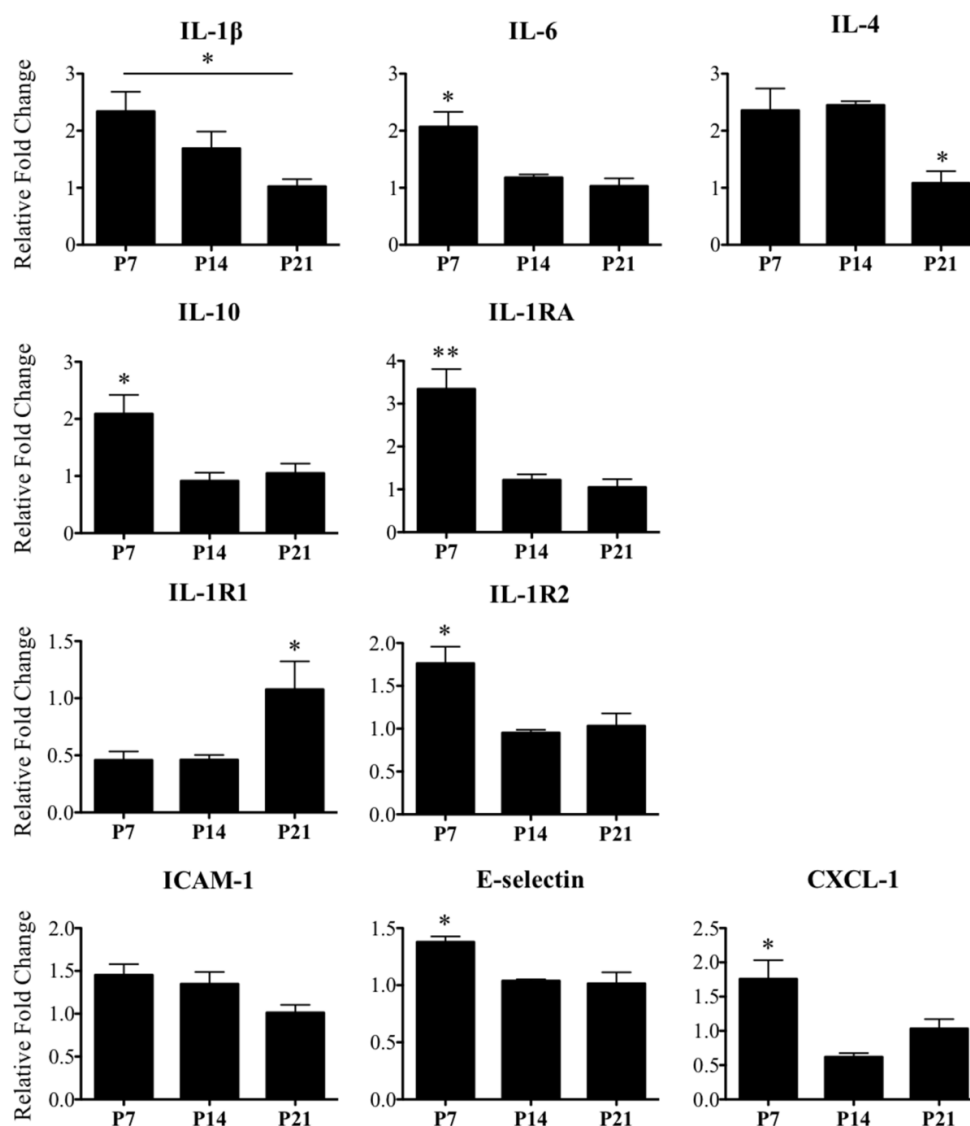


Figure 3.4 mRNA expression of pro- and anti-inflammatory mediators in the brain during postnatal development. IL-1 β , IL-6, IL-4, IL-10, IL-1RA, IL-1R1, IL-1R2, ICAM-1, E-selectin and CXCL-1 mRNA was measured by RT-qPCR in the naïve contralateral striatum of C57Bl/6 mice at P7, P14 and P21. The expression of most inflammatory mediators measured, including IL-1 β , IL-6, IL-10, IL-1RA, IL-1R2, E-selectin and CXCL-1, was higher at P7 than at P14 and P21. IL-4 was upregulated at P7 and P14 compared to P21 and IL-1R1 was increased at P21 and not at P7 and P14. Data are represented as relative fold change from expression levels of P21 naïve mice. Samples were compared with two housekeeping genes (Zpf91 and Cdc40). Bars represent mean $\pm$ SEM. * p <0.05 and ** p <0.01. Statistics: one-way ANOVA with Tukey's multiple comparison test. n =4 each group.

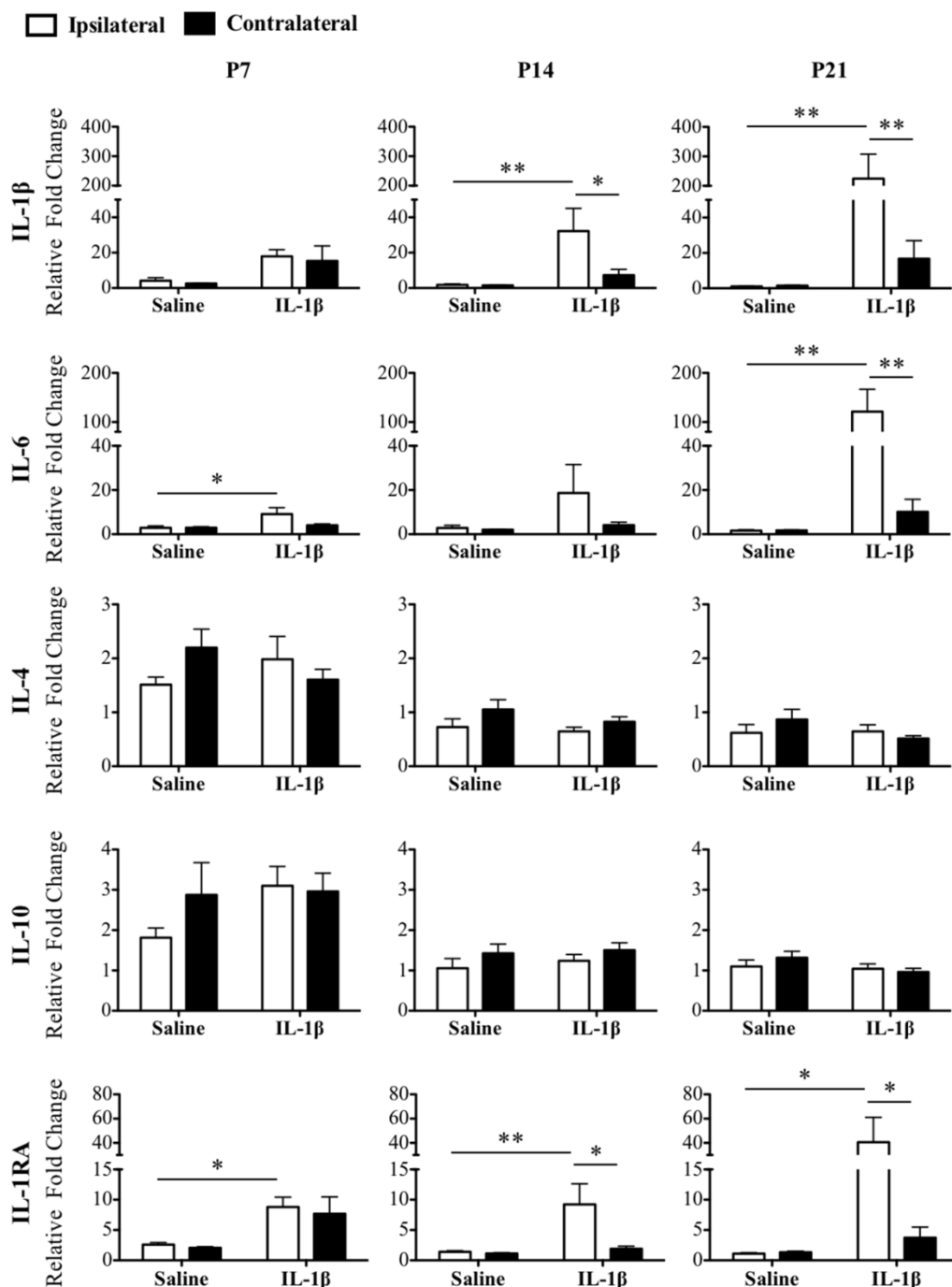


Figure 3.5 mRNA expression of pro- and anti-inflammatory cytokines in the brain after focal inflammation during postnatal period. IL-1 β , IL-6, IL-4, IL-10, and IL-1RA mRNA was measured by RT-qPCR in the ipsilateral and contralateral striatum from mice that received i.c. injection of saline or 1ng IL-1 β at P7, P14 or P21. The expression of IL-4 and IL-10 did not change at any time point but the expression of IL-1 β , IL-6, and IL-1RA was upregulated in the ipsilateral striatum injected with 1ng IL-1 β of older mice. Data are represented as relative fold change from contralateral expression levels of P21 naïve mice. Samples were compared with two housekeeping genes (Zpf91 and Cdc40). Bars represent mean $\pm$ SEM. * p <0.05 and ** p <0.01. Statistics: two-way ANOVA with Bonferroni–Dunn post-hoc test. At least $n=8$ each group.

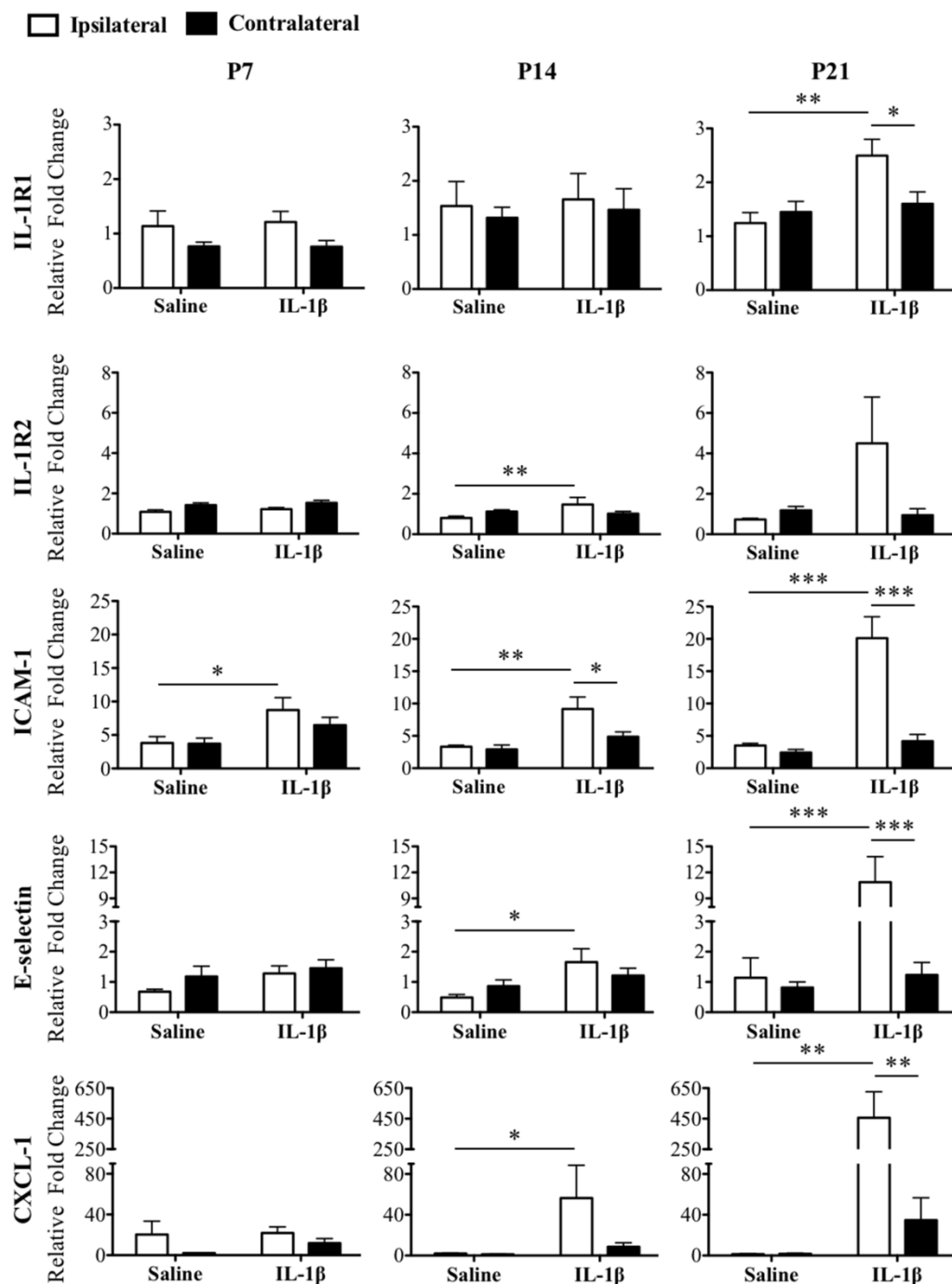


Figure 3.6 mRNA expression of IL-1β receptors, adhesion molecules and chemokines in the brain after focal inflammation during postnatal period. IL-1R1, IL-1R2, ICAM-1, E-selectin and CXCL-1 mRNA was measured by RT-qPCR in the ipsilateral and contralateral striatum from mice that received i.c. injection of saline or 1ng IL-1β at P7, P14 or P21. The expression of IL-1R1, ICAM-1, E-selectin and CXCL-1 was upregulated in the ipsilateral striatum injected with 1ng IL-1β of older mice. Data are represented as relative fold change from contralateral expression levels of P21 naïve mice. Samples were compared with two housekeeping genes (Zpf91 and Cdc40). Bars represent mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001. Statistics: two-way ANOVA with Bonferroni–Dunn post-hoc test. At least n=8 each group.

3.2.3 Microglia responded to inflammation from P14 onwards

After showing that upregulation of inflammatory mediators does not clearly correspond with the "window of susceptibility" to 1ng IL-1 β at P14, other contributors to neuroinflammation were assessed. The Iba-1 antibody was used to analyse microglia population in the striatum at the different postnatal ages (Figure 3.7). The number of microglia in the naïve striatum significantly ($p < 0.001$) increased throughout the postnatal period (P7: 82 ± 4 cells/mm², P14: 129 ± 4 cells/mm² and P21: 161 ± 6 cells/mm²; Figure 3.7A and B). The number of microglia did not change after the 1ng IL-1 β injection into the striatum (Figure 3.7B). At P7, the morphology of microglia did not change after i.c. injection of IL-1 β at P7, but these cells presented a very different morphology when compared to naïve and saline injected brains at later age. P7 microglia were dark stained, rounded and with very few thick long processes and changed towards a morphology with many long and thin processes throughout postnatal development (Figure 3.7C). The morphology of the cells acquired an activated amoeboid state from P14 onwards after focal inflammation. These cells had fewer, shorter processes than the cells in saline injected brains (Figure 3.7C).

3.2.4 A specific cell population was identified at P14

Microglia response to focal inflammation was not shown to be correlated with BBB permeability or neutrophil recruitment at P14. However, Iba-1⁺ large flattened irregular perivascular cells (IPCs) were observed around vessels in P14 naïve brains (Figure 3.8A) both in the striatum and the cortex. Iba-1 (brown) and GLUT-1 (purple) double immunohistochemistry showed that more IPCs per mm² of striatum were observed around vessels in naïve P14 animals when compared to P7 and P21 ($p < 0.05$, P14: 4.1 ± 1.0 /mm² vs. P7: 0.7 ± 0.2 /mm² and P21: 0.5 ± 0.1 /mm²) (Figure 3.8B and C). These cells

were still present after i.c. injection of 1ng IL-1 β in the left striatum, and they were found to be associated with neutrophil recruitment, which was observed after double immunohistochemistry for Iba-1 (brown) and neutrophils (purple) (Figure 3.8D). CD68 and CD206 antibodies were also used to label macrophages in the brain in order to verify if the specific perivascular macrophage population could also be immunoreactive for these markers. CD68⁺ and CD206⁺ cells with the same perivascular morphology as IPCs in close association with vessels were counted in the striatum. Very few CD68⁺ IPCs were present in the brain at any time point of postnatal development and the number of these cells did not vary between P7, P14 and P21 (P7: $1.3 \pm 1.0/\text{mm}^2$, P14: $1.5 \pm 0.6/\text{mm}^2$ and P21: $2.0 \pm 0.7/\text{mm}^2$) (Figure 3.8E). Regarding CD206 marker, the number of positive cells were $0.4 \pm 0.1/\text{mm}^2$ at P7, $1.9 \pm 0.3/\text{mm}^2$ at P14 and $2.1 \pm 0.3/\text{mm}^2$ at P21. Once again, very few CD206⁺ IPCs were observed in every age group, but it significantly increased from P7 to P21 ($p < 0.05$) (Figure 3.8F).

3.3 Discussion

The results in this chapter clearly demonstrated that susceptibility to acute inflammation changed throughout postnatal development in mice. A "window of susceptibility" to focal inflammation was found at P14 and this was associated with the presence of age-specific Iba1⁺ IPCs. More neutrophils were recruited to the brain parenchyma and BBB permeability to endogenous IgG was visible at P14. While the presence of a novel macrophage population at P14 was the only unique finding to explain the vulnerability of the brain at this age, it is likely that other aspects of the developmental stage of the brain also contribute to this "window of susceptibility".

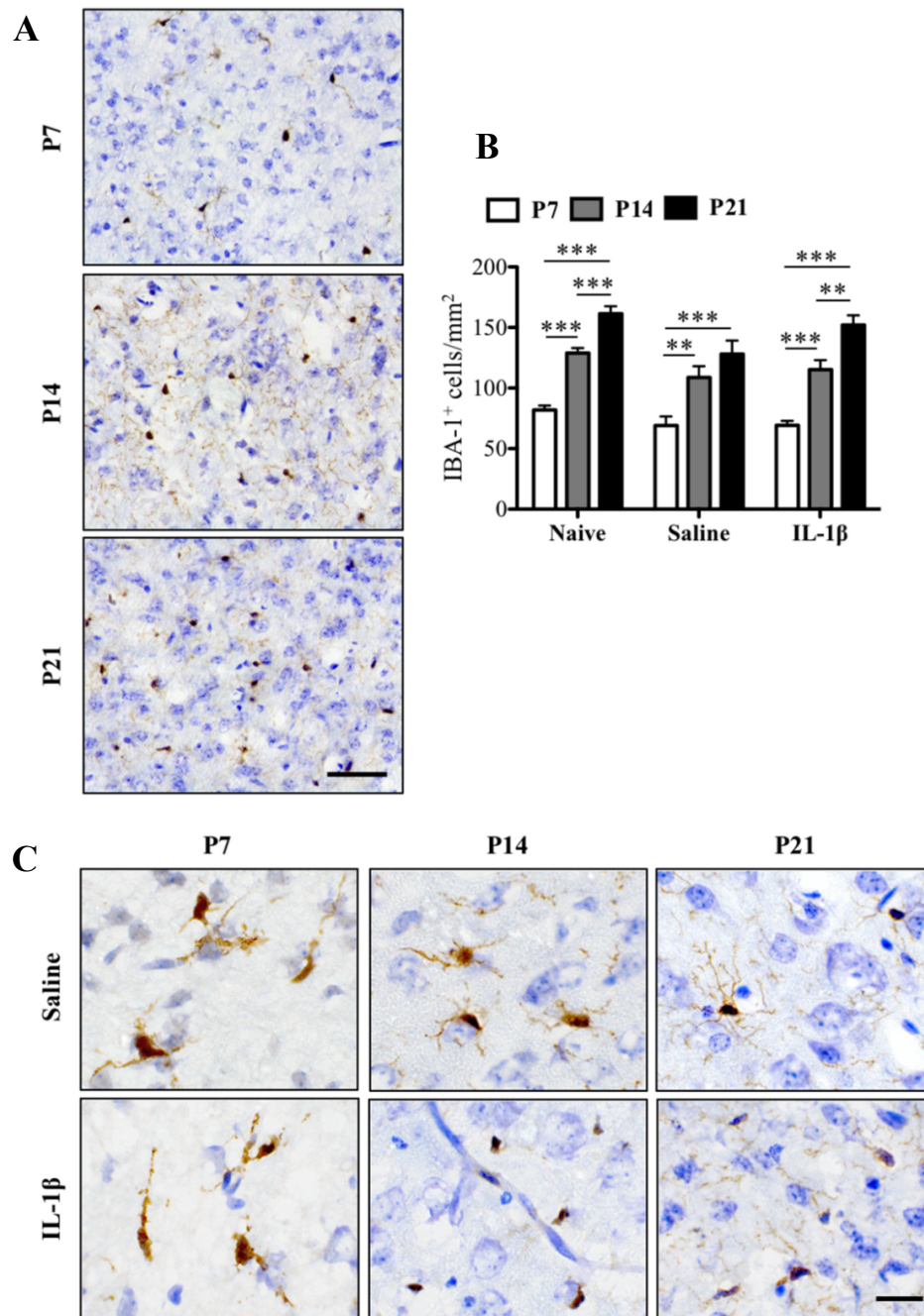


Figure 3.7 Microglia population in naïve mice and after focal inflammation during postnatal development. Microglia population at P7, P14 and P21 was studied after immunohistochemistry using Iba-1 antibody. The number of microglia increased with age. This can be seen in the micrographs showing microglia in the naïve brain (A) and graph showing the results of blinded cell counts (B). The number of cells did not change after i.c. injection of 1ng IL-1 β at P7, P14 or P21 (B). Data are represented as number of microglia per mm². ***p<0.001. Statistics: two-way ANOVA with Bonferroni–Dunn post-hoc test. P7: n=6 for naïve, n=4 for saline and 1ng IL-1 β ; P14: n=10 for naïve, n=5 for saline and 1ng IL-1 β ; P21: n=11 for naïve, n=4 for saline and 1ng IL-1 β . P7 microglia in control brains showed a distinct amoeboid morphology from P14 and P21 (C). Microglia morphology changed to a more amoeboid and activated state from saline to IL-1 β injected brains at P14 and P21 (C). Scale bar = 50 μ m.

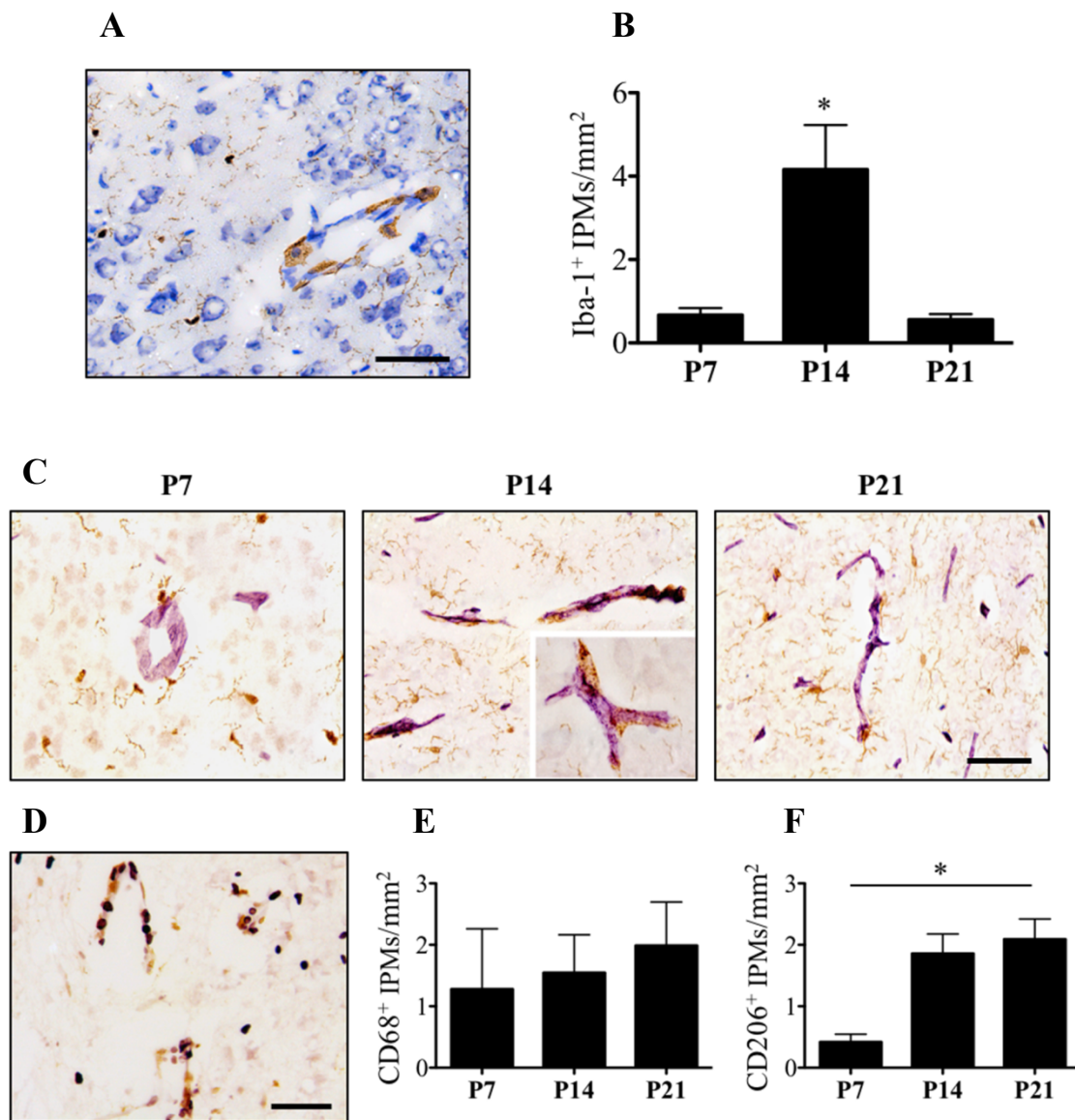


Figure 3.8 Perivascular macrophage population in the brain during postnatal development. Immunohistochemistry for microglia using Iba-1 antibody revealed not only Iba-1⁺ cells in the parenchyma but also around vessels, which were identified by cresyl violet counterstain, in the naïve P14 brains (A). The number of these cells, which were large, flattened and irregular (IPCs), was obtained after double immunohistochemistry for Iba-1 (brown) and vessels (GLUT-1; purple) in P7, P14 and P21 naïve brains. The number of these cells significantly increased in the naïve brain at P14 when compared to P7 and P21 (B and C) and there were in close association with neutrophils (D – double immunohistochemistry for Iba-1 (brown) and neutrophils (purple)) after focal inflammation (D). Quantification of perivascular cells after immunohistochemistry for CD68 and CD206 was also performed but this did not reveal any specific IPC population in the naïve brain at P14 (E and F). Data are represented as number of cells per mm² of striatum. * $p < 0.05$. P7: $n = 5$. P14: $n = 8$ and P21: $n = 9$. Scale bar = 50 μ m. IPCs: Large flattened irregular perivascular cells.

BBB permeability to endogenous IgG was detected after IL-1 β injection in the left striatum at P14. IgG molecules are approximately 155,000Da in size and can be used to look for increases in BBB permeability (Saunders et al., 2015). While a small percentage (0.1%) of IgG molecules can normally cross the BBB (Obermeier et al., 2013), the use of naïve and saline controls in these experiments clearly showed that the presence of IgG after inflammation results from the IL-1 β injection. IgG as a marker of BBB permeability has the advantage of being an endogenous molecule and, therefore, avoids any adverse effects from injecting exogenous molecules. However, the use of a combination of different sized molecules is important to dissect the properties of BBB permeability. Biotin or fluorophore labelled-dextran are polysaccharides of glucose molecules that can be purchased in a range of sizes, from 1500 to 2,000,000Da. These can be injected into the circulation and, consequently, detected in brain parenchyma when BBB permeability increases. Moreover, radiolabelled molecules, such as sucrose (342.3Da) and inulin (7000Da), can be quantified in the brain and, therefore, can be used to discover the extent of BBB permeability, which is not achieved with IgG or dextran (Saunders et al., 2015).

The increased BBB permeability at P14 was correlated with increased number of neutrophils recruited to brain parenchyma in the ipsilateral striatum injected with 1ng IL-1 β . Neutrophils are major contributors during acute inflammation and they quickly migrate to the injury site in order to resolve the damage (Kolaczowska and Kubes, 2013). They can also produce high levels of ROS that may affect BBB integrity by activation of matrix metalloproteinases, oxidative damage, modulation of tight junction proteins, cytoskeletal reorganization, and induction of inflammatory mediators (Pun et al., 2009). Previous work has shown that 1ng IL-1 β injected to the left striatum of P21 rats promoted an acute increase in BBB permeability in a neutrophil-dependent manner (Anthony et al., 1998, 1997). Shaftel et al. (2007) have also shown that IL-1 β can

increase BBB permeability and leukocyte recruitment in mice, however, in their model of chronic and sustained (for 2 weeks) IL-1 β production in the hippocampus, the change in BBB permeability was not neutrophil dependent. The respective acute and chronic assessment is likely to be the major explanation for the different outcomes reported in these studies. In an acute inflammatory lesion, neutrophils are expected to be major and only contributors; however, other type of cells can be recruited and, consequently, may be responsible for the increased BBB permeability, as the inflammatory lesion develops. Indeed, Shaftel et al. (2007) have shown the presence of other leukocytes, including dendritic cells and CD3⁺ T lymphocytes in the hippocampus after chronic expression of IL-1 β , but did not specifically investigate their role in BBB leakage. These previous results (Anthony et al., 1998, 1997; Shaftel et al., 2007) support the hypothesis that the impact of neutrophils on the BBB integrity may vary between acute and chronic models of inflammation and, therefore, it is likely that neutrophils are a major contributor to the BBB disruption in our acute brain inflammation mice model. However, depletion of neutrophils before the i.c. injection of IL-1 β at P14 should be performed in order to confirm this assumption.

BBB breakdown can be characterized by different features, including reduced tight junctions expression and redistribution, impaired efflux and influx function and, disrupted basement membrane (Obermeier et al., 2013). In this study, no reduction in mRNA levels of efflux, influx and vesicular transporters as well as tight junctions were observed. However, it is important to consider that brain inflammation can decrease efflux transporters activity without changing their expression during development (Harati et al., 2012). Likewise, though mRNA expression levels for tight junction proteins were not changed, the protein distribution and structure might have been affected (Coisne and Engelhardt, 2011). Previous data have shown that occludin and ZO-1 were absent from

vessels presenting extensive neutrophil recruitment in the striatum of P21 rats that received i.c. injection of IL-1 β (Bolton et al., 1998). Therefore, future experiments ought to study tight junction distribution and transport activity in order to discover if these are really not affected by i.c. injection of 1ng IL-1 β .

This is not the first study to show a developmental susceptibility to inflammation, but adds data to support the observation that susceptibility to inflammation may vary with type, site of injury, as well as species. Anthony et al. (1997) showed that IL-1 β injection in the striatum of only P21 Lewis rats resulted in neutrophil recruitment and BBB disruption when compared to P0 and adult rats. Lawson and Perry (1995) have shown that LPS injection in the striatum of BALB/c mice promoted an increased neutrophil recruitment to the brain at P7 and not at P0 (Lawson and Perry, 1995). LPS injection peripherally in Sprague-Dawley rats transiently increased BBB permeability in the first postnatal week (Stolp et al., 2005). In addition, i.p. injection of LPS followed by hypoxia-ischemia increased neutrophils recruitment to the brain and BBB permeability at P12 and not at P1 in Lewis rats (Brochu et al., 2011). These differences may result not only from the immunogen-specific molecular cascades but also from the distinct brain development timeline between rats and mouse (Semple et al., 2013; Workman et al., 2013). This may explain, in part, the differences in the “window of susceptibility” to i.c. injection of IL-1 β observed in this chapter (P14 mice) and in previous data (P21 rats; (Anthony et al., 1997)).

These results showed, for the first time, that a Iba-1⁺ IPC population was present at the susceptible time point during brain development. However, these cells were not revealed by the other markers used, including CD68, which may be expressed by microglia in steady state and upregulated when these cells are activated (Perry and Teeling, 2013), or CD206, which was been shown to be specifically expressed by perivascular macrophages

(Galea et al., 2005; Goldmann et al., 2016). This observation suggests that the specific Iba-1⁺ IPCs found at P14 may not express either CD68 or CD206. The fact that almost no CD206⁺ IPCs were found at P14 suggests that Iba-1⁺ IPCs may not be the typical perivascular macrophages found in normal adult mouse brain. In fact, Goldmann et al. (2016) have demonstrated that perivascular cells could be distinguishable from microglia on the basis of their expression of CD206 in the adult brain. Moreover, Iba-1⁺ IPCs found at P14 presented bigger and larger cell bodies than the adult CD206⁺ perivascular macrophages found by Goldmann et al. (2016). Thus, here it is hypothesized that there is a specific developmental pattern of blood-borne monocytes/macrophages recruitment to the perivascular space during postnatal period, which may alter the responsiveness of the brain or BBB to inflammation. After i.c. injection of IL-1 β , neutrophils were observed in very close association with these cells. The correlation between the influx of Iba-1⁺ IPCs and neutrophil recruitment observed in this study strongly supports the hypothesis of a common pathway for cellular entry to the brain parenchyma and, consequently, for BBB disruption in response to the focal inflammation at P14. Previous evidence has shown that neutrophils are preferentially extravasated from inflamed dermal venules in close proximity to perivascular macrophages in adult mice (Abtin et al., 2014). In addition, perivascular macrophages have been suggested to be important for the recruitment of leukocytes in response to injury as these cells can express chemokines and other inflammatory mediators (Nourshargh and Alon, 2014). However, it still remains unclear why the increased number of Iba-1⁺ IPCs is transient during the postnatal period. This could be the result of different cues from the CNS, resulting from the developing stage of the brain, and/or haematopoiesis system age-specific features. No published data was found regarding age-specific perivascular cells appearance. In order to determine whether this cell population contributes to the "window of susceptibility", it will be necessary to

further characterize them and experimentally manipulate the population to ascertain whether the "window of susceptibility" is altered. Depletion of blood-borne monocytes/macrophages at P14 may represent a first step to study their role in the response to focal inflammation in the brain. In the next chapter, different approaches were used in order to deplete the Iba-1⁺ IPCs found at P14. Regarding characterization of these cells, more work is needed. Double or triple immunofluorescence and flow cytometry using a large number of markers for myeloid cells may be used to further characterise macrophages/microglia during postnatal development. Laser capture microdissection may also be used to isolate age-specific Iba-1⁺ IPCs and, consequently, study their unique pattern of gene expression, which may then be used to differentiate them from other cells and to specifically target them.

The data in this chapter also showed that the immune responsiveness of the brain varied over development. P7 mice showed a muted response to i.c injection of 1ng IL-1 β , with minimal changes in expression of inflammatory genes, and no signs of altered BBB permeability or cellular injury. This is despite a comparatively higher basal level of cytokine expression at this age, which is thought to be due to these inflammatory mediators having an important role in regulating normal developmental processes (Bauer et al., 2007; Garay and McAllister, 2010; Stolp, 2013), such as neurogenesis, neuronal and glial cell migration, proliferation and differentiation, and synaptic maturation and pruning (Bilbo and Schwarz, 2012). Inflammatory mediators, including IL-1 β , IL-6, E-selectin and CXCL-1, were increased in the striatum of naïve P7 mice and their expression decreased throughout postnatal period suggesting their importance for early brain development. Previous data have shown that IL-1 β production in cortex of rats peaked at E18 and decreased after until it became undetectable in adults (Giulian et al., 1988). In the hippocampus of rats, IL-1 β was increased at P0 when compared to adult

(Schwarz et al., 2012). Another study has shown that IL-6 was highly expressed in the embryonic cortex (E18) compared to P2, P20 and the adult rat (Pousset, 1994). All these studies and the observation in this thesis support the hypothesis that cytokines are extremely important for brain development and, therefore, are highly expressed early in development. Consequently, their expression decrease as the brain mature with age. Chemokines have also been implicated in different neural processes, including neurotransmission and neural stem cells migration (Bilbo and Schwarz, 2012). Anti-inflammatory mediators, such as IL-4, IL-10, IL-1RA and IL-1R2, were also highly expressed in younger mice, which suggest, not only that they may have a role in development, but also that the dynamic balance between pro- and anti- inflammatory mediators is essential for brain homeostasis in the newborn. This well controlled balance may also restrict the response to inflammation in the brain at early postnatal time points. The results in this chapter showed that microglia in control animals had large and rounded cell bodies with a very few thick long processes at P7 and changed with age towards a more ramified morphology, where microglia had many long thin processes. At P14, microglia exhibited a more ramified morphology than at P7 but most of the cells were still not fully mature as at P21. In addition, the number of microglia increased from P7 onwards. It is known that microglia have a ramified morphology in the adult naïve brain. They have thin and long processes, and small cell bodies. It is also known that microglia develop and mature during the first postnatal month towards a ramified morphology. Between P5 and P15 the number of these cells increases dramatically and at the end of this period the entire parenchyma is filled by microglia (Harry, 2013; Lawson et al., 1990; Perry et al., 1985). Early in postnatal development microglia are known to have a large, rounded and amoeboid-like morphology, which is similar to the adult morphology after injury (Cuadros and Navascués, 1998). However, such amoeboid morphology was not

associated with an increased inflammatory response in terms of brain expression of inflammatory mediators and leukocyte recruitment after i.c. injection of IL-1 β in P7 mice. Many previous studies have shown that microglia produce many inflammatory cytokines and chemokines, and are thought to play an important role in hypoxia-ischemia induced in neonatal rodents (P5-P10) (reviewed in Lai et al., 2016). The contradictory observations in these studies and in this thesis, indicate that microglia response is highly dependent on the type of injury occurring early in postnatal period. In contrast to P7, P14 and P21 brains expressed higher levels of inflammatory mediators after focal inflammation as the number of microglia increased and their morphology changed towards a ramified, adult-like one. These cells may be then well developed and, consequently, capable of responding to injury as observed in this chapter, where microglia presented an activated amoeboid morphology in P14 and P21 brains 4 hours after i.c. injection of 1ng IL-1 β . Consequently, many inflammatory mediators were highly expressed in the ipsilateral striatum after focal inflammation at P14 and P21 when compared to controls. At P21, there was no BBB disruption, and the number of neutrophils in the parenchyma resembled P7. While a significant pro-inflammatory response to the IL-1 β injection was observed in these animals, there were also significant increases in IL-1RA mRNA expression, which is known to inhibit the activation of IL-1R1 and can limit neutrophil recruitment to inflamed tissues (Kolaczowska and Kubes, 2013). This suggests that there was a controlled immune response after inflammation at P21, avoiding substantial recruitment of neutrophils, which may partially explain why BBB injury is transient during brain development and, consequently, why the "window of susceptibility" to injury closes.

3.4 Conclusion

The data in this chapter showed a developmental phenomenon that may contribute to the “window of susceptibility” for postnatal brain inflammation. A population of Iba-1⁺ IPCs was present in the naïve brain of P14 animals and may have thus provided a nexus for further leukocyte recruitment at this time point. Targeting this cellular population may intervene in the leukocyte-mediated damage that can occur during the “window of susceptibility”. The developmental stage of the brain and its capacity to respond to inflammation were also shown to determine the resulting injury. The immune response in the brain gradually developed from an immature stage at P7 to a high and controlled response at P21. Between these ages, there was a high susceptibility to inflammation that increased BBB permeability and recruitment of high number of neutrophils, which may increase injury and contribute to a worst outcome. These results suggest that the brain respond differently to injury during development and that age-specific therapies to neurodevelopmental disorders may need to be considered.

CHAPTER 4

TARGETING PERIVASCULAR CELLS TO RESOLVE THE "WINDOW OF SUSCEPTIBILITY"

4.1 Introduction and aims

In the previous chapter, it was discovered that P14 mice were more susceptible to focal inflammation in the brain than before or after this developmental "window". This "window of susceptibility" was characterised by increased recruitment of neutrophils to the brain and increased BBB permeability. An Iba-1⁺ IPC population was specifically present in the brain at P14, which suggested that a certain monocyte/macrophage population may be recruited to the brain at around P14 and, consequently, generate a niche for neutrophil recruitment after focal inflammation by altering local/focal characteristics of the inflammatory response. However, in order to confirm this hypothesis, it is necessary to experimentally inhibit the recruitment of these perivascular cells to the brain during development and, consequently, remove them by P14. Using this experimental paradigm, it would be possible to test whether the "window of susceptibility" to inflammation in the brain is dependent on the presence of this new perivascular macrophage population.

In this chapter, two experimental approaches were tested in order to deplete Iba-1⁺ IPCs and, consequently, to inhibit the recruitment of these cells to the perivascular space in the brain. In the first of these paradigms, an antibody against CSF1R was used. CSF1R is expressed by monocytes/macrophages and it is essential for their production, proliferation, differentiation and function. The antibody used, M279, is a rat immunoglobulin G1 (IgG1) mAb that blocks the ability of CSF-1 to maintain proliferation of a CSF-1-dependent cell line and does not effectively elicit complement-

mediated lysis. This antibody was shown to effectively deplete peripheral macrophages, but leave microglia intact when injected i.p. (MacDonald et al., 2010). In the second paradigm, clodronate-filled liposomes were used to kill the peripheral monocytes/macrophages by apoptosis. Liposomes can be administered through i.v. injection and are phagocytosed by circulating macrophages. Once inside the phagocytic cells, the phospholipid bilayer of the liposomes is disrupted and the clodronate is released. Clodronate is toxic and induces cell death through an apoptotic pathway (Van Rooijen et al., 1996). Immunohistochemistry was performed on liver and brain sections to determine the effectiveness of both targeting systems on macrophage/microglia populations, including the newly identified Iba-1⁺ IPCs. Liver was used as an indicator of the effectiveness of both treatments in depleting peripheral macrophages due to their permissive architecture. Blood entering the hepatic parenchyma flows through a network of sinusoids capillaries that include a fenestrated layer of liver sinusoidal endothelial cells. Kupffer cells sit on the surface of these cells and, therefore, are readily exposed to the circulating content of the blood (Ishibashi et al., 2009; Si-tayeb et al., 2010).

4.2 Results

4.2.1 Anti-CSF1R treatment did not deplete peripheral macrophages but affected microglia population

Anti-CSF1R (120 μ g) was injected i.p., twice per week from P0 to P14. Naïve mice and mice injected with rat IgG were used as controls. To test the effectiveness of the treatments, Iba-1⁺ cell populations were counted in the liver and the brain. Anti-CSF1R treatment was insufficient to deplete macrophages in the liver as all three groups of mice showed the same number of Iba-1⁺ positive macrophages (naïve: 425 $\pm$ 13 cells/mm², rat IgG: 405 $\pm$ 39 cells/mm² and anti-CSF1R: 401 $\pm$ 19 cells/mm²) (Figure 4.1A and B).

Analysis of the brains after Iba-1 immunohistochemistry showed that the average number of microglia did not significantly change in all three groups (naïve: 129 ± 4 cells/mm², IgG: 160 ± 14 cells/mm² and anti-CSF1R: 108 ± 63 cells/mm²) (Figure 4.2A). However, surprisingly, almost no microglia and no perivascular cells were observed in the brain of two out of the four anti-CSF1R treated animals (Figure 4.2B, C and D). The remaining microglia found in these animals were restricted to the corpus callosum (Figure 4.2D). In the other two brains, Iba-1⁺ cells, including microglia and perivascular macrophages, were present as they were in the control animals.

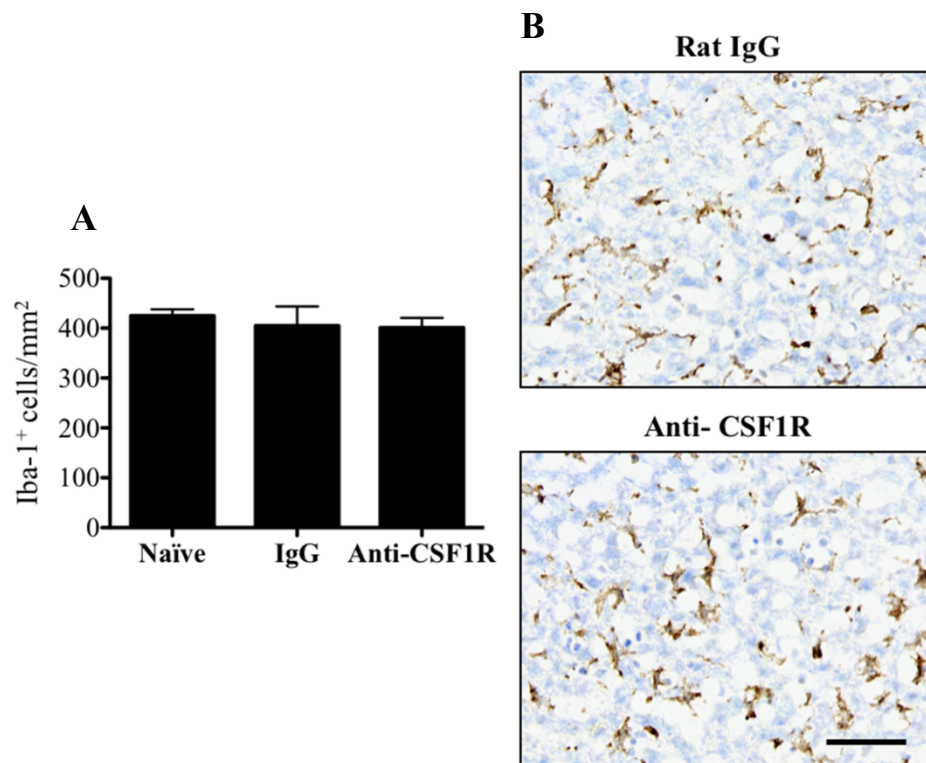


Figure 4.1 Impact of anti-CSF1R treatment on macrophage population in the liver. Immunohistochemistry using Iba-1 antibody was performed to stain macrophages in the naïve liver (P14) and liver from mice that received i.p. injection of rat anti-CSF1R or rat IgG twice per week, from P0 to P14. Quantification of the number of macrophages showed that anti-CSF1R did not affect these cells (A). Data are represented as number of Iba-1⁺ cells per mm². Statistics: one-way ANOVA with Tukey's multiple comparison test. Naïve: n=3, IgG: n=3 and anti-CSF1R: n=4. Representative pictures of Iba-1⁺ macrophages in the liver of mice injected with rat IgG or anti-CSF1R are shown in B. Scale bar = 50µm.

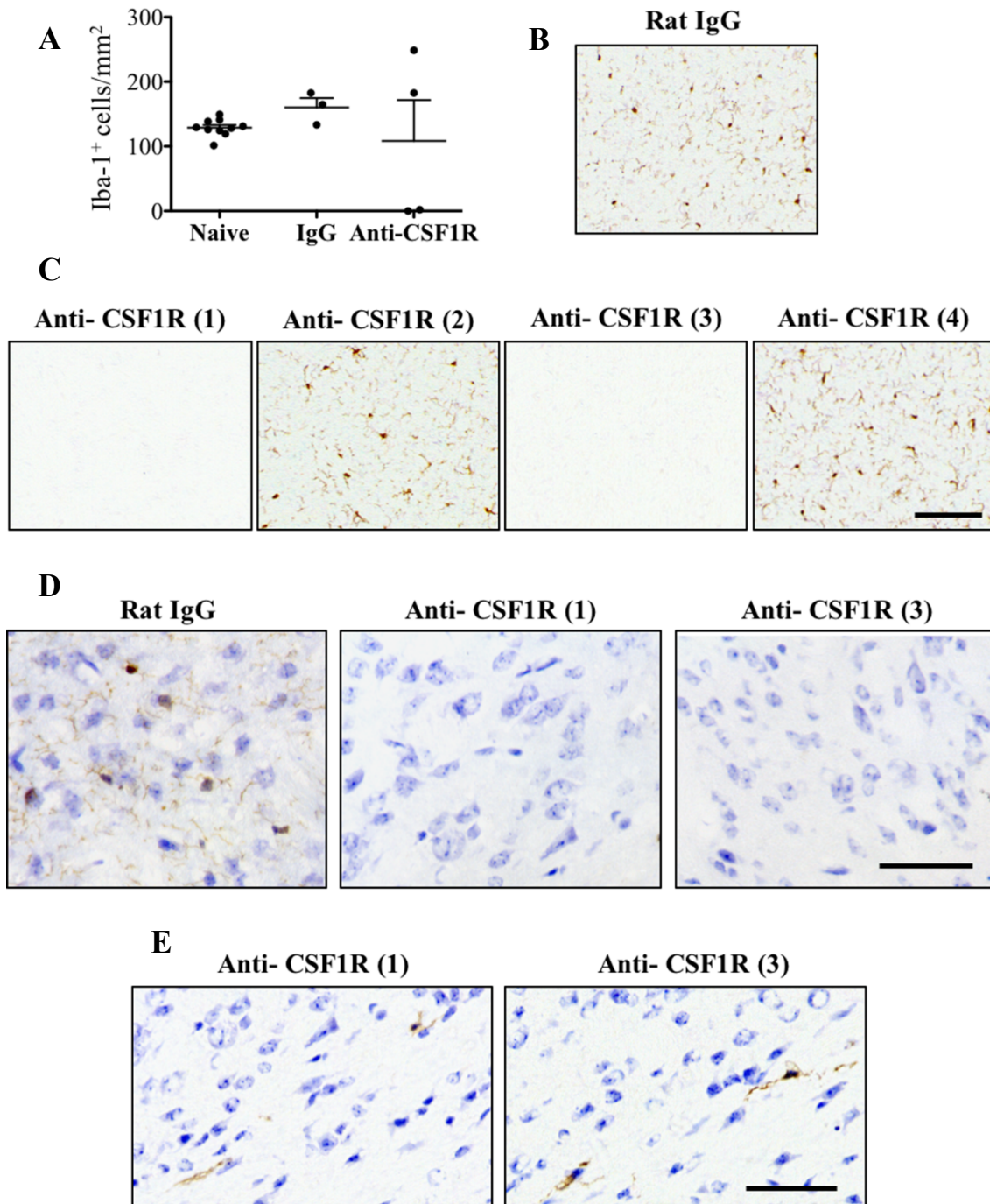


Figure 4.2 Microglia population after anti-CSF1R treatment. Microglia were analysed after immunohistochemistry for Iba-1 with or without cresyl violet counterstain in brains from P14 naïve mice, and from mice that received i.p. injection of rat anti-CSF1R or rat IgG twice per week, from P0 to P14. Quantification of the number of microglia in the striatum did not show any significantly changes between both treatment groups (A) but two out of the four anti-CSF1R treated animals show almost no microglia and no perivascular cells in the brain (striatum in B, C and D). The remaining few microglia in these two animals were restrictive to corpus callosum (E). Scale bar = 50µm. Data in A are represented as number of Iba-1⁺ cells per mm². Statistics: Kruskal-Wallis with Dunn's multiple comparisons test. Naïve: n=10, IgG: n=3 and anti-CSF1R: n=4.

4.2.2 Clodronate liposomes depleted peripheral macrophages and significantly decrease the number of Iba⁺ perivascular cells in the brain

Clodronate containing liposomes were injected into the tail vein of C57Bl/6 mice either 24 hours or 48 hours before histological assessment at P14 (i.e. on P13 or P12 respectively). Vehicle control animals were treated with PBS filled liposomes and were injected 24 hours before histological assessment at P14 (on P13), and baseline values were assessed in naïve animals at P14. Immunohistochemistry for Iba-1⁺ macrophages in the liver showed that clodronate treatment 24 or 48 hours before P14 resulted in a significant ($p < 0.001$) depletion of macrophages (Figure 4.3A and B). The number of Iba-1⁺ macrophages in the liver were $425 \pm 13/\text{mm}^2$ in naïve animals, $438 \pm 36/\text{mm}^2$ in mice injected with PBS liposomes at 24 hours before P14, $35 \pm 13/\text{mm}^2$ in mice injected with clodronate-filled liposomes at 24 hours before P14 and $86 \pm 17/\text{mm}^2$ in animals that received clodronate liposomes at 48 hours before P14. Conversely, the number of microglia did not change in the brains of any of the four treatment groups (Figure 4.4A). Double immunohistochemistry for Iba-1 and vessels (laminin 1 + 2) was performed and the total number of Iba-1⁺ IPCs were counted in each brain. By P14, the number of Iba-1⁺ IPCs was significantly ($p < 0.05$) decreased from naïve animals to mice that were injected with clodronate-filled liposomes at P12 (48 hours) (naïve: $36 \pm 8/\text{mm}^2$ vs. clodronate liposomes 48 hours: $15 \pm 2/\text{mm}^2$) (Figure 4.4C and D). The number of these cells also decreased at P14 when clodronate-filled liposomes were injected at P13 (24 hours) though this was not statistically significant (Figure 4.4C).

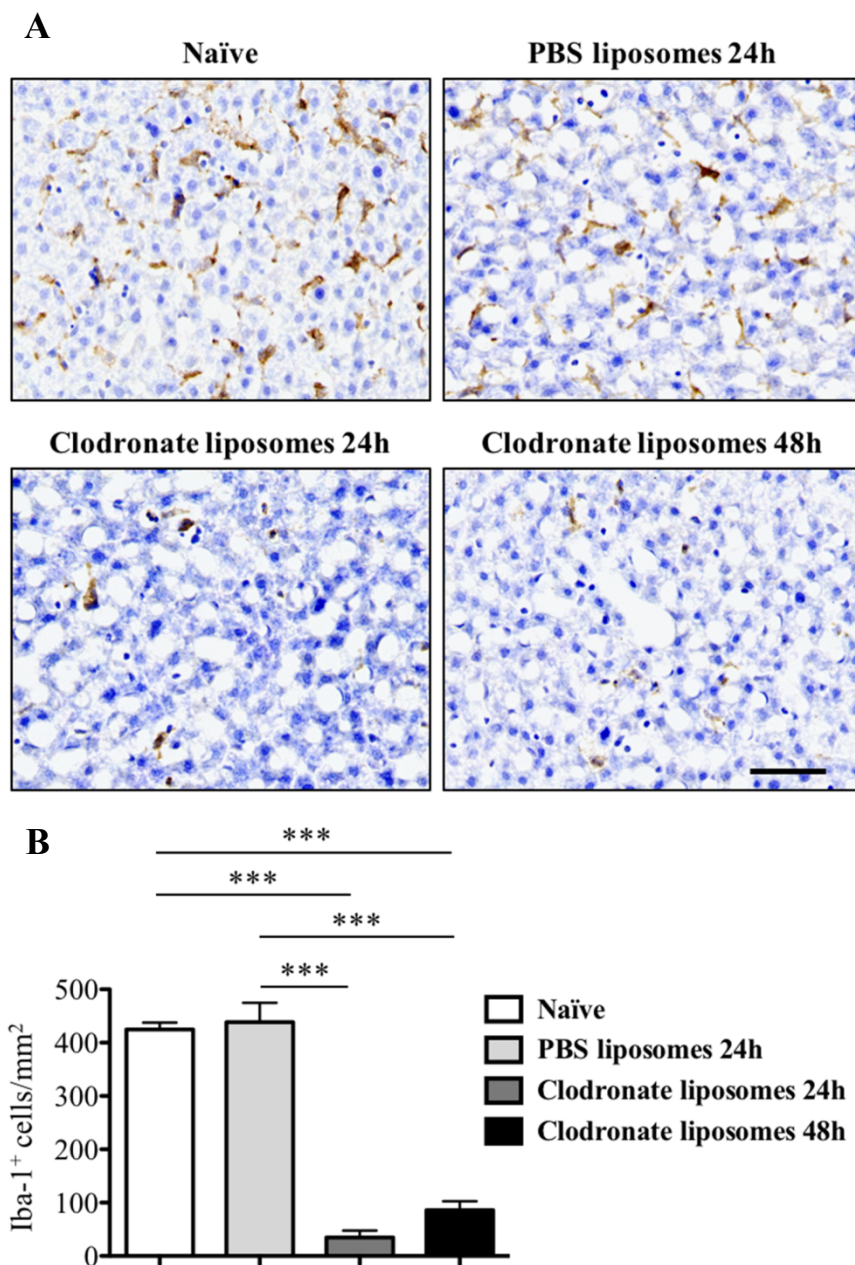


Figure 4.3 Macrophages in the liver after injection of clodronate-filled liposomes. Immunohistochemistry using Iba-1 antibody was performed to stain macrophages in naïve livers (P14) and, livers from P14 mice that received i.v. injection of liposomes filled with PBS or clodronate 24 or 48 hours earlier (A). Quantification of the number of macrophages showed that clodronate liposomes were efficient in depleting macrophages in the liver at P14 when they were injected 24 or 48 hours before (B). Data are represented as number of Iba-1⁺ cells per mm². Statistics: one-way ANOVA with Tukey's multiple comparison test. Naïve: n=3, PBS liposomes P13: n=3, clodronate liposomes 24h: n=3 and clodronate liposomes 48h: n=4. Scale bar = 50µm.

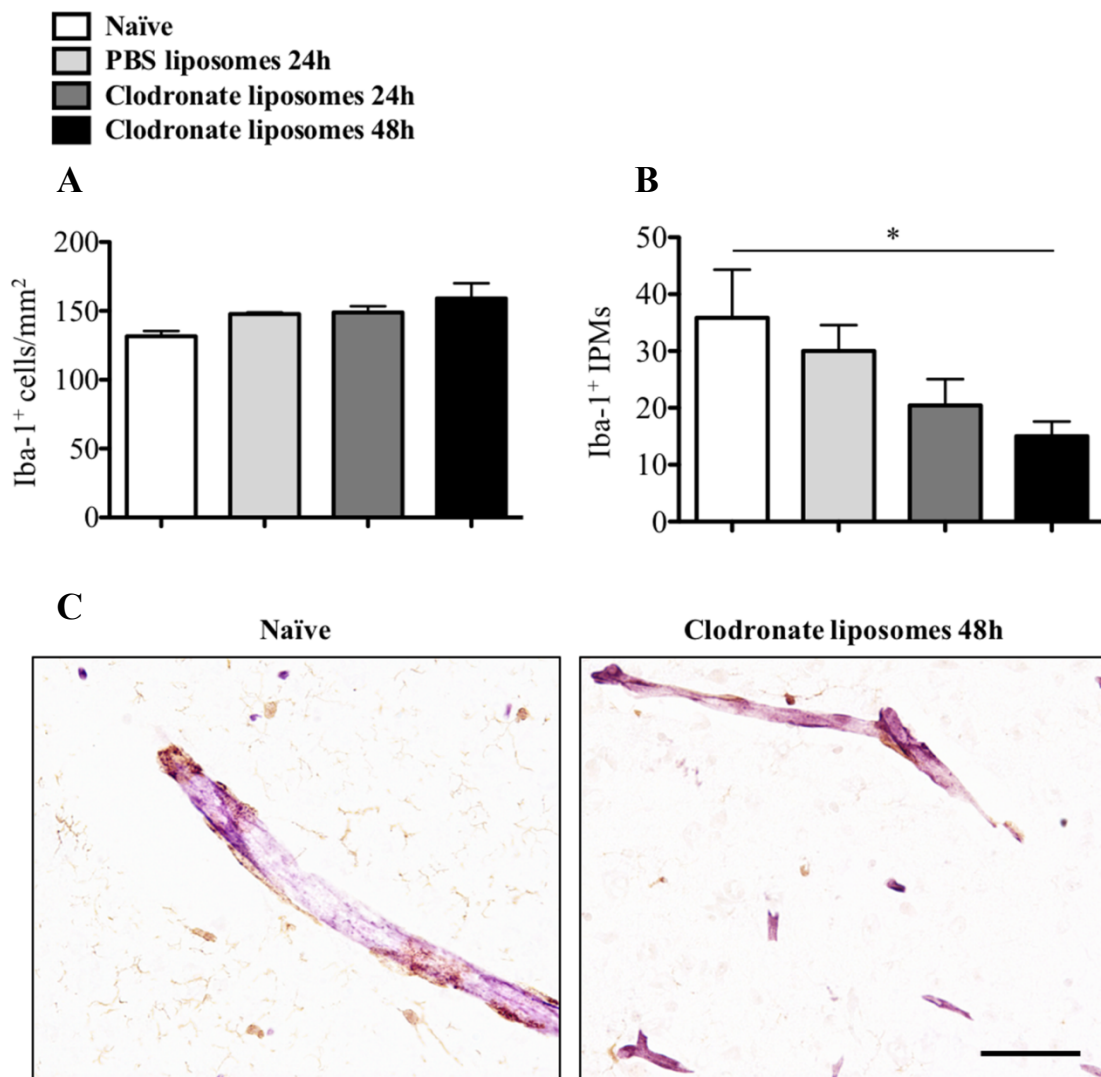


Figure 4.4 Microglia and Iba-1⁺ perivascular cell (IPCs) populations after injection of clodronate-filled liposomes. Microglial cells and IPCs were analysed after double immunohistochemistry for Iba-1 (brown) and vessels (GLUT-1) using brains from naïve mice (P14), and from P14 mice that received i.v. injection of liposomes filled with PBS or clodronate 24 or 48 hours earlier. Quantification of parenchymal Iba-1⁺ cells showed that liposomes filled with clodronate did not affect the number of microglia (A). Data are represented as number of Iba-1⁺ cells per mm². Clodronate liposomes injected 48 hours before assessment at P14 significantly reduced the number of Iba-1⁺ IPCs when compared to naïve brains (B and C). Scale bar = 50µm. Data are represented as total average number of Iba-1⁺ IPCs in two entire brain coronal sections that were approximately 120µm apart. Statistics: one-way ANOVA with Tukey's multiple comparison test for A and Kruskal-Wallis with Dunn's multiple comparisons test for B. Naïve: n=7, PBS liposomes 24h: n=3, clodronate liposomes 24h: n=3 and clodronate liposomes 48h: n=4. IPCs: Large flattened irregular perivascular macrophages.

4.3 Discussion

The data in this chapter demonstrates for the first time that liposomes filled with clodronate can be used to decrease the number of Iba-1⁺ IPCs without affecting microglial population during postnatal development. This was an improvement over the anti-CSF1R method as this treatment affected the number of microglia population and, therefore, could not be used to specifically deplete Iba-1⁺ IPCs.

In the anti-CSF1R experiments, there was no reduction of Iba-1⁺ cells in the liver but Iba-1⁺ cells were almost entirely depleted in the brain of two out of the four anti-CSF1R treated mice when compared to naïve or IgG-treated mice. This result contradicts previous observations in adult mice, which indicates that peripheral anti-CSF1R treatment causes depletion of resident macrophages in the liver, but does no effect brain microglia (MacDonald et al., 2010). The authors have shown that the half-life of 400µg of the CSF1R antibody injected in the peritoneal cavity of adult mice was 68 hours and that injections thrice per week for three weeks in adult mice was sufficient to maintain the antibody in circulation and to promote an almost complete depletion of macrophages in the liver without interfering with microglia (MacDonald et al., 2010). In this study, this experimental design was replicated, but the dose was adjusted to the weight of P14 mice (120µg). Anti-CSF1R treatment did not deplete macrophages in the liver at P14 suggesting that there are other trophic factors in circulation that keep macrophages alive at this stage of postnatal development or that the dose of the antibody was inefficient to overcome macrophage repopulation in the liver from self-renewal or blood-borne monocytes (Sieweke and Allen, 2013; Varol et al., 2015), which may be faster and more efficient than in adults as P14 liver is still growing (Alexander et al., 1997; Walthall et al., 2005). However, this is speculative as there is no published data regarding this aspect during postnatal development. In order to determine if this age specific effect is real,

future experiments should include further examination of peripheral macrophage populations after anti-CSF1R treatment at different ages and doses.

One interpretation of the contradictory results between P14 and adult mice regarding the brain is that the antibody was able to cross the BBB in neonatal mice, but not in adults. Once in the brain the antibody against CSF1R would be able to bind to the receptor in microglia, inhibit microglia proliferation and lead to their death. CSF1R is known to be expressed in microglia, and regulate cell proliferation and survival (reviewed in Chitu et al., 2016). *CSF1R*-null mice brains were shown to be almost devoided of microglia either at E18, P1 and adulthood (Erblich et al., 2011; Ginhoux et al., 2010). Moreover, inhibition of CSF1R by PLX3397, which was administered mixed in the normal rodent diet, was shown to deplete all microglia in the regions analysed (hippocampus, thalamus and cortex) by day 21 of treatment in adult mice, without compromising the BBB (no increased permeability to Evans blue observed). The same authors have also shown that many microglia were positively stained for caspase-3, a classic marker of apoptosis, during treatment (Elmore et al., 2014). It is possible that the specific composition of the BBB at P14 may facilitate the transfer of the antibody against CSF1R more readily than in the adult brain. It is known that tight junctions and many of the transport mechanisms, including solute-linked carrier and efflux transporters, are present in the BBB very early in the development and that this translates into functional effectiveness of the BBB (reviewed in Ek et al., 2012 and Saunders et al., 2012). However, different patterns of expression of proteins associated with transporters (influx and efflux), ion channels, and metabolic and transduction pathways were also observed in the neonate when compared to the adult brain (Daneman et al., 2010). Indeed, expression of influx transporters for nutrients such as amino acids, glucose and trace metals was shown to be upregulated in the mouse neonate endothelial cells (Daneman et al., 2010) and some were shown to be

more active than in adult brain (Braun et al., 1980; Cornford and Cornford, 1986; Cornford et al., 1982; Lefauconnier and Trouvé, 1983; Pardridge and Mietus, 1982). Some amino acid transporters and transferrin receptors were also shown to facilitate the transport of toxic compounds such as heavy metals and different drugs (reviewed in Ek et al., 2012 and Saunders et al., 2012, 2016). Therefore, changes in function of different transporters may determine the composition of the brain environment essential for development and allow the entry of the CSF1R antibody. Another hypothesis for microglia depletion at P14 could be that anti-CSF1R would affect microglia influx to the brain. However, it is now thought that resident microglia population is well established by P14 and that proliferation of these resident cells is responsible for the increasing number of microglia during development (Alliot et al., 1999; Ginhoux et al., 2010; Kierdorf et al., 2013; Schulz et al., 2012). Future experiments are necessary in order to confirm the observation that anti-CSF1R treatment reduces microglia, as only two out of four treated mice showed almost complete depletion of Iba-1⁺ cells. It is important to study how microglia are depleted and if these cells repopulate the brain after being removed at P14. Previous studies have already shown that microglia repopulate from brain-resident progenitor cells after being depleted by PLX3397 in adult mice (Elmore et al., 2014), though no data is available regarding young mice. It would also be interesting to examine repopulation follow CSF1R-mediated depletion to discover whether the morphology and distribution of the cells return to normal. In addition, mice at other time points during postnatal development could be treated with the antibody in order to verify if this phenomenon of microglia depletion occurs throughout this period and to discover the specific time point when CSF1R is no longer able to access the brain. Even if complete depletion had been observed in this study, these results suggest that anti-CSF1R treatment is not the best option to specifically deplete Iba-1⁺ perivascular cells.

The second approach used in this chapter for Iba-1⁺ IPCs was the injection of liposomes filled with clodronate, which are known to cause macrophage apoptosis. These liposomes were shown to be engulfed by macrophages and once inside the cells, clodronate was released and, consequently, induced apoptosis (Van Rooijen et al., 1996). However, liposomes cannot cross the vascular barriers formed by capillary walls in many organs and are, therefore, restricted in their targeting of tissue specific macrophages. In order to overcome this, liposomes need be administrated through a tissue specific route in order to encounter target macrophage populations (Rooijen and Sanders, 1994; van Rooijen and van Kesteren-Hendrikx, 2002). Injection of 0.1ml of the liposome suspension, which contains 5mg/ml of clodronate per 10 grams of animal weight, was shown to deplete circulating and, consequently, liver macrophages within 24 hours when injected via i.v. route (Rooijen and Hendrikx, 2010; Van Rooijen and Sanders, 1996, 1998; Van Rooijen et al., 1990; Sunderkotter et al., 2004; Xue et al., 1999). I.v. administration of liposomes filled with clodronate in rats was also shown to reduce infiltration of macrophages to the brain in EAE without affecting perivascular macrophages, and, therefore, suggesting that liposomes do not cross the BBB (Bauer et al., 1995b; Huitinga et al., 1990). This method was implemented in the current study in order to deplete circulating monocytes/macrophages and, consequently, limit migration of these cells to the perivascular space in the brain. Injection of clodronate-filled liposomes into mice 24 hours before they reached P14 induced an almost complete depletion of Iba-1⁺ macrophages in the liver at P14 when compared to naïve animals and those injected with PBS liposomes. This indicated that the treatment successfully depleted Kupfer cells in the liver. The injected liposomes did not affect the resident microglial population owing to the fact that liposomes injected peripherally do not cross BBB (Bauer et al., 1995b; Claassen and Van Rooijen, 1984; Huitinga et al., 1990). When clodronate liposomes were

injected at P13 (24 hours before reaching P14), mice showed a reduction of Iba-1⁺ IPCs when compared to naïve animals and those injected with PBS liposomes, though this effect was not significant. For this reason, clodronate liposomes were also injected 48 hours before reaching P14, which promoted a further reduction and this time significant when compared to naïve mice. This suggests that liposomes successfully reduced Iba-1⁺ IPCs and, consequently, may be used to study the effects of focal inflammation in the absence of Iba-1⁺ IPCs.

Liposomes filled with clodronate did not completely deplete the specific Iba-1⁺ cell population associated with vessels at P14, which suggests that these cells may migrate from the circulation to the perivascular space at earlier time points prior to P12. It would be expected that the injection of clodronate liposomes even earlier than P12 may completely deplete these cells. However, it is important to consider that macrophages repopulation in the liver starts 4 days after liposomes injection (Van Rooijen et al., 1990; Yamamoto et al., 1996) and, therefore, repeated injections might have a greater and longer effect on the number of Iba-1⁺ cells being recruited to the perivascular space in the brain than single injections. It is also known that adult perivascular cells are bone marrow derived and these cells are continuously replaced by blood-borne monocytes (Williams et al., 2001). Previous findings have shown a constant turnover of adult perivascular macrophages in physiologic conditions (Bechmann et al., 2001). Therefore, it is important that liposomes also deplete monocytes in circulation. Previous studies have shown that monocytes were almost completely depleted in the circulation 18 hours after i.v. injection of liposomes filled with clodronate and that they started to repopulate 24 hours after the injection (Sunderkotter et al., 2004). Others have also shown that monocytes were only partially depleted in the circulation 48 hours after i.v. injection of clodronate liposomes (Zhang-Hoover et al., 2000). In my experiments, circulating

monocytes may not be completely depleted following injection of clodronate liposomes both at P12 or P13. This would then result in the consequent migration of those cells into the perivascular space in the brain where they could differentiate into IPCs (Van Rooijen et al., 1997). Based on the data observed here and previous studies, future experiments should include i.v. injections of liposomes filled with clodronate every day from P10 or P11 to P13 and blood should be analysed to verify the absence of monocytes. If this were to completely deplete the Iba-1⁺ IPCs population, it would be then possible to assess if absence of Iba-1⁺ IPCs would protect the brain from neutrophil recruitment and BBB disruption following focal inflammation. Perivascular macrophages can be completely depleted by intraventricular injection of liposomes filled with clodronate (Polfliet et al., 2002, 2001). This protocol would allow us to test if, in fact, Iba-1⁺ IPCs are present in the perivascular space but it wouldn't allow us to test our main hypothesis that the age-specific IPC population is transiently recruited from the periphery to the brain at P14.

4.4 Conclusion

The results in this chapter showed that the antibody against CSF1R (M279) failed to deplete macrophages in the liver and to protect microglia in the brain, contrary to expectations from previous work in adult mice. Therefore, this antibody may not be appropriate for use in young animals to study the influence of the specific absence of peripheral macrophages on the brain response to inflammation. Injection of liposomes filled with clodronate was more effective in depleting peripheral macrophages and, consequently, limiting the recruitment of macrophages into the brain without interfering with the microglial population. These findings highlight the importance of the developmental stage of the animals on their response to exogenous molecules and that extrapolation of data from studies using adult animals may not be straightforward.

Finally, i.v. injection of liposomes containing clodronate was shown to be a good model to reduce the number of Iba-1⁺ IPCs in the brain and, consequently, to assess the importance of these cells in the "window of susceptibility" to inflammation in the brain, which may be used in the future.

CHAPTER 5

EXPERIMENTAL ACTIVATION OF THE ACUTE PHASE RESPONSE AFTER FOCAL BRAIN INJURY INVERTS THE "WINDOW OF SUSCEPTIBILITY"

5.1 Introduction and aims

In this chapter, the relationship between brain inflammation and the peripheral inflammatory response during postnatal development was studied. It is known that inflammation in the CNS is followed by a peripheral APR that is coordinated mainly by the liver in adult rodents (Anthony and Couch, 2014; Anthony et al., 2012). However, this has not been examined during the first weeks after birth in comparison with the adults. It was our suspicion that the altered APR during development might, in some way, account for the appearance of the "window of susceptibility". Low or high doses of IL-1 β were injected in the striatum of P7, P14, P21 and 8-week-old C57Bl/6 mice and the inflammatory response in the liver was studied after 4 hours. Naïve and saline injected mice were used as controls and to study the baseline immune environment in the liver during the postnatal period. With these experiments the variability in the liver immune response to neuroinflammation during postnatal development, and the relationship between the severity of brain and peripheral inflammatory responses during development were explored. Finally, the impact of activating the liver immune response on the susceptibility of the brain to inflammation was studied. For all these experiments, histology and gene expression analysis were performed in order to investigate the recruitment of leukocytes and expression of inflammatory mediators in the liver and in the brain.

5.2 Results

5.2.1 Basal expression of key inflammatory mediators and the number of neutrophils in the liver were highest in younger mice

Before studying the immune response in the liver following brain focal inflammation, it was important to examine the liver immune status during development. Therefore, mRNA expression of inflammatory mediators (Figure 5.1) and leukocyte content (Figure 5.2) in the liver were analysed in the naïve C57Bl/6 mice at different postnatal ages (P7, P14, P21 and 8 weeks). Expression levels of all transcripts were calculated as relative fold change from expression levels of naïve 8-week-old mice. In general, the expression of most of the pro- and anti- inflammatory mediators measured were higher in young animals than in older mice. Hepatic IL-1 β expression levels did not change throughout development. However, IL-6 expression was higher ($p < 0.01$) in the liver at P7 (5.96 ± 0.47) compared to the other ages. The expression levels of this cytokine decreased from P7 to 2.98 ± 0.61 at P14. The levels observed at P7 and P14 were significantly higher than those measured at P21 (1.34 ± 0.23) and 8 weeks (1.00 ± 0.03). TNF and IL-10 exhibited a similar expression pattern. These cytokines were elevated in the liver at P7 (TNF- α : 2.90 ± 0.33 , IL-10: 2.03 ± 0.28) though, only significantly different from P21 (TNF- α : 1.09 ± 0.53 and IL-10: 0.93 ± 0.22) and 8 weeks (TNF- α : 1.03 ± 0.17 and IL-10: 1.00 ± 0.04). At P14, the expression levels of TNF and IL-10 were 1.86 ± 0.30 and 1.21 ± 0.21 , respectively, and these were not significantly different from other ages. Expression of the adhesion molecule ICAM-1 and the chemokines CXCL-1, CXCL-2 and CXCL-5 were also measured in the naïve liver. ICAM-1 mRNA expression was significantly ($p < 0.05$) higher at P7 (1.84 ± 0.10) and P14 (1.79 ± 0.18) when compared to 8 weeks (1.00 ± 0.03) but not to P21 (1.43 ± 0.23). CXCL-1 and CXCL-2 expression

did not differ during development. However, CXCL-5 was significantly ($p < 0.05$) higher during the first three weeks after birth (P7: 3.51 ± 0.17 , P14: 2.97 ± 0.34 and P21: 3.22 ± 0.41) when compared to 8-week-old mice (1.10 ± 0.32) (Figure 5.1).

The presence of leukocytes in the liver during normal development was evaluated by looking at mRNA expression of CD11b, a marker for leukocytes, including monocytes and macrophages, and by immunohistochemistry for neutrophils. The expression of CD11b decreased throughout development though this was not statistically significant (P7: 1.77 ± 0.06 , P14: 1.41 ± 0.15 , P21: 1.24 ± 0.24 and 8 weeks: 1.00 ± 0.06) (Figure 5.2A). The number of neutrophils in the liver was quantified after immunohistochemistry using MBS (Figure 5.2B and C). At P7, 205.5 cells/mm^2 were present in the liver and this number was significantly ($p < 0.001$) higher than in P14, P21 and 8-week-old livers. The number of neutrophils significantly decreased from 65.51 cells/mm^2 at P14 to 38.63 cells/mm^2 at P21 and to 21.84 cells/mm^2 at 8 weeks. Specifically, at P7, many neutrophil clusters were observed throughout the liver (Figure 5.2C).

5.2.2 Expression of key inflammatory mediators and the number of leukocytes in the liver increased after injection of a high dose of IL-1 β in the brain and were highest at P7 and P14

mRNA expression of the same inflammatory mediators studied above was also measured in the liver after injection of saline, 1ng or 100ng IL-1 β in the striatum of P7, P14, P21 and 8-week-old C57Bl/6 mice (Figure 5.3 and 5.4). Expression levels of all transcripts were calculated as relative fold change from expression levels of naïve 8-week-old mice. There were no significant differences between the expression levels of all inflammatory mediators between naïve and saline injected striatum except CXCL-1 mRNA expression, which significantly increased in saline injected animals at P7 (1.03 ± 0.13 naïve vs. 96.97

± 9.39 saline), P14 (1.05 ± 0.10 naïve, 80.97 ± 8.00 saline) and 8 weeks (1.07 ± 0.24 naïve vs. 60.96 ± 16.86 saline) and ICAM-1 mRNA expression at P7 (1.84 ± 0.10 naïve vs. 3.65 ± 0.41 saline) (Figure 5.4).

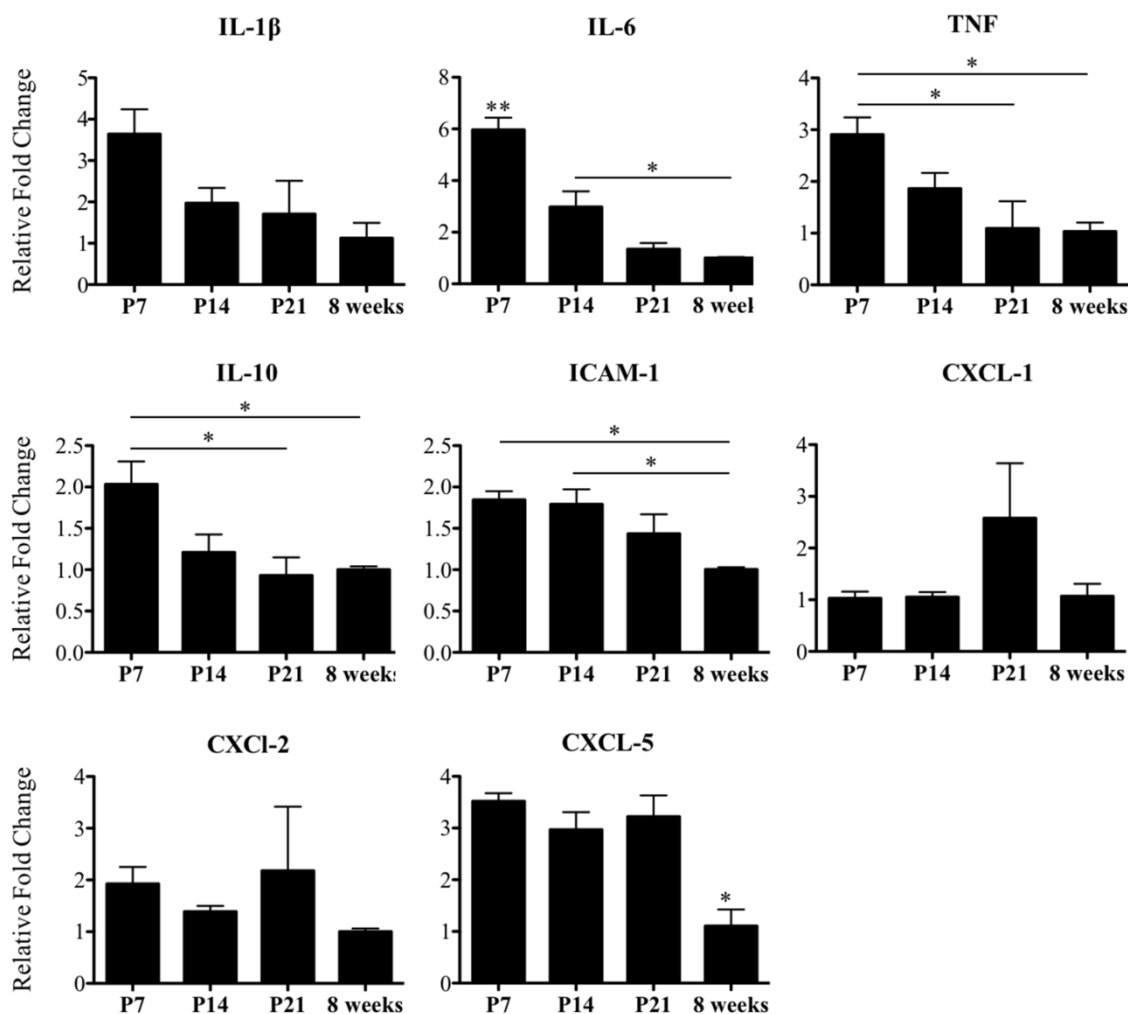


Figure 5.1 mRNA expression of pro- and anti-inflammatory cytokines in the liver during postnatal development. IL-1 β , IL-6, TNF, IL-10, ICAM-1, CXCL-1, CXCL-2 and CXCL-5 mRNA was measured by RT-qPCR in the naïve liver of mice at different postnatal ages (P7, P14, P21 and 8 weeks). IL-6, TNF- α , IL-10 were highly expressed in younger animals and decreased throughout development. ICAM-1 and CXCL-5 were highly expressed during the first 3 weeks after birth when compared to adult mice while CXCL-1 and CXCL-2 expression did not change throughout development. Data are represented as relative fold change from expression levels of 8-week-old naïve mice. Samples were compared with two housekeeping genes (*Csnk2a2* and *Pak1ip1*). Bars represent mean $\pm$ SEM. * $p < 0.05$. Statistics: one-way ANOVA with Tukey's multiple comparison test. P7: $n=4$, P14: $n=4$, P21 $n=4$ and 8 weeks: $n=3$.

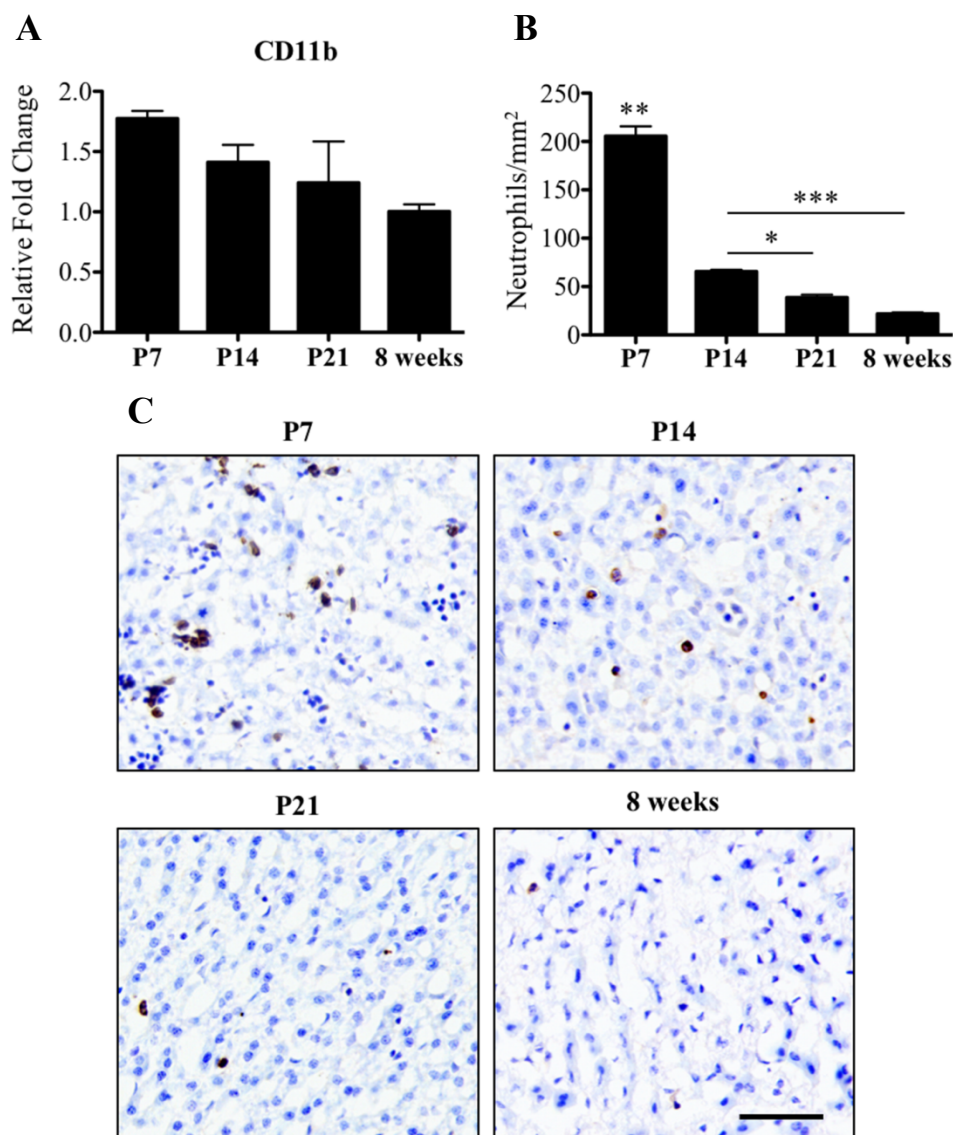


Figure 5.2 mRNA expression of CD11b and the number of neutrophils in the liver during postnatal development. CD11b mRNA was measured by RT-qPCR and the number of neutrophils were counted after immunohistochemistry for neutrophils (MBS) in the naïve liver of C57Bl/6 mice at different postnatal ages (P7, P14, P21 and 8 weeks). CD11b expression did not significantly change during development (A) and the number of neutrophils was highest at P7 (B). Representative pictures of immunohistochemistry for neutrophils (brown) with cresyl violet counterstain in the liver at the different ages are shown in C. Scale bar = 50µm. Data are represented as relative fold change from expression levels of 8-week-old naïve mice in A and as number of neutrophils per mm² in B. Samples were compared with two housekeeping genes (Csnk2a2 and Pak1ip1) in A. Bars represent mean ± SEM. *p<0.05 and ***p<0.001. Statistics: one-way ANOVA with Tukey's multiple comparison test. P7: n=4, P14: n=4, P21 n=4 and 8 weeks: n=4.

The mRNA expression of most pro- and anti- inflammatory mediators did not change in the liver after i.c. injection of 1ng IL-1 β . Only TNF significantly increased between saline (0.91 ± 0.06) and 1ng IL-1 β (1.53 ± 0.09) at 8 weeks (Figure 5.3).

The i.c injection of 100ng IL-1 β increased the expression of most of the inflammatory mediators in the liver in an age-dependent manner (Figure 5.3 and 5.4). In detail, IL-1 β expression was significantly upregulated at P7 (10.30 ± 1.04 vs 4.07 ± 0.64 saline and 3.48 ± 0.69 1ng IL-1 β) and P14 (7.53 ± 0.45 vs. 1.74 ± 0.09 saline and 1.14 ± 0.27 1ng IL-1 β) but not at P21 and 8 weeks. IL-6 expression significantly increased after i.c. injection of 100ng IL-1 β in the liver of P7 (104.40 ± 10.23 vs. 7.40 ± 2.22 saline and 4.73 ± 0.84 1ng IL-1 β), P14 (84.85 ± 5.17 vs. 1.42 ± 0.34 saline and 1.52 ± 0.50 1ng IL-1 β), P21 (8.81 ± 2.21 vs. 0.98 ± 0.29 saline and 1.06 ± 0.16 1ng IL-1 β) and 8-week-old (3.65 ± 0.44 vs. 0.88 ± 0.14 saline, 1.00 ± 0.14 1ng IL-1 β) mice. TNF expression was significantly upregulated in the liver only when 100ng IL-1 β was injected in the brain at P7 (7.86 ± 0.41 vs. 4.42 ± 0.83 saline and 3.79 ± 0.37 1ng IL-1 β), P14 (6.42 ± 0.60 vs. 1.99 ± 0.25 saline and 2.32 ± 0.82 1ng IL-1 β) and P21 (2.34 ± 0.09 vs. 0.58 ± 0.02 saline and 0.58 ± 0.12 1ng IL-1 β). IL-10 was only significantly upregulated in the liver at P21 after i.c. injection of 100ng IL-1 β (2.05 ± 0.13 vs. 0.77 ± 0.13 saline, 0.70 ± 0.08 1ng IL-1 β) (Figure 5.3). ICAM-1 expression was significantly induced in the liver at P14 (3.82 ± 0.21 vs. 2.10 ± 0.32 saline and 1.46 ± 0.31 1ng IL-1 β), P21 (4.23 ± 0.60 vs. 0.97 ± 0.33 saline and 1.26 ± 0.086 1ng IL-1 β) and 8-week-old (3.20 ± 0.22 vs. 1.01 ± 0.05 saline and 0.90 ± 0.06 1ng IL-1 β) mice. CXCL-1 mRNA expression significantly increased at P14 (150.80 ± 4.65 vs. 80.97 ± 8.00 saline and 62.15 ± 14.53 1ng IL-1 β) and P21 (125.2 ± 17.76 vs. 32.54 ± 12.56 saline and 34.88 ± 10.67 1ng IL-1 β), and CXCL-2 was significantly upregulated in the liver after i.c. injection of 100ng IL-1 β at P7 ($82.99 \pm$

18.00 vs. 3.14 ± 0.63 saline and 3.63 ± 0.45 1ng IL-1 β), P14 (46.22 ± 6.57 vs. 3.08 ± 0.54 saline and 2.07 ± 0.61 1ng IL-1 β), P21 (46.35 ± 19.50 vs. 2.51 ± 0.20 saline and 2.37 ± 0.24 1ng IL-1 β) and 8 weeks (3.77 ± 0.77 vs. 1.52 ± 0.23 saline and 1.33 ± 0.17 1ng IL-1 β). Finally, CXCL-5 expression significantly increased at P7 (10.60 ± 1.28 vs. 3.28 ± 0.70 saline and 2.31 ± 0.29 1ng IL-1 β) and P14 (12.60 ± 0.85 vs. 2.45 ± 0.34 saline and 1.15 ± 0.31 1ng IL-1 β). This chemokine was also upregulated at P21 (5.41 ± 0.84), though only significantly different from 1ng IL-1 β group (2.01 ± 0.31), and at 8 weeks (2.35 ± 0.29) when compared to 1ng IL-1 β (1.35 ± 0.09) group of mice (Figure 5.4). The magnitude of increase in the expression of a number of inflammatory mediators in the liver after i.c. injection of 100ng IL-1 β was significantly higher at P7 when compared to the other ages, which was shown by a two-way ANOVA analysis with Bonferroni–Dunn post-hoc test. Indeed, mRNA expression of IL-1 β , IL-6, TNF, IL-10, ICAM-1 and CXCL-2 in the liver after 100ng IL-1 β injection in the brain was highest at P7 and decreased with age. CXCL-1 expression exhibited a very different pattern when compared to the other inflammatory mediators. After 100ng IL-1 β injection in the brain, the liver expressed higher levels of this chemokine at P14 and P21 than at P7 or 8 weeks. Finally, i.c. injection of 100ng IL-1 β induced higher levels of CXCL-5 in the liver at P7 and P14 when compared to P21 and 8 weeks ($p < 0.01$) (Table 5.1).

In order to determine if the increase in mRNA expression of the inflammatory mediators in the liver correlated with the presence of leukocytes, CD11b mRNA expression and the number of neutrophils in the liver were quantified after injection of 100ng IL-1 β in the brain. CD11b expression was significantly upregulated in the liver at P7 (5.34 ± 0.23 vs. 1.78 ± 0.06 naïve and 2.67 ± 0.28 saline), P14 (3.36 ± 0.18 vs. 1.41 ± 0.15 naïve and 1.87 ± 0.13 saline) and P21 (2.26 ± 0.15 vs. 1.24 ± 0.34 naïve and 0.91 ± 0.16 saline). When a

comparison was made between between ages, CD11b expression in the liver after i.c. injection of 100ng IL-1 β was highest at P7 and significantly decreased throughout development (Figure 5.5A). Immunohistochemistry was performed to detect neutrophils in the liver and the number of these cells per mm² of liver was quantified (Figure 5.5B and C). The number of neutrophils in the liver significantly increased at all ages after injection of 100ng IL-1 β in the brain. The number of cells increased from approximately 205 $\pm$ 10/mm² (naïve) and 186 $\pm$ 8/mm² (saline) to 362 $\pm$ 40/mm² at P7, 65 $\pm$ 2/mm² (naïve) and 83 $\pm$ 11/mm² (saline) to 338 $\pm$ 52/mm² at P14, 39 $\pm$ 3/mm² (naïve) and 74 $\pm$ 3/mm² (saline) to 237 $\pm$ 8/mm² at P21, and from 22 $\pm$ 1/mm² (naïve) and 31 $\pm$ 3/mm² (saline) to 155 $\pm$ 18/mm² at 8 weeks. The two-way ANOVA with Bonferroni-Dunn post-hoc test used also showed that the number of neutrophils in the liver was highest at P7 and P14 (p<0.001), and significantly decreased to P21 and from P21 to 8 weeks in response to i.c. injection of 100ng IL-1 β (Figure 5.5B). Figure 5.6 shows representative pictures of the data described above, including livers from saline and 100ng IL-1 β injected mice.

In summary, injection of a high dose of IL-1 β in the brain increased the number of neutrophils in the liver at all time points, but it was greatest at the earlier time points (P7 and P14). In addition, it is clear that the APR at P14 was not very different from P7 except that CXCL-1 was increased. Finally, there was not an evident APR when a low dose of IL-1 β was injected in the left striatum at any time point and, therefore, may not account to the "window of susceptibility" found in chapter 3.

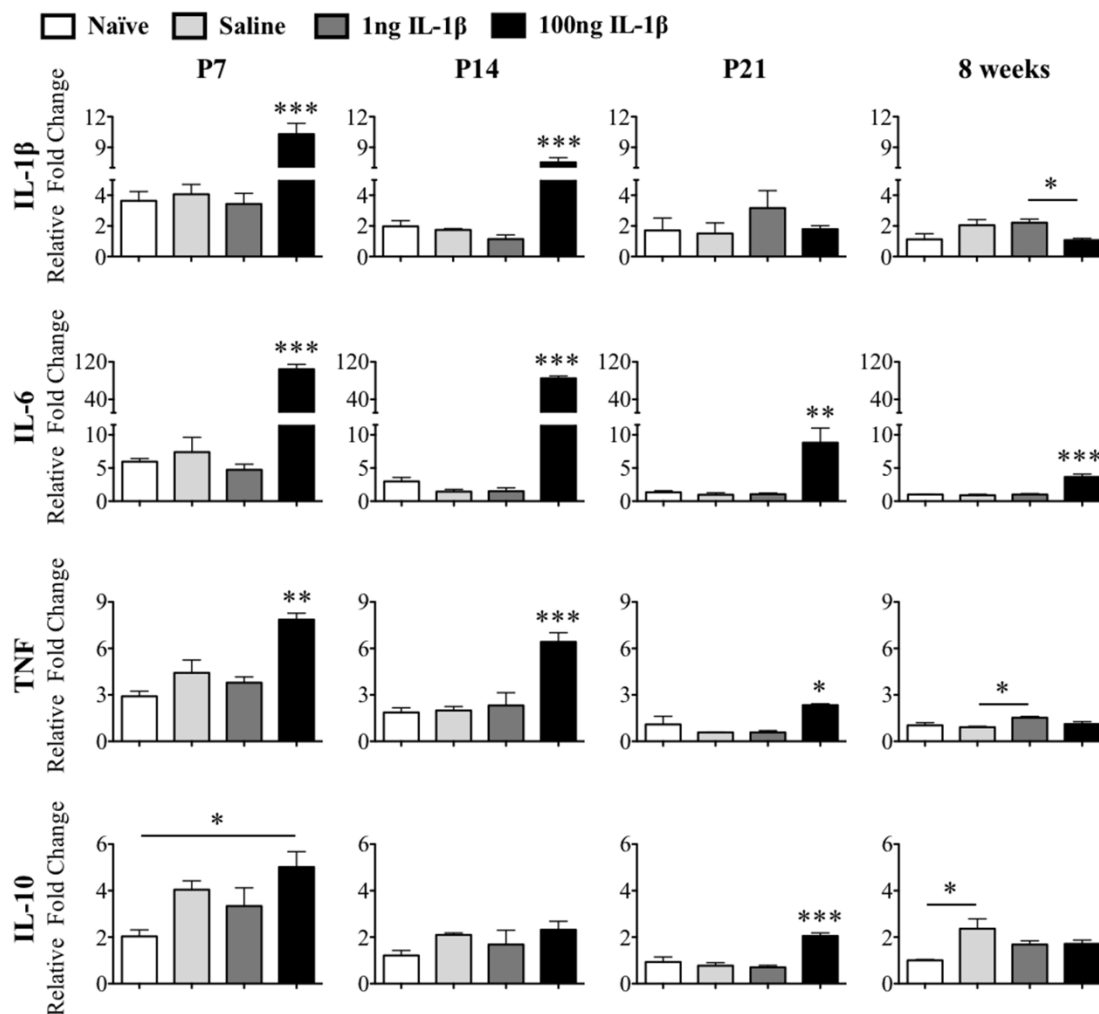


Figure 5.3 Effect of low and high doses of IL-1 β in the brain on mRNA expression of pro- and anti-inflammatory cytokines in the liver. IL-1 β , IL-6, TNF and IL-10 mRNA was measured by RT-qPCR in the liver of naïve mice and mice that received i.c. injection of saline or 1ng IL-1 β or 100ng IL-1 β at different postnatal ages (P7, P14, P21 and 8 weeks). Injection of 1ng IL-1 β in the brain did not induced the expression of any of the cytokines, but injection of 100ng IL-1 β in the brain promoted the expression in the liver: IL-1 β at P7 and P14, IL-6 throughout development, TNF at P7, P14 and P21, and IL-10 at P7 and P21. Data are represented as relative fold change from expression levels of 8-week old naïve mice. Samples were compared with two housekeeping genes (Csnk2a2 and Pak1ip1). Bars represent mean $\pm$ SEM. *p<0.05, **p<0.01 and ***p<0.001. Statistics: one-way ANOVA with Tukey's multiple comparison test. P7: n=4 for saline, n=5 for 1ng and 100ng IL-1 β ; P14: n=4 for saline, n=3 for 1ng IL-1 β , n=5 for 100ng IL-1 β , P21: n=4 for saline, 1ng IL-1 β and 100ng IL-1 β ; 8 weeks: n=4 for saline and 1ng IL-1 β , n=5 for 100ng IL-1 β .

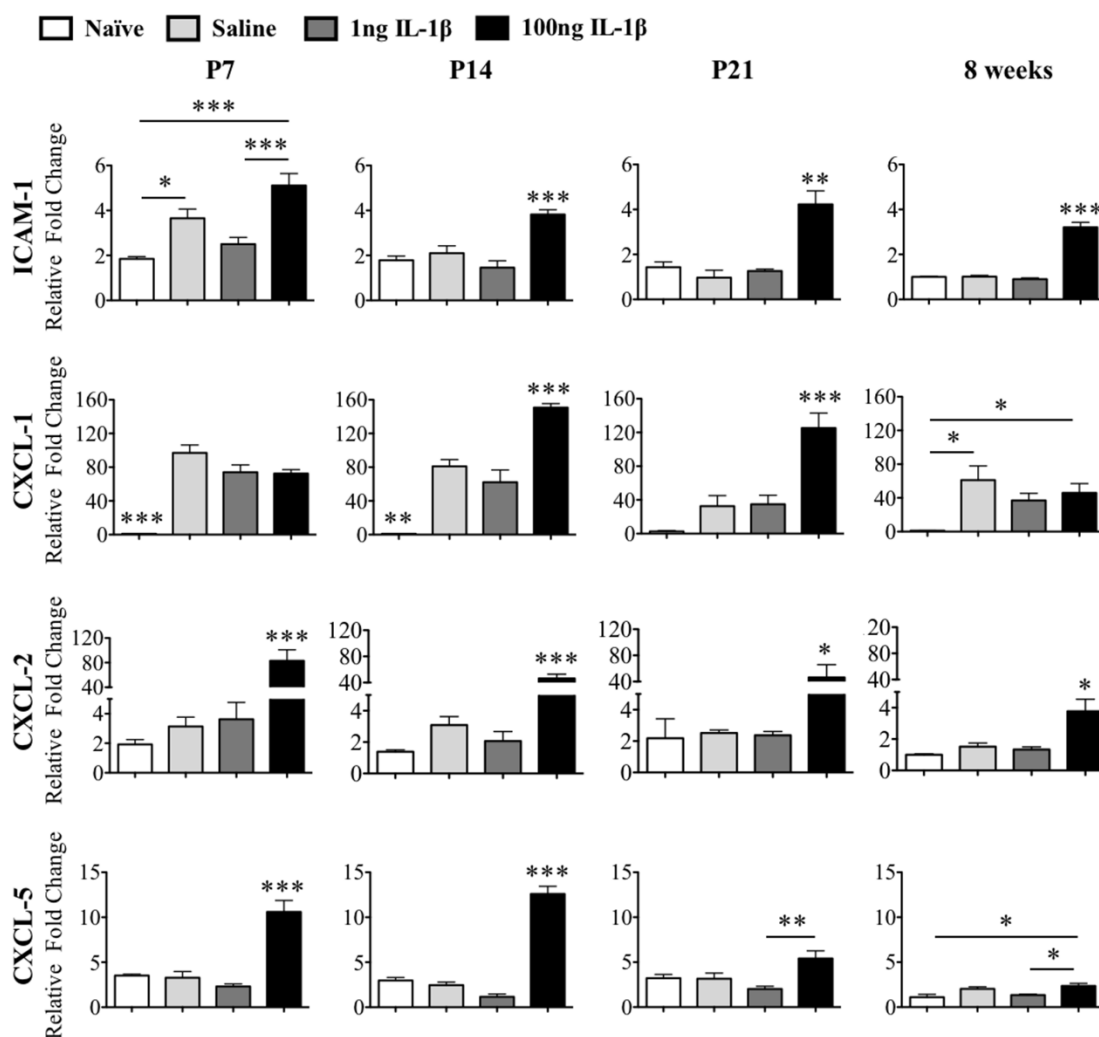


Figure 5.4 Effect of low and high doses of IL-1 β in the brain on mRNA expression of adhesion molecules and chemokines in the liver. ICAM-1, CXCL-1, CXCL-2 and CXCL-5 mRNA was measured by RT-qPCR in the liver of naïve mice and mice that received i.c. injection of saline or 1ng IL-1 β or 100ng IL-1 β at different post-natal ages (P7, P14, P21 and 8 weeks). Injection of 1ng IL-1 β in the brain did not induced the expression of any of the inflammatory mediators, but injection of 100ng IL-1 β in the brain promoted the expression in the liver: ICAM-1 at P14, P21 and 8 weeks, CXCL-1 at P14 and P21, CXCL-2 throughout development, and CXCL-5 at P7 and P14. Data are represented as relative fold change from expression levels of 8-week old naïve mice. Samples were compared with two housekeeping genes (*Csnk2a2* and *Pak1ip1*). Bars represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Statistics: one-way ANOVA with Tukey's multiple comparison test. P7: $n = 4$ for saline, $n = 5$ for 1ng and 100ng IL-1 β ; P14: $n = 4$ for saline, $n = 3$ for 1ng IL-1 β , $n = 5$ for 100ng IL-1 β , P21: $n = 4$ for saline, 1ng IL-1 β and 100ng IL-1 β ; 8 weeks: $n = 4$ for saline and 1ng IL-1 β , $n = 5$ for 100ng IL-1 β .

Table 5.1 Hepatic mRNA expression levels of inflammatory mediators after injection of 100ng IL-1 β in the brain at different time points during postnatal period.

	P7	P14	P21	8 weeks	P < 0.05
IL-1 β	10.30 $\pm$ 1.04	7.53 $\pm$ 0.45	1.79 $\pm$ 0.23	1.07 $\pm$ 0.12	P7 vs. P14, P21 and 8 weeks P14 vs. P7, P21 and 8 weeks
IL-6	104.40 $\pm$ 10.23	84.85 $\pm$ 5.17	8.81 $\pm$ 2.21	3.65 $\pm$ 0.44	
TNF	7.86 $\pm$ 0.41	6.42 $\pm$ 0.60	2.34 $\pm$ 0.09	1.11 $\pm$ 0.16	
IL-10	5.02 $\pm$ 0.66	2.32 $\pm$ 0.37	2.05 $\pm$ 0.13	1.71 $\pm$ 0.16	P7 vs. P14, P21 and 8 weeks
ICAM-1	5.11 $\pm$ 0.53	3.82 $\pm$ 0.21	4.23 $\pm$ 0.60	3.20 $\pm$ 0.22	P7 vs. P14 and 8 weeks
CXCL-1	72.41 $\pm$ 4.70	150.80 $\pm$ 4.65	125.2 $\pm$ 17.76	45.90 $\pm$ 11.10	P7 vs. P14 and P21 8 weeks vs. P14 and P21
CXCL-2	82.99 $\pm$ 18.00	46.22 $\pm$ 6.57	46.35 $\pm$ 19.50	3.77 $\pm$ 0.77	P7 vs. P14, P21 and 8 weeks P14 vs. P7 and 8 weeks P21 vs. P7 and 8 weeks
CXCL-5	10.60 $\pm$ 1.28	12.60 $\pm$ 0.85	5.41 $\pm$ 0.84	2.35 $\pm$ 0.29	P7 vs. P21 and 8 weeks P14 vs. P7, P21 and 8 weeks P21 vs. 8 weeks

Data are represented as relative fold change from expression levels of 8-week old naïve mice. Samples were compared with two housekeeping genes (Csnk2a2 and Pak1ip1). Statistical significance was calculated by a two-way ANOVA analysis with Bonferroni–Dunn post-hoc test, where all conditions (naïve, saline, 1ng IL-1 β and 100ng IL-1 β) and ages were compared.

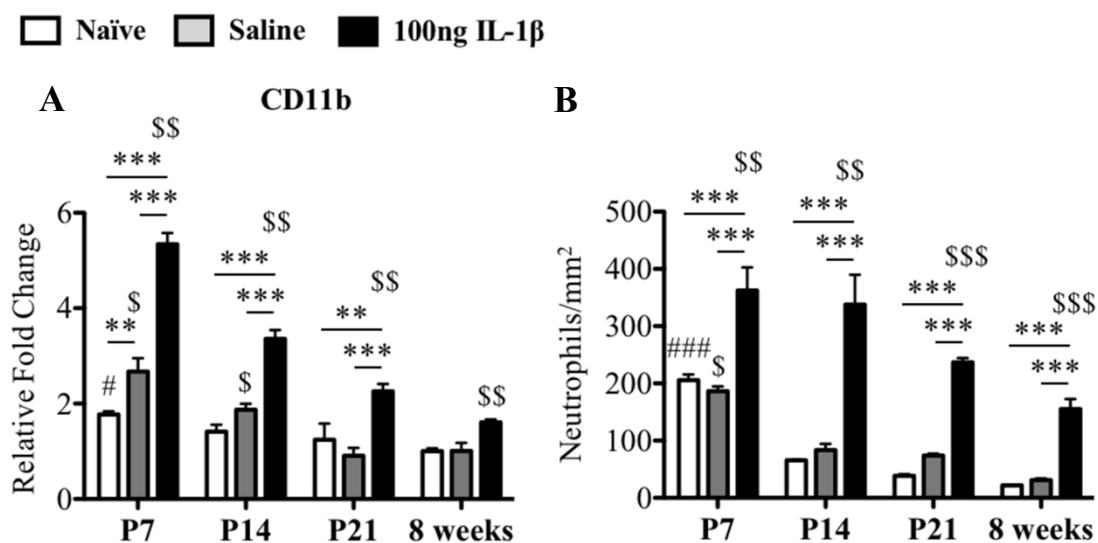


Figure 5.5 mRNA expression of CD11b and the number of neutrophils in the liver after injection of 100ng IL-1 β in the brain. CD11b mRNA was measured by RT-qPCR and the number of neutrophils were counted after immunohistochemistry for neutrophils (MBS) in the liver of naïve mice and mice that received i.c. injection of saline or 100ng IL-1 β at different postnatal ages (P7, P14, P21 and 8 weeks). Injection of 100ng IL-1 β in the left striatum significantly induced CD11b expression in the liver at P7, P14 and P21. CD11b expression after brain inflammation was highest at P7 and decreased throughout development (A). The number of neutrophils in the liver increased after i.c. injection of 100ng IL-1 β in all ages and this increase was higher at P7 and P14 than at P21 and 8 weeks (B). Data are represented as relative fold change from expression levels of 8-week old naïve mice in A and as number of neutrophils per mm² in B. Samples were compared with two housekeeping genes (Csnk2a2 and Pak1p1) in A. Bars represent mean $\pm$ SEM. **p<0.01 and ***p<0.001. For A) #p<0.05 vs. naïve 8 weeks, \$ significantly different from all saline values, \$\$ significantly different from all 100ng values. For B) ###p<0.001 vs. all naïve values, \$ significantly different from all saline values, SS significantly different from 100ng P21 and 8 weeks, \$\$\$ significantly different from all 100ng values. Statistics: two-way ANOVA with Bonferroni–Dunn post-hoc test. For A) P7: n=4 for naïve and saline, n=5 for 100ng IL-1 β ; P14: n=4 for naïve and saline, n=5 for 100ng IL-1 β ; P21 n=4 each group and 8 weeks: n=4 for naïve and saline, n=5 for 100ng IL-1 β . For B) P7: n=3 each group, P14: n=3 each group, P21: n=3 each group and 8 weeks: n=4 for naïve and saline, n=7 for 100ng IL-1 β .

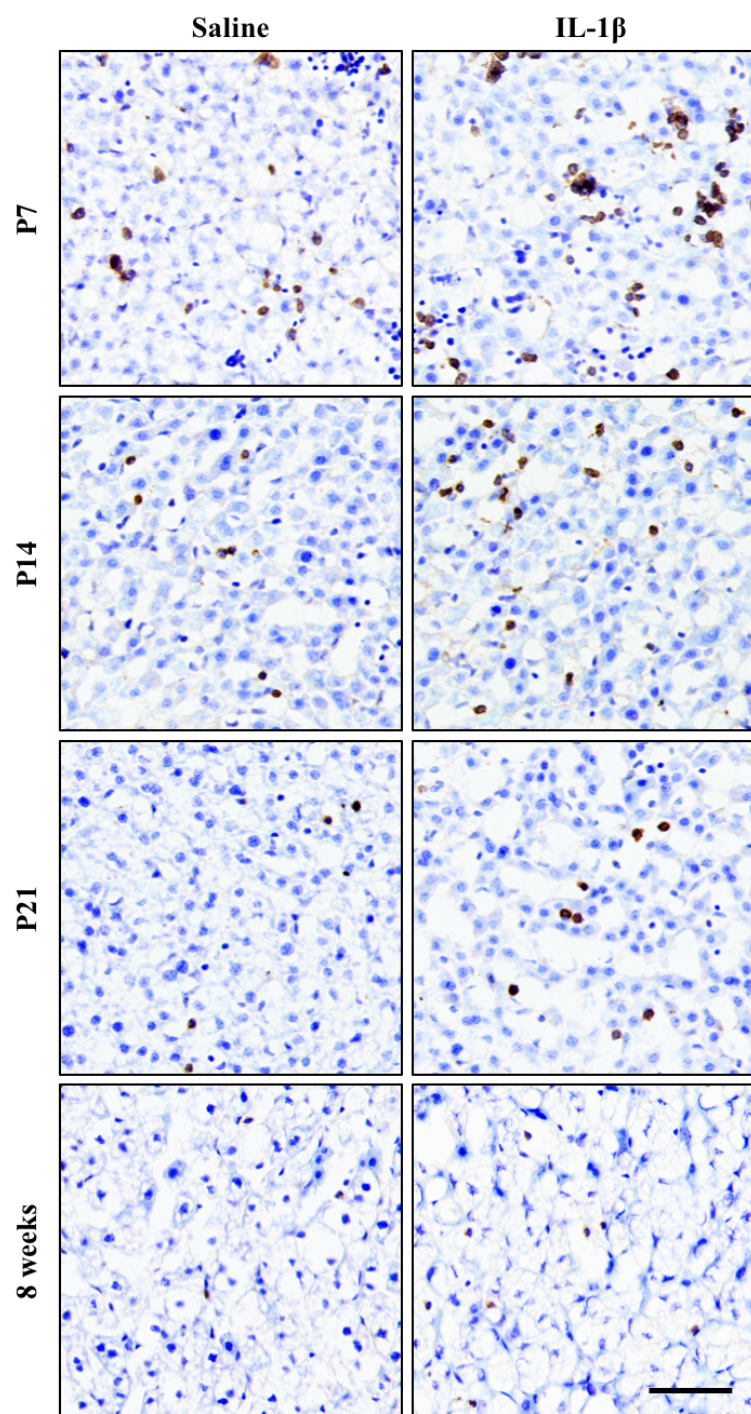


Figure 5.6 Neutrophil recruitment to the liver after injection of 100ng IL-1 β in the brain. Representative pictures of neutrophil (MBS) immunohistochemistry with cresyl violet counterstain using liver sections from mice that received i.c. injection of saline or 100ng IL-1 β at P7, P14, P21 or 8 weeks. Scale bar = 50 μ m.

5.2.3 The "window of susceptibility" in the brain disappeared after 100ng IL-1 β injection in the brain

In chapter 3, it was shown that 1ng IL-1 β injection in the left striatum increased BBB permeability and neutrophil recruitment to the parenchyma at P14. In addition, i.c. injection of 1ng IL-1 β did not produce peripheral inflammation. Given that the 100ng dose of IL-1 β affected the liver inflammatory response, experiments were performed to determine if this dose of IL-1 β would have the same effect in the brain as 1ng IL-1 β . Previous studies have shown that increasing doses of IL-1 β injected in the brain in adult rats not only induced the APR in the liver, but also a dose-dependent leukocyte recruitment to the brain (Campbell et al., 2003). Double immunohistochemistry for neutrophils (MBS) and vessels (Glut-1) and immunohistochemistry for endogenous IgG were performed using brain sections of naïve and saline or 1ng IL-1 β or 100ng IL-1 β injected mice. As discussed in chapter 3, no neutrophils were observed in naïve or saline injected mice. Representative spatial maps of neutrophil distribution in brains injected with 1ng IL-1 β at P7, P14, P21 and 8 weeks showed that, as indicated previously in chapter 3, large number of neutrophils were recruited to the ipsilateral hemisphere at P14. Very few neutrophils were found in the brain of P7, P21 and 8week-old mice injected with 1ng IL-1 β . Representative spatial maps of neutrophil recruitment to the brain injected with 100ng IL-1 β showed that very few neutrophils were present in any of the time points of development (Figure 5.7). To confirm these observations, neutrophils were counted in all ipsilateral striatum to determine their presence in the brain parenchyma or vessels (Figure 5.8). There was a decrease in the number of neutrophils in the parenchyma from i.c. injection of 1ng IL-1 β to 100ng IL-1 β at P7 (23 ± 10 cells/mm² and 0.14 ± 0.14 cells/mm², respectively), P14 (85 ± 21 cells/mm² and 1 ± 0.1 cells/mm², respectively) and P21 (13 ± 4 cells/mm² and 1 ± 1 cells/mm², respectively), though only significantly

($p < 0.001$) different at P14 (Figure 5.8B). At 8 weeks the brain parenchyma had 0.1 ± 0.1 cells/mm² when 1ng IL-1 β was injected and 1 ± 1 cells/mm² after i.c. injection of 100ng IL-1 β , but this was not statistically different (Figure 5.8B). In vessels, there was a significant decrease on the number of cells from 1ng IL-1 β to 100ng IL-1 β at P7 (11 ± 3 cells/mm² and 2 ± 0.5 cells/mm², respectively), P14 (8 ± 1 cells/mm² and 2 ± 0.2 cells/mm², respectively) and P21 (8 ± 2 cells/mm² and 1 ± 0.5 cells/mm², respectively). There was no significant decrease at 8 weeks (1ng IL-1 β : 0.1 ± 0.1 cells/mm² and 100ng IL-1 β : 1 ± 1 cells/mm²) (Figure 5.8C). The Two-way ANOVA analysis with Bonferroni–Dunn post-hoc test used for these results also showed that the number of neutrophils in vessels significantly decreased from P7, P14 and P21 to 8 weeks and that the number of neutrophils in the parenchyma was higher at P14 than at P7, P21 and 8 weeks 4 hours after the i.c. injection of 1ng IL-1 β . However, these changes were no longer statistically significant when 100ng IL-1 β was injected in the striatum, showing that the number of cells either in vessels or in the brain parenchyma is similar between the different ages (Figure 5.8B and C). The absence of neutrophils at P14 after i.c. injection of 100ng IL-1 β when compared to 1ng IL-1 β injection in the brain was correlated with BBB permeability. As observed in chapter 3, 1ng IL-1 β injection in the brain increased BBB permeability to endogenous IgG only at P14 when compared to P7 and P21. No increased BBB permeability was observed at 8 weeks when compared to saline controls. After i.c. injection of 100ng IL-1 β , no increased BBB permeability was detected in any time point when compared with saline control (Figure 5.9).

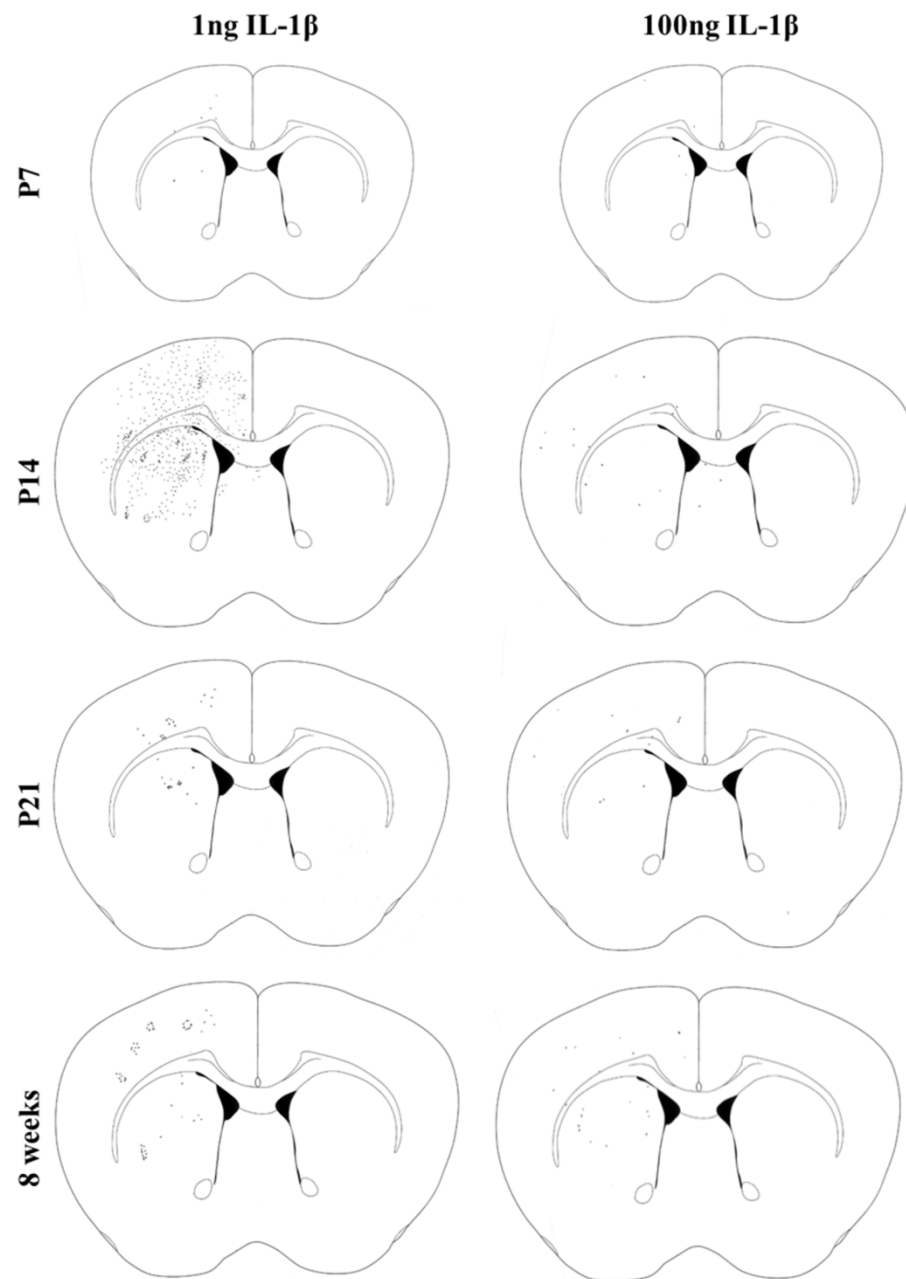


Figure 5.7 Spatial maps of neutrophil distribution after injection of 1ng or 100ng IL-1 β in the brain. Double immunohistochemistry for neutrophils (MBS) and vessels (GLUT-1) was performed using brain sections from mice that received i.c. injection of 1ng or 100ng IL-1 β at P7, P14, P21 or 8 weeks. Representative spatial maps were obtained and these showed that more neutrophils were present in the ipsilateral hemisphere at P14 after i.c. injection of 1ng IL-1 β when compared to P7, P21 and 8-week-old mice. Very few neutrophils were present in the brain at any time point during postnatal development when 100ng IL-1 β was injected in the striatum.

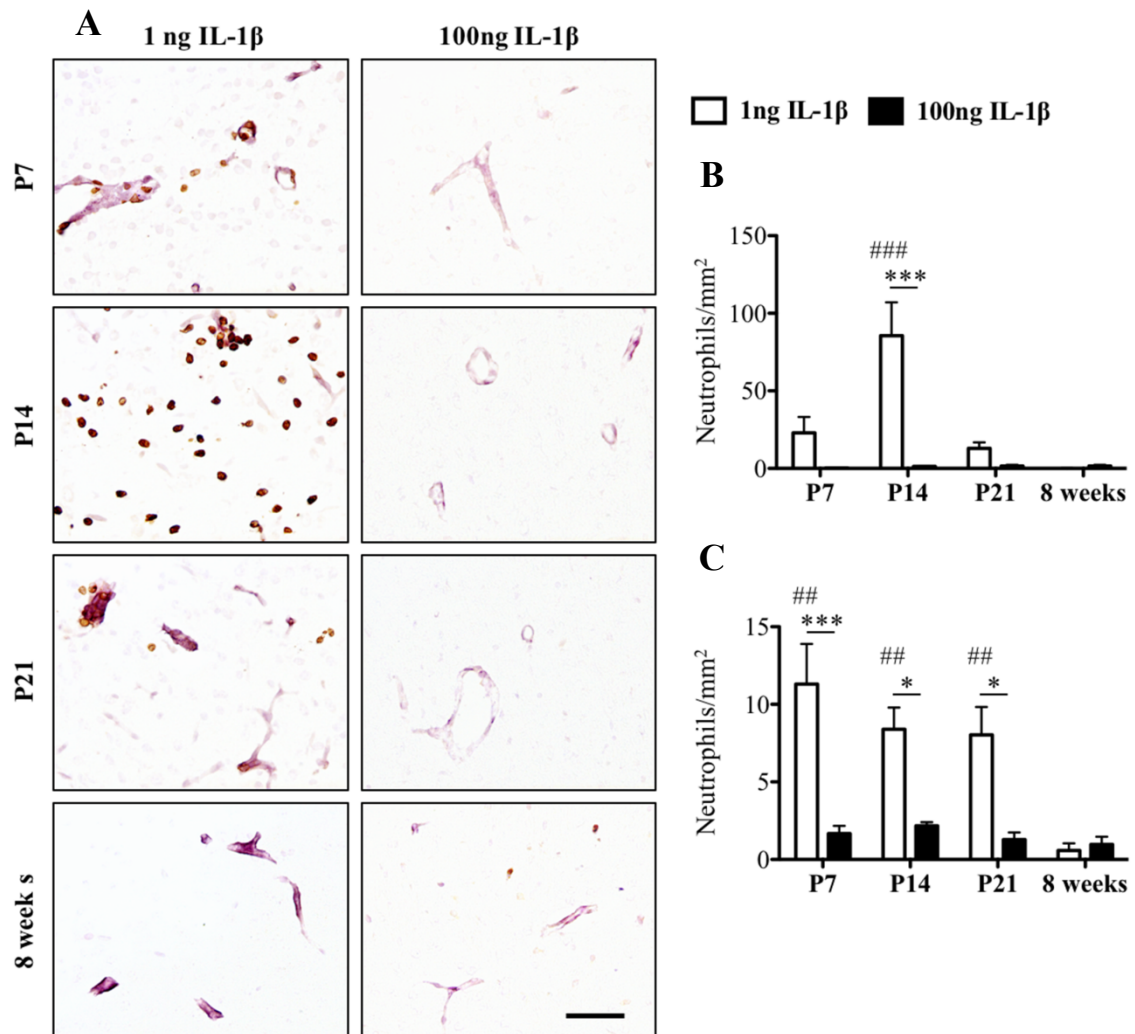


Figure 5.8 Recruitment of neutrophils to the brain after injection of 1ng or 100ng IL-1 β in the brain. The number of neutrophils were counted in brains injected with 1ng or 100ng IL-1 β at P7, P14, P21 or 8 weeks after double immunohistochemistry for neutrophils (MBS, brown) and vessels (laminin 1+2, purple) on the basis of their presence in the parenchyma or in vessels (A). More neutrophils were recruited to the brain parenchyma after i.c. injection of 1ng IL-1 β at P14 when compared to P7, P21 and 8-week-old mice and this difference disappeared after 100ng IL-1 β injection in the brain (B). In vessels, more neutrophils were counted at P7, P14 and P21 than at 8 weeks when 1ng IL-1 β was injected in the brain and all ages had approximately the same number of neutrophils after i.c. injection of 100ng IL-1 β (C). Data are represented as number of neutrophils per mm² of striatum. * p <0.05, *** p <0.001, ### p <0.001 vs. all 1ng values and ## p <0.01 vs. 1ng 8weeks mean. Statistics: two-way ANOVA with Bonferroni–Dunn post-hoc test. P7: n =4 each group; P14: n =5 for 1ng IL-1 β , n =3 for 100ng IL-1 β ; P21: n =4 for 1ng IL-1 β , n =3 for 100ng IL-1 β ; 8 weeks: n =3 each group. Scale bar = 50 μ m.

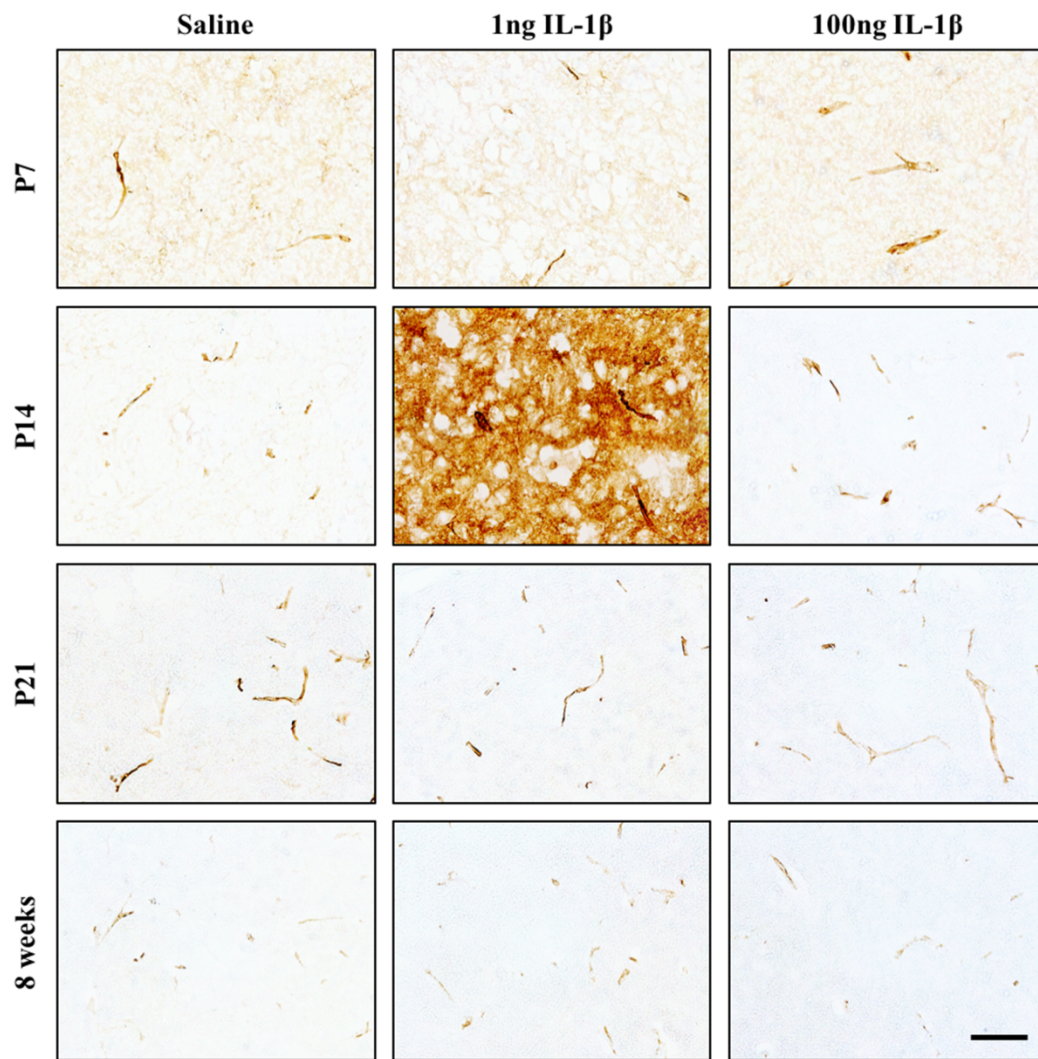


Figure 5.9 Effects of 1ng or 100ng IL-1 β in the brain on BBB permeability. BBB permeability to endogenous IgG was measured in brains injected with saline or 1ng or 100ng IL-1 β at P7, P14, P21 or 8 weeks. Representative pictures of BBB permeability in the ipsilateral striatum of each treatment group of mice are shown. Significant increase in BBB permeability was observed only at P14 after i.c. injection of 1ng IL-1 β and not after 100 ng IL-1 β . Scale bar = 50 μ m. P7: n=4 each group; P14: n=4 for saline, n=5 for 1ng IL-1 β , n=3 for 100ng IL-1 β ; P21: n=4 for saline and 1ng IL-1 β , n=3 for 100ng IL-1 β ; 8 weeks: n=3 each group.

5.2.4 Blood-brain barrier permeability and neutrophil recruitment to the brain decreased while brain expression of inflammatory mediators increased with the concentration of IL-1 β injected in the brain

In light of the findings described above, a more extensive dose response experiment was performed at P14, repeating the previous findings together with an additional intermediate dose of 10ng. BBB permeability, neutrophil recruitment and expression of IL-1 β and ICAM-1 were examined and compared to naïve and saline injected mice. After 4 hours, IgG positive stain was found in the ipsilateral striatum after 1ng IL-1 β injection, consistent with previous results. This decreased when 10ng IL-1 β was injected in the brain, and almost no IgG was visible after i.c. injection of 100ng IL-1 β (Figure 5.10).



Figure 5.10 Effects of increasing doses of IL-1 β on BBB permeability during susceptible period. BBB permeability to endogenous IgG was measured in brains injected with 1ng, 10ng or 100ng IL-1 β at P14. BBB permeability was highest in the ipsilateral striatum injected with 1ng IL-1 β and this decreased with the increasing concentration of IL-1 β injected in the brain. $n=3$ each group. Representative pictures are shown here.

This result was correlated with the number of neutrophils recruited to the brain. After immunohistochemistry for neutrophils (MBS) and vessels (Glut-1), it was observed that the number of neutrophils in the ipsilateral striatum decreased with the severity of the inflammation (Figure 5.11A and B). In brains injected with 1ng IL-1 β , 37 ± 18 neutrophils per mm² of striatum were observed in vessels and 126 ± 41 neutrophils per

mm² of striatum were found in the parenchyma, indicating that from the total number of neutrophils recruited to the brain more of these cells were able to reach the parenchyma ($p < 0.05$) (Figure 5.11C). When 10ng ($14 \pm 7/\text{mm}^2$ in vessels and $10 \pm 7/\text{mm}^2$ in the parenchyma) or 100ng IL-1 β ($5 \pm 1/\text{mm}^2$ in vessels and $2 \pm 0.2/\text{mm}^2$ in the parenchyma) were injected in the striatum, not only were there fewer neutrophils found all together, but a smaller percentage were able to cross the BBB. The difference between the number of cells in the parenchyma was not statistically significant when 10ng or 100ng IL-1 β were injected in the brain, and the same was observed for the number of cells in vessels. Consequently, the number of neutrophils in the parenchyma was significantly ($p < 0.01$) higher when 1ng IL-1 β was injected in the brain when compared to 10ng and 100ng IL-1 β (Figure 5.11C).

mRNA expression of IL-1 β and ICAM-1 was measured in the ipsilateral and contralateral striatum of naïve mice and saline, 1ng, 10ng or 100ng IL-1 β injected P14 mice (Figure 5.12). Expression levels of both transcripts were represented as relative fold change from contralateral expression levels of naïve P14 mice. Both inflammatory mediators presented similar expression pattern. There were no significant differences between ipsilateral and contralateral expression of IL-1 β and ICAM-1 in any of the control or treatment groups. Expression of both inflammatory mediators in the ipsilateral striatum did not change from naïve (IL-1 β : 1.08 ± 0.06 ; ICAM-1: 1.24 ± 0.15) to saline injected animals (IL-1 β : 4.57 ± 2.03 ; ICAM-1: 1.11 ± 0.12). Expression levels of IL-1 β and ICAM-1 in the ipsilateral side of the brain significantly increased from naïve and saline injected brains to 1ng IL-1 β injected striatum (IL-1 β : 278.30 ± 151.10 ; ICAM-1: 18.80 ± 9.36) and from 1ng IL-1 β to 10ng IL-1 β (IL-1 β : 588.70 ± 27.42 ; ICAM-1: 52.67 ± 2.10) and 100ng (IL-1 β : 729.80 ± 159.10 ; ICAM-1: 65.80 ± 2.99) injected brains. The mRNA expression of both inflammatory mediators in the ipsilateral striatum did not

significantly differ between 10ng and 100ng IL-1 β treatment groups. In the contralateral striatum, the expression of IL-1 β and ICAM-1 in naïve mice (IL-1 β : 1.03 ± 0.14 ; ICAM-1: 1.00 ± 0.7), and saline (IL-1 β : 1.34 ± 0.22 ; ICAM-1: 0.93 ± 0.10) or 1ng IL-1 β (IL-1 β : 36.88 ± 10.38 ; ICAM-1: 4.38 ± 0.92) injected animals did not significantly differ from each other. The expression of both inflammatory mediators in the contralateral side significantly increased from controls and 1ng IL-1 β injected striatum to 10ng IL-1 β injected brains (IL-1 β : 319.90 ± 34.03 ; ICAM-1: 31.34 ± 6.38) and from 10ng IL-1 β to 100ng IL-1 β (IL-1 β : 669.60 ± 91.44 ; ICAM-1: 64.43 ± 5.68) injected mice (Figure 5.12).

5.2.5 The inverse relationship between the severity of the brain cellular injury and the magnitude of the hepatic acute phase response

In the previous section, a dose response was observed within the P14 brain, which was characterised by a disconnect between the local inflammatory response and the measures of BBB permeability and neutrophil recruitment. Thus, the APR in the liver was assessed in order to establish if an equivalent relationship exists between the brain and peripheral response. Immunohistochemistry for neutrophils (MBS) (Figure 5.13) and RT-qPCR for IL-6, TNF, ICAM-1, CXCL-2 and CXCL-5 (Figure 5.14) were performed. The number of neutrophils in the liver increased as the concentration of IL-1 β injected in the brain increased (Figure 5.13A). The number of cells was unchanged between naïve (54 ± 6 cells/mm²), saline (62 ± 13 cells/mm²) and 1ng IL-1 β (64 ± 4 cells/mm²) injected mice. However, i.c. injection of 10ng IL-1 β significantly induced the recruitment of neutrophils to the liver (150 ± 36 cells/mm²). The number of neutrophils in the liver was even higher ($p < 0.001$) after 100ng IL-1 β injection (319 ± 18 /mm²) (Figure 5.13B). IL-6, TNF, ICAM-1, CXCL-2 and CXCL-5 showed similar mRNA expression pattern. The expression of all these inflammatory mediators in the liver increased from control and 1ng IL-1 β

injected mice to 10ng and 100ng IL-1 β injected animals though, it was only statistically significant after the injection of 100ng IL-1 β into the brain (Figure 5.14).

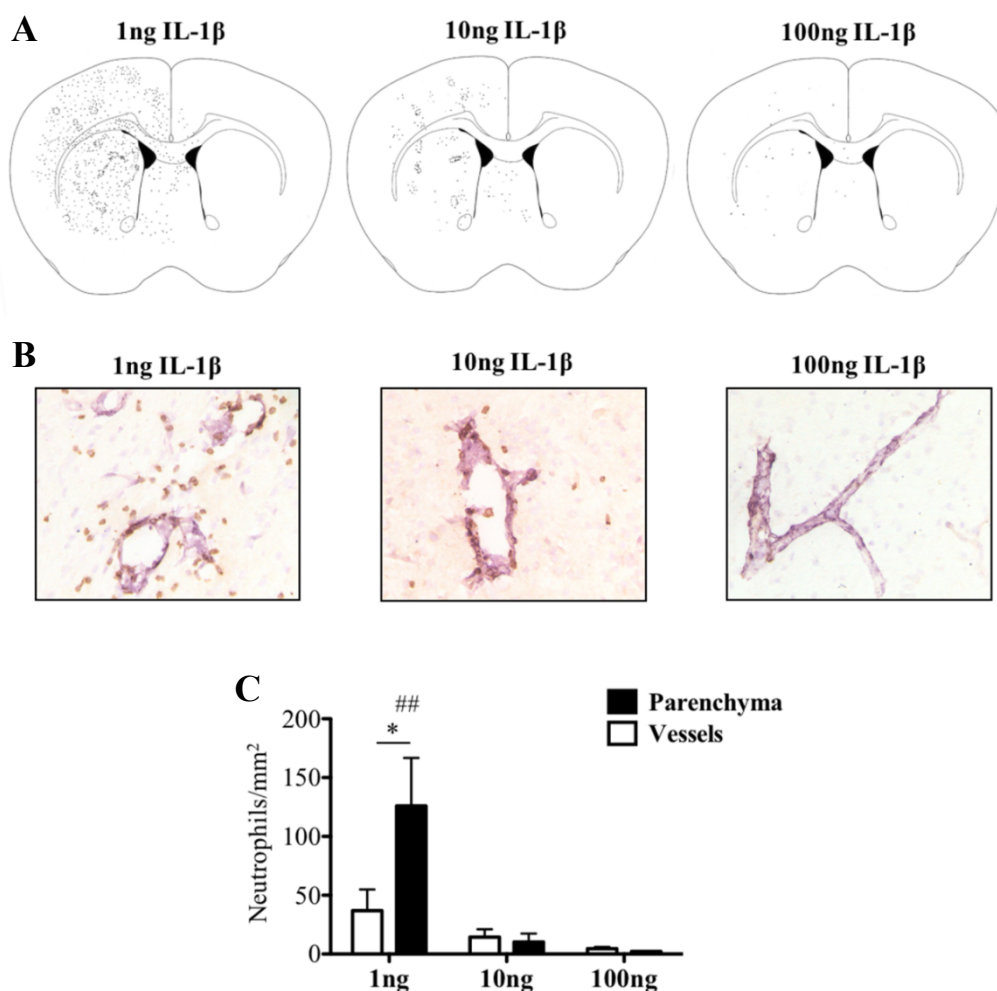


Figure 5.11 Neutrophil recruitment after injection of 1ng, 10ng or 100ng IL-1 β in the brain during susceptible period. Representative spatial maps of neutrophil distribution in the brain (A) and double immunohistochemistry for neutrophils (MBS, brown) and vessels (laminin 1+2, purple) (B) using brain sections from mice injected with 1ng, 10ng or 100ng IL-1 β at P14 showed that the number of neutrophils in the brain decreased with the dose of IL-1 β . Quantification of neutrophils on the basis of their presence in the parenchyma or in vessels showed that more cells were recruited to the brain parenchyma after i.c. injection of 1ng IL-1 β when compared to 10ng or 100ng IL-1 β (C). Data are represented as number of neutrophils per mm² of striatum. *p<0.05 and ##p<0.01 vs all parenchyma values. Statistics: two-way ANOVA with Bonferroni–Dunn post-hoc test. n=3 each group. Scale bar = 50 μ m.

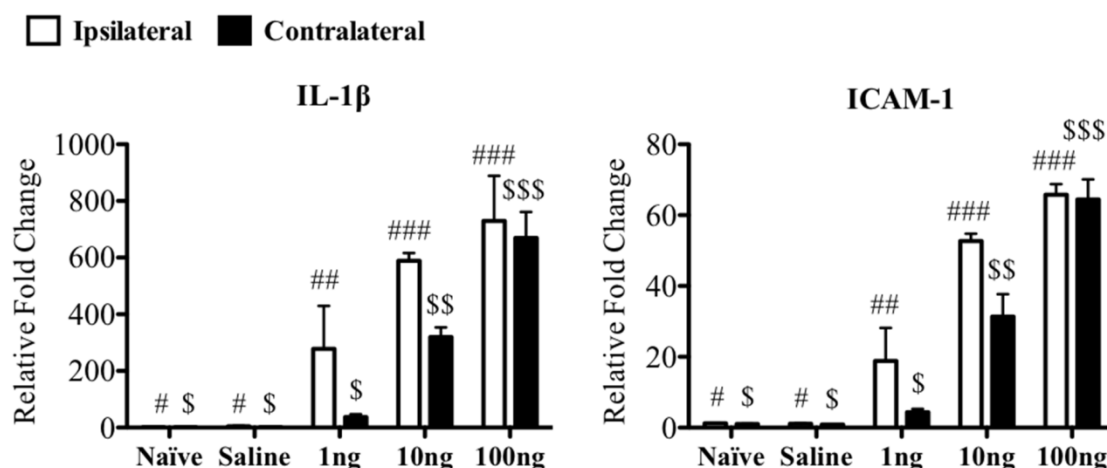


Figure 5.12 Effect of increasing focal inflammation on the expression of IL-1 β and ICAM-1 in the brain. IL-1 β and ICAM-1 mRNA was measured by RT-qPCR in the ipsilateral and contralateral striatum of naïve mice and mice that received i.c. injection of saline or 1ng, 10ng or 100ng IL-1 β at P14. The expression of both inflammatory mediators in the brain increased from control animals to 1ng IL-1 β injected mice and continued to increase with the concentration of IL-1 β injected in the striatum. Data are represented as relative fold change from contralateral expression levels of P14 naïve mice. Samples were compared with two housekeeping genes (Zpf91 and Cdc40). Bars represent mean $\pm$ SEM. # significantly different from 1ng, 10ng and 100ng ipsilateral values. ## significantly different from naïve, saline, 10ng and 100ng ipsilateral values, ### significantly different from naïve, saline and 1ng ipsilateral values, \$ significantly different from 10ng and 100ng contralateral values, \$\$ significantly different from naïve, saline, 1ng, 100ng contralateral values, \$\$\$ significantly different from naïve, saline, 1ng, 10ng contralateral values. Statistics: one-way ANOVA with Tukey's multiple comparison test. n=4 each group.

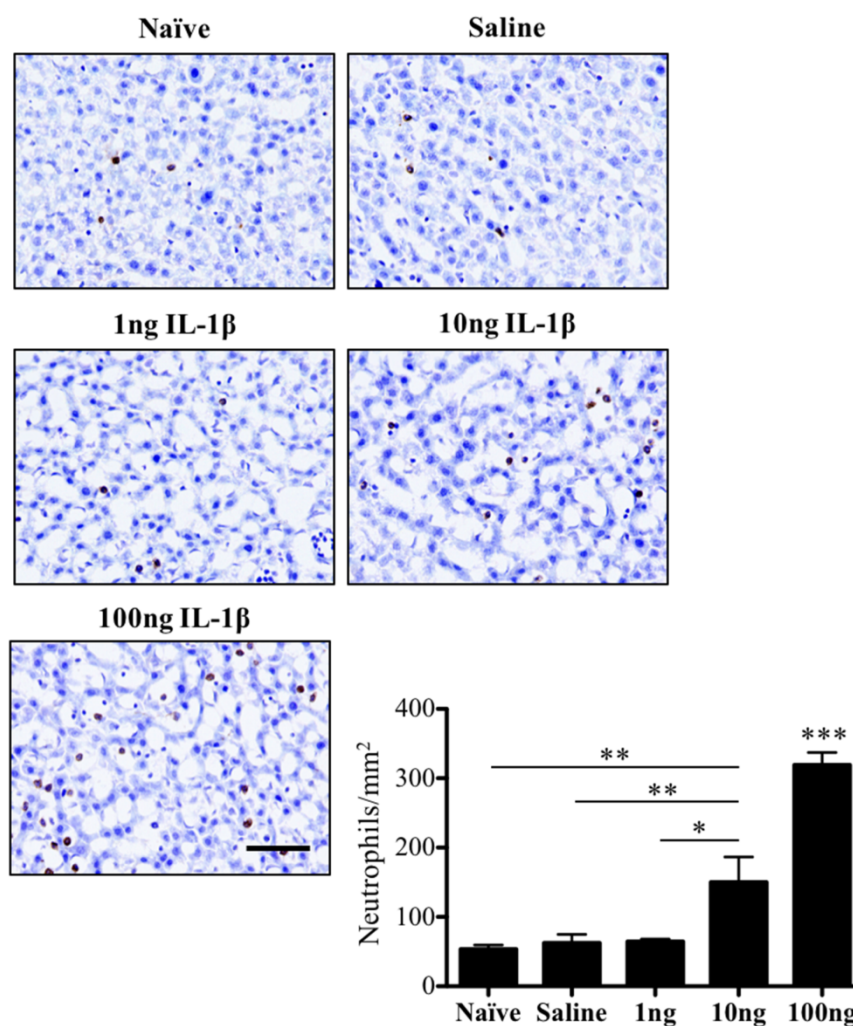


Figure 5.13 Neutrophil recruitment to the liver after injection of 1ng, 10ng or 100ng IL-1 β in the brain during susceptible period. The number of neutrophils were counted after immunohistochemistry for neutrophils (MBS, brown) with cresyl violet counterstain (blue) in the liver of naïve mice and mice that received i.c. injection of saline or 1ng, 10ng or 100ng IL-1 β at P14 (A). Scale bar = 50 μ m. The number of neutrophils did not change when 1ng IL-1 β was injected in the brain but increased after i.c. injection of 10ng IL-1 β and even more after 100ng IL-1 β injection (B). Data are represented as number of neutrophils per mm². * p <0.05, ** p <0.01 and *** p <0.001. Statistics: one-way ANOVA with Tukey's multiple comparison test. n =3 each group.

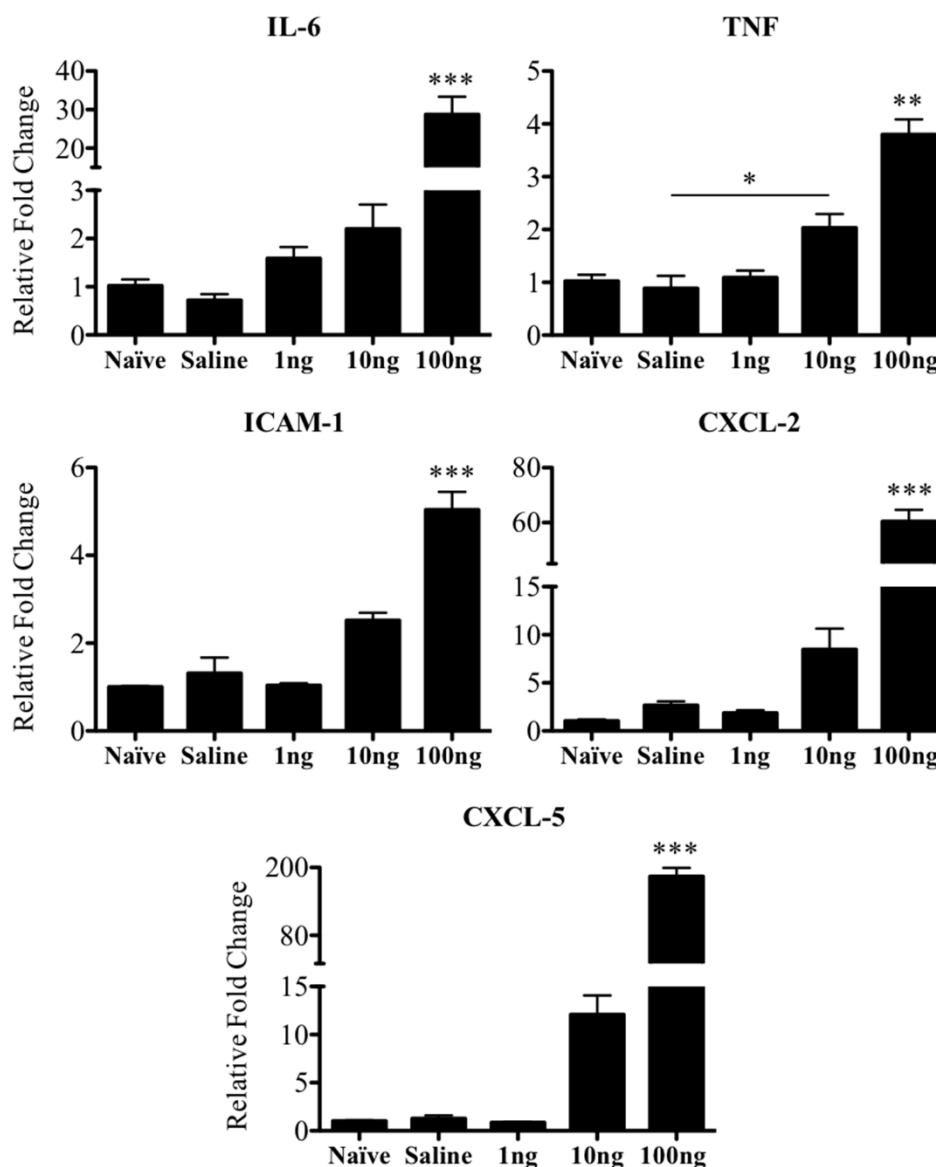


Figure 5.14 The expression of inflammatory mediators in the liver after i.c. injection of 1ng, 10ng or 100ng IL-1 β during susceptible period. IL-6, TNF, ICAM-1, CXCL-2 and CXCL-5 mRNA was measured by RT-qPCR in the liver of naïve mice and mice that received i.c. injection of saline or 1ng, 10ng or 100ng IL-1 β at P14. Injection of 100ng IL-1 β in the brain significantly induced the expression of all inflammatory mediators. Data are represented as relative fold change from expression levels of P14 naïve mice. Samples were compared with two housekeeping genes (Csnk2a2 and Pak1p1). Bars represent mean $\pm$ SEM. * p <0.05 and *** p <0.001. Statistics: one-way ANOVA with Tukey's multiple comparison test. n =4 for naïve, 1ng, 10ng and 100ng and n =8 for saline.

5.2.6 Peripheral injection of IL-1 β after injection of a low dose of IL-1 β induced the hepatic acute phase response

The results described above showed an inverse relationship between the severity of the brain cellular response to inflammation and the magnitude of the APR in the liver, which suggested that the response in the liver to high concentrations of IL-1 β injected in the brain may contribute to the decreased neutrophil recruitment to the brain parenchyma. To test this hypothesis, P14 mice received an i.v. injection of 100ng IL-1 β immediately after i.c. injection of 1ng IL-1 β in order to activate the liver immune response to a degree observed within previous experiments. Two other groups of mice were used as controls, one received i.c. injection of saline and i.v. injection of 100ng IL-1 β and the other received i.c. injection of 1ng IL-1 β and i.v. injection of saline. Immunohistochemistry for neutrophils (MBS) in the liver was performed (Figure 15A). The number of neutrophils recruited to the liver significantly increased in both groups where i.v. injection of 100ng IL-1 β was performed ($255 \pm 20/\text{mm}^2$ saline i.c. / 100ng i.v. and $195 \pm 11/\text{mm}^2$ 1ng i.c. / 100ng i.v.) when compared to 1ng i.c. / saline i.v. ($45 \pm 3/\text{mm}^2$). A comparison was also made between the number of neutrophils in the liver of these three groups to mice from previous experiments where 100ng IL-1 β was injected in the striatum. This animal group had more neutrophils (328 ± 25 cells/ mm^2) in the liver than all three groups that received i.v. injections (Figure 5.15B). Although the injection of 100ng IL-1 β in the brain significantly increased the number of neutrophils in the liver when compared to saline i.c. / 100ng i.v., 1ng i.c. / saline i.v. and 1ng i.c. / 100ng i.v. groups of animals, these experiments showed that injection of 100ng IL-1 β in the periphery was able to increase the number of neutrophil recruited to the liver and, consequently, closely mimic liver response after injection of a high dose of IL-1 β in the brain.

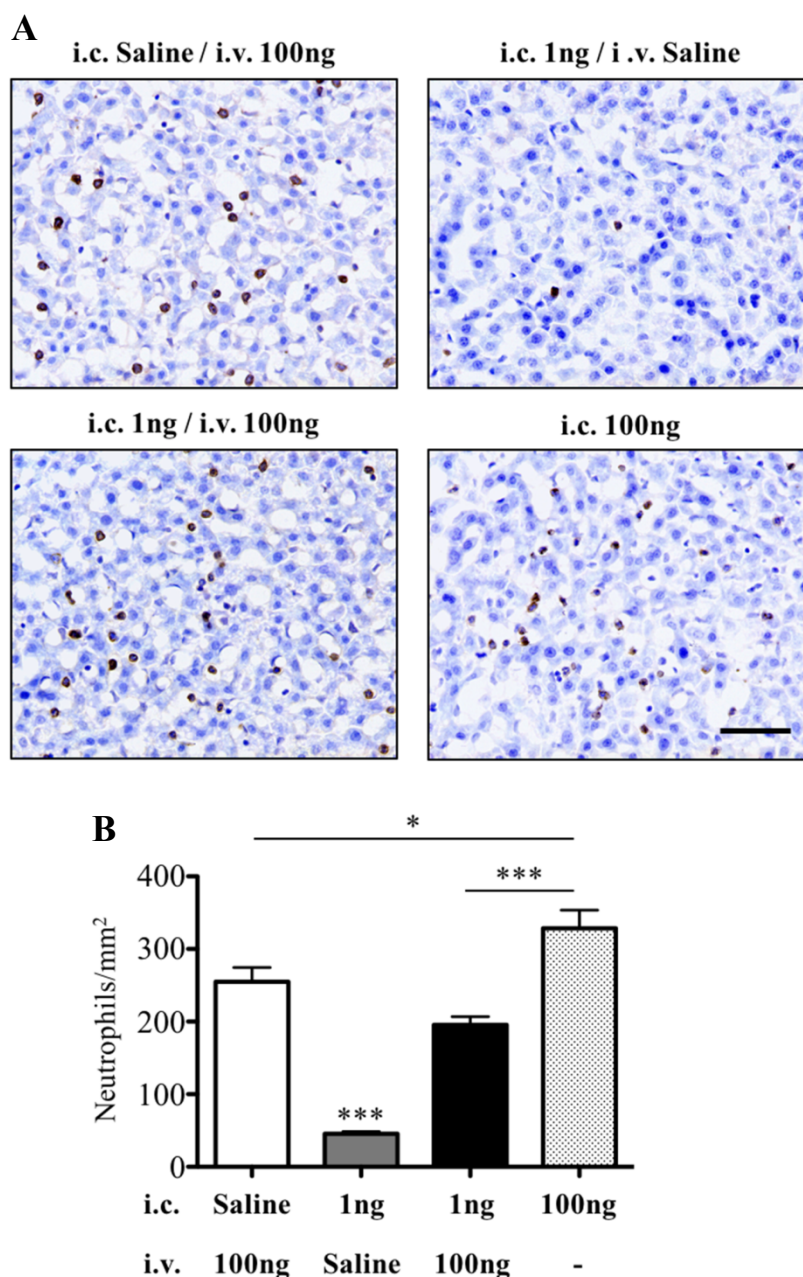


Figure 5.15 Effect of experimental activation of the APR on the number of neutrophils recruited to the liver after injection of 1ng IL-1 β in the brain. Immunohistochemistry for neutrophils (MBS) with cresyl violet counterstain was performed using livers from P14 mice that received an i.v. injection of saline or 100ng IL-1 β immediately after i.c. injection of 1ng IL-1 β (1ng i.c. / saline or 100ng i.v.) and mice that received i.c. injection of saline followed by i.v. injection of 100ng IL-1 β (saline i.c. / 100ng i.v.) (A). Representative picture of neutrophil presence in the liver of mice injected with 100ng IL-1 β in the brain obtained from previous experiments is also showed in A. Scale bar = 50 μ m. Quantification of neutrophils showed that injection of 100ng IL-1 β in periphery after injection of 1ng IL-1 β in the brain increased the number of neutrophils recruited to the liver, mimicking the APR in the liver after injection of 100ng IL-1 β in the brain (B). Data are represented as number of neutrophils per mm². *p<0.05 and ***p<0.001. Statistics: one-way ANOVA with Tukey's multiple comparison test. saline i.c. / 100ng i.v.: n=3, 1ng i.c. / saline i.v.: n=8, 1ng i.c. / 100ng i.v.: n=8 and 100ng i.c.: n=6.

The expression levels of many inflammatory mediators also supported the concept that systemic administration of 100ng IL-1 β induces the hepatic APR as observed when 100ng IL-1 β was injected in the left striatum. IL-6, TNF and CXCL-5 were upregulated in the liver of 1ng i.c. / 100ng i.v. when compared to 1ng i.c. / saline i.v. group of mice (Figure 5.16). In detail, mRNA expression of IL-6, TNF, ICAM-1, CXCL-2 and CXCL-5 was measured in the liver of the saline i.c. / 100ng i.v., 1ng i.c. / saline i.v. and 1ng i.c. / 100ng i.v. groups of mice (Figure 5.16). Expression levels of all transcripts were represented as relative fold change from expression levels of 1ng i.c. / saline i.v. group of mice. IL-6 and TNF were significantly upregulated in livers of saline i.c. / 100ng i.v. (IL-6: 19.13 ± 2.81 ; TNF- α : 3.64 ± 0.27) and 1ng i.c. / 100ng i.v. (IL-6: 13.03 ± 2.70 ; TNF- α : 2.83 ± 0.43) animals when compared to the 1ng i.c. / saline i.v. (IL-6: 1.05 ± 0.14 ; TNF- α : 1.14 ± 0.22) group of mice and, did not significantly differ from each other. ICAM-1 mRNA expression was significantly increased in saline i.c. / 100ng i.v. (3.88 ± 0.29) compared to 1ng i.c. / saline i.v. (1.26 ± 0.30) livers. Mice that received 1ng i.c. followed by 100ng i.v. showed increased expression of ICAM-1 in the liver (2.53 ± 0.48) though not significantly different from the other two groups. CXCL-2 was significantly higher in the liver of saline i.c. / 100ng i.v. (17.08 ± 4.86) mice than 1ng i.c. / saline i.v. (1.08 ± 0.18) and 1ng i.c. / 100ng i.v. (4.45 ± 0.96) animals. Once again, CXCL-2 was increased in 1ng i.c. / 100ng i.v. livers, but not statically significant. Finally, CXCL-5 expression was upregulated in the livers from saline i.c. / 100ng i.v. (147.80 ± 18.24) and 1ng i.c. / 100ng i.v. (65.60 ± 7.62) when compared to 1ng i.c. / saline i.v. (1.06 ± 0.15). Also, CXCL-5 expression was significantly higher in saline i.c. / 100ng i.v. than in 1ng i.c. / 100ng i.v. mice (Figure 5.16).

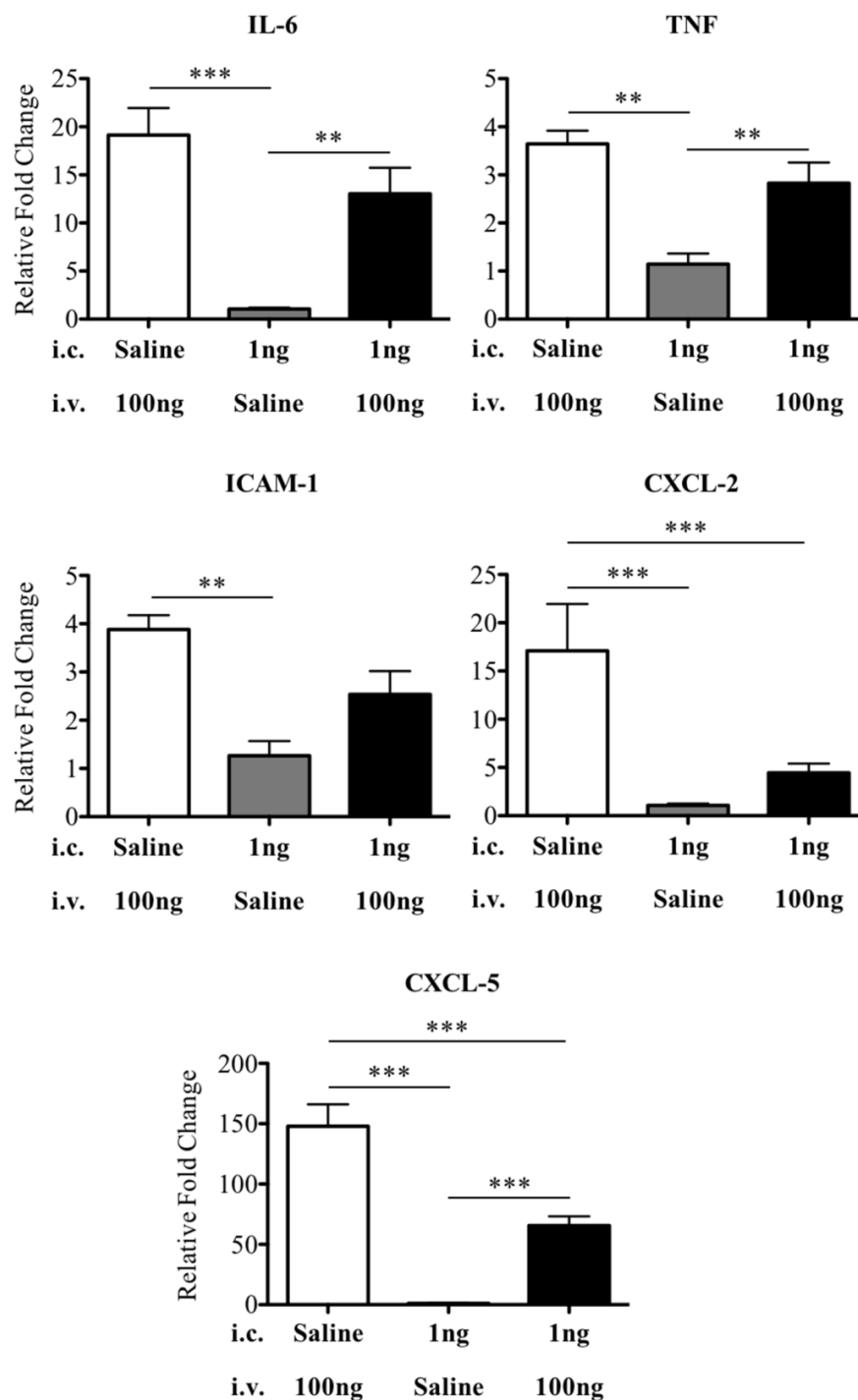


Figure 5.16 Effect of experimental activation of the APR on the hepatic expression of inflammatory mediators after injection of 1ng IL-1 β in the brain. IL-6, TNF, ICAM-1, CXCL-2 and CXCL-5 mRNA was measured by RT-qPCR in the liver of three different groups of P14 mice: saline i.c. / 100ng i.v., 1ng i.c. / saline i.v. and 1ng i.c. / 100ng i.v.. The injection of IL-1 β in the periphery after i.c. injection of 1ng IL-1 β increased the expression of IL-6, TNF and CXCL-2 in the liver when compared to a sole i.c. injection of 1ng IL-1 β in the brain. Samples were compared with two housekeeping genes (Csnk2a2 and Pak1ip1). Data are represented as relative fold change from expression levels of 1ng i.c. / saline i.v. group of animals. Bars represent mean $\pm$ SEM. ** p <0.01 and *** p <0.001. Statistics: one-way ANOVA with Tukey's multiple comparison test. saline i.c. / 100ng i.v.: n =3, 1ng i.c. / saline i.v.: n =8 and 1ng i.c. / 100ng i.v.: n =8.

5.2.7 Experimental activation of the acute phase response after intracerebral injection of a low dose of IL-1 β protected the brain from neutrophil recruitment and increased BBB permeability

Activation of the APR in the liver together with focal inflammation in the brain promoted by injection of 1ng IL-1 β was characterised by the increased number of neutrophils, and expression of inflammatory cytokines and chemokines in the liver. This outcome was equivalent to that achieved by a single i.c. injection of 100ng IL-1 β . As the i.c. injection of 100ng IL-1 β had been previously shown to reduce BBB breakdown and recruitment of neutrophils, further assessment was performed to determine if this phenomenon was recapitulated in the brains of 1ng i.c. / 100ng i.v. treated animals. Immunohistochemistry for neutrophils (MBS) and endogenous IgG was performed using brain sections of mice that received i.c. injection of 1ng IL-1 β followed by i.v. injection of saline or 100ng IL-1 β and from mice of previous experiments that receive i.c. injection of 100ng IL-1 β . Representative spatial maps of neutrophil distribution in the brain showed that activation of the APR in the liver after injection of 1ng IL-1 β in the brain induced an almost complete depletion of neutrophils in the brain and this resembled brains that received i.c. injection of 100ng IL-1 β (Figure 5.17A). Immunohistochemistry (Figure 5.17B) and quantification of the number of neutrophils per mm² of striatum (Figure 5.17C) confirmed this observation. The number of neutrophils in the striatum significantly decreased ($p < 0.001$) in mice that received i.c. injection of 1ng IL-1 β followed by i.v. injection of saline (162 ± 13 cells/mm²) compared with mice that received injection of 1ng IL-1 β injection in the brain followed by i.v. injection of 100ng IL-1 β (4 ± 0.4 cells/mm²) and mice where 100ng IL-1 β was injected in the brain (5 ± 1 cells/mm²). The number of neutrophils in the brain was almost the same in the striatum of mice where

APR was activated in the liver after 1ng IL-1 β injection in the striatum and of mice that received 100ng IL-1 β in the striatum (Figure 5.17B and C).

BBB permeability to IgG decreased in mice that received i.v. injection of 100ng IL-1 β when compared to those that received i.v. injection of saline after 1ng IL-1 β injection in the brain (Figure 5.17D).

mRNA expression of IL-1 β , ICAM-1, CXCL-2 and CXCL-5 was also measured in the ipsilateral and contralateral striatum of animals that received i.c. injection 1ng IL-1 β followed by i.v. injection of saline or 100ng IL-1 β , and of mice that only received i.v. injection of 100ng. Expression levels of these inflammatory mediators were calculated as relative fold change from expression levels of 1ng i.c. / saline i.v. group of mice in order to discover if experimental acute phase activation after i.c. injection of 1ng IL-1 β would change the expression of inflammatory mediators in the brain. None of these inflammatory mediators showed any changes in their expression levels (Figure 5.18), suggesting that experimental activation of the hepatic APR does not induce the expression of cytokines, chemokines and adhesion molecules in the brain.

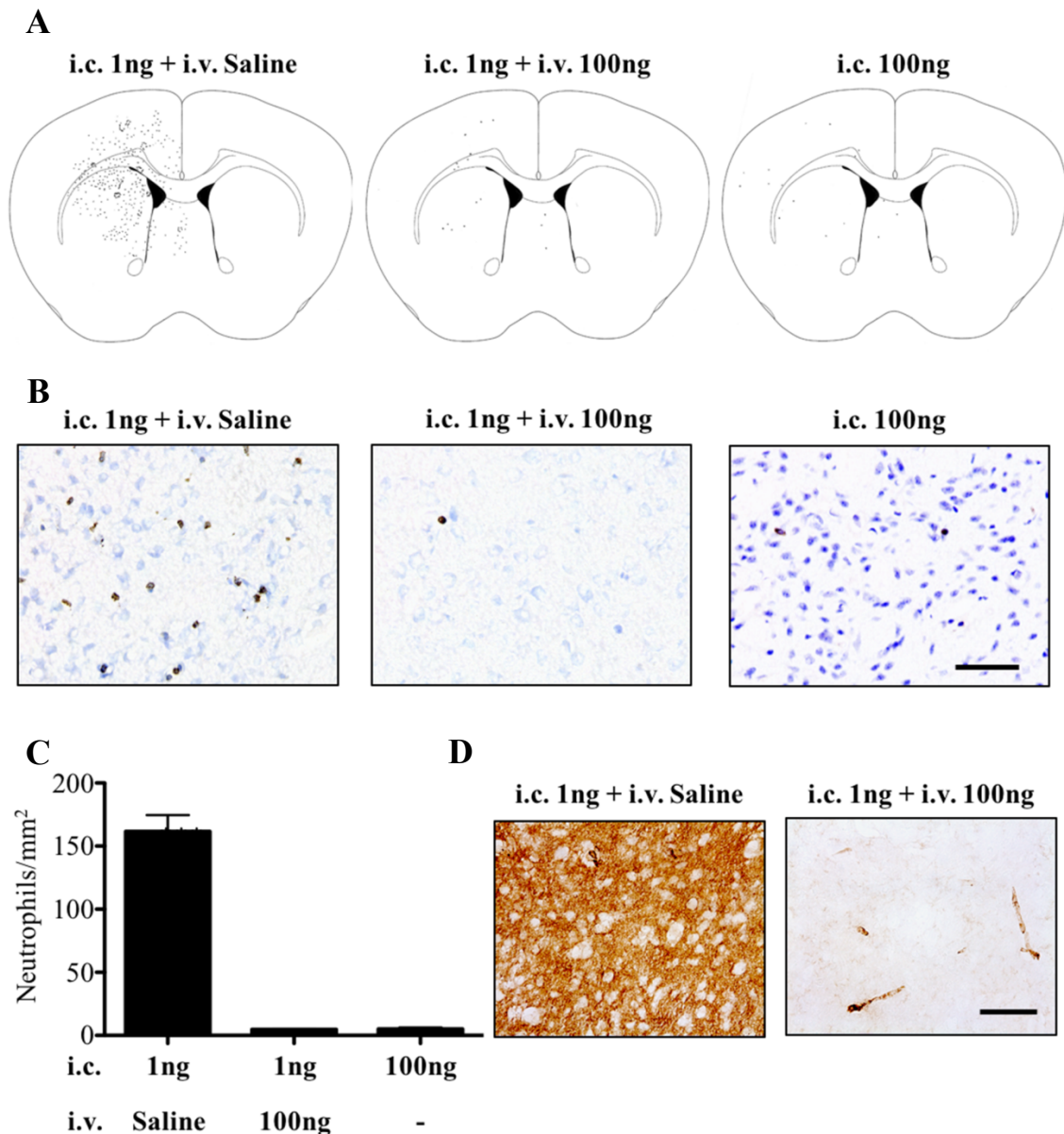


Figure 5.17 Effect of experimental activation of the APR on the number of neutrophils recruited to the brain and BBB permeability after injection of 1ng IL-1 β in the brain. Representative spatial maps of neutrophil distribution in the brain (A) and immunohistochemistry for neutrophils (MBS, brown) with cresyl violet counterstain (B) using brain sections from P14 mice that received an i.v. injection of saline or 100ng IL-1 β immediately after i.c. injection of 1ng IL-1 β (1ng i.c. / saline or 100ng i.v.) and from P14 mice injected with 100ng IL-1 β in the brain (from previous experiments). The recruitment of neutrophils to the brain decreased when the APR in the liver was activated after injection of 1ng IL-1 β in the brain when compared to brains injected with 1ng IL-1 β alone. The number of neutrophils in the brain resembled the number of cells observed in the brain of animals that received i.c. injection of 100ng IL-1 β . Data are represented as number of neutrophils per mm² of striatum (C). * $p < 0.05$ and *** $p < 0.001$. Statistics: one-way ANOVA with Tukey's multiple comparison test. BBB permeability to endogenous IgG was measured in brains (D). BBB permeability decreased when the APR in the liver was activated after injection of 1ng IL-1 β in the brain when compared to brains injected with 1ng IL-1 β alone. Scale bar = 50 μ m. 1ng i.c. / saline i.v.: $n = 3$, 1ng i.c. / 100ng i.v.: $n = 4$ and 100ng i.c.: $n = 6$.

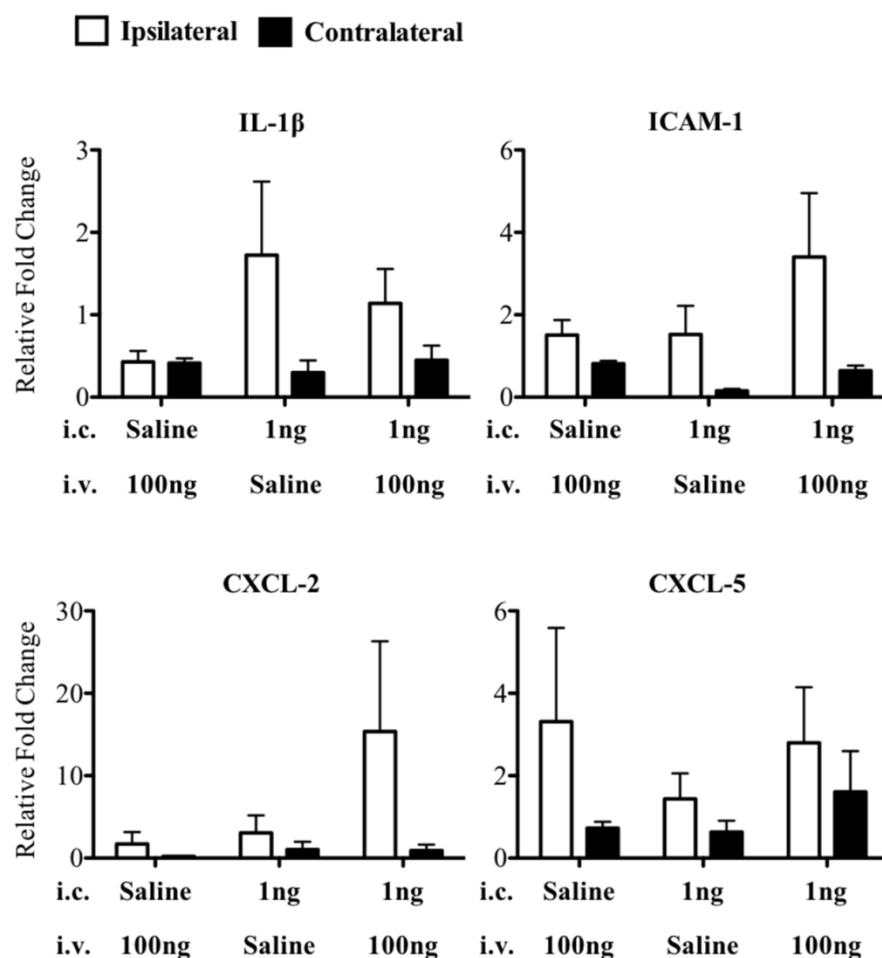


Figure 5.18 Effect of experimental activation of the APR on the expression of inflammatory mediators in the striatum after injection of 1ng IL-1 β in the brain. IL-1 β , ICAM-1, CXCL-2 and CXCL-5 mRNA was measured by RT-qPCR in the ipsilateral and contralateral striatum of three different groups of mice: saline i.c. / 100ng i.v., 1ng i.c. / saline i.v. and 1ng i.c. / 100ng i.v.. No changes were observed in the expression of any of the inflammatory mediators. Samples were compared with two housekeeping genes (Zfp91 and Cdc40). Data are represented as relative fold change from expression levels of 1ng i.c. / saline i.v. group of mice. Bars represent mean $\pm$ SEM. Statistics: two-way ANOVA with Bonferroni–Dunn post-hoc test. saline i.c./100ng i.v.: n=3, 1ng i.c./saline i.v.: n=4 and 1ng i.c./100ng i.v.: n=4.

5.3 Discussion

The results in this chapter showed that injection of a high dose of IL-1 β in the brain induced an APR in the liver, which decreased during postnatal development, and that the immunological background of the liver varied with age and therefore, may contribute to the differential hepatic response to brain inflammation. During the "window of susceptibility" at P14, an inverse relationship was shown between the cellular response in the brain and the hepatic APR to focal inflammation in the brain. The results also showed that the brain susceptibility to inflammation at P14 can be ameliorated by activation of the peripheral inflammatory response in the liver.

Inflammation in the CNS is known to be followed by a peripheral APR that is coordinated mainly by the liver in adult rodents. The liver was shown to respond to acute brain injury by producing cytokines, chemokines and other APPs. These chemical mediators are then release to the circulation and able to induce the recruitment of leukocytes to the blood, liver and brain in order to resolve the initial injury or contribute to further damage (reviewed in Anthony and Couch, 2014). Data presented here showed that P7 mice expressed more IL-6, TNF and IL-10 in their livers at baseline than P14, P21 and 8-week-old mice. In rodents, the liver undergoes a complex maturation process during the first four weeks after birth, where extensive structure remodelling happens in order to achieve a mature architecture (Alexander et al., 1997; Walthall et al., 2005). The liver weight also increases during the first four postnatal weeks as a result of the extensive hepatic cell proliferation (Apte et al., 2007; Behrens et al., 2002; Septer et al., 2012). TNF and IL-1 have been shown to have an important role in hepatocytes growth during this postnatal period by activating the NF-kB pathway (Kinoshita and Miyajima, 2002; May, 1999). These two cytokines were also shown to be important for initiation and progression of liver regeneration after partial hepatectomy (Böhm et al., 2010; Fausto et al., 2006; Taub,

2004). Other studies have shown that IL-10 represses liver regeneration by limiting inflammatory cytokines, such as TNF, IL-6 and CCL-2, that activate regeneration after liver injury (Louis et al., 1998; Rai et al., 1997; Yin et al., 2011). Therefore, it is possible that during early postnatal development high levels of pro- and anti-inflammatory cytokines may coordinate functions to precisely regulate, synchronize and limit liver growth. The results in this chapter also showed that ICAM-1 and CXCL-5 were highly expressed during the first two or three weeks after birth, respectively, when compared to adult mice and that the number of neutrophils in the liver was highest at P7 and decreased throughout the development. There are no published data showing the direct role of adhesion molecules, chemokines and neutrophils in liver development. However, ICAM-1 and neutrophils were shown to have a crucial role in liver regeneration after partial hepatectomy and this may indicate its importance in liver growth during development. Selzner et al. (2003) have suggested that the expression of ICAM-1 by Kupffer cells and endothelial cells after partial hepatectomy promotes neutrophil recruitment, which activates the release of IL-6 and TNF by the Kupffer cells (Selzner et al., 2003). Another study has examined the foetal liver, which is known to be the main hematopoietic organ during embryonic development, from E12 to P0 in mice. The authors have shown that neutrophils start to appear at E15 and their number increases until P0, where they represent several foci (Ayres-silva et al., 2011). As a consequence of liver maturation, mature or immature neutrophils leave the liver as its environment is no longer favourable for them (Ayres-silva et al., 2011; Kinoshita et al., 1999; Si-tayeb et al., 2010). This concept supports the data in this chapter showing that the number of neutrophils decrease from P7 to 8 weeks, by which point very few neutrophils were found. Indeed, it is described that neutrophils are largely absent from the adult healthy liver (Campbell et al., 2003; Gregory et al., 1996). Finally, another explanation for the increased expression of

inflammatory mediators early in postnatal period may be directly linked to the increased number of neutrophils, which are able to express these mediators, observed in the liver at P7 and P14.

The increased expression of inflammatory mediators and leukocytes number in the naïve liver at P7 may, not only contribute to liver growth and development, but also to the inflammatory response after acute injury in the brain. The APR in the liver to inflammation in the brain was highest at P7 and decreased throughout development after the injection of 100ng IL-1 β in the striatum. This high dose of IL-1 β in the brain induced the expression of different inflammatory mediators, including IL-1 β , IL-6, TNF, ICAM-1, CXCL-2 and CXCL-5, increased expression of CD11b and the recruitment of neutrophils to the liver of P7 and P14 mice when compared to P21 and 8-week-old mice. Although the hepatic response was higher in younger animals, adult mice showed an APR in the liver characterised by upregulation of IL-6, ICAM-1 and CXCL-2 and, increased number of neutrophils in the liver. Previous studies are in concordance with this data as they have also shown that the liver acutely responds to different brain injuries in adult rodents. Stereotaxic injection in the striatum of TNF (1000ng) or IL-1 β (1000ng) in adult rats induced APR in the liver by upregulating the expression of chemokines and increasing the recruitment of leukocytes, including neutrophils, 4 hours after brain injection (Campbell et al., 2003, 2005). Stereotaxic injection of IL-1 β (1ng) in the striatum also induced the expression of APPs, including serum amyloid A and P, in the liver 6 hours after the injection in adult C57Bl/6 mice (Wilcockson et al., 2002). Moreover, 4 hours after experimental stroke in adult mice, CXCL-1 expression was upregulated in the liver (Chapman et al., 2009). These studies only examined the APR in adult animals and did not compare them to other ages. There are very few published papers that studied the hepatic immune response in young animals. Bonestroo et al.

(2013) have shown that cerebral hypoxia-ischemia caused a hepatic APR after 3 hours in P7 rats characterized by upregulation of CXCL-1 and downregulation of TNF, IL-1 β and CCL-2. However, this study did not explore the hepatic response in other postnatal ages or in adults as has been done here. It is clear from the data in this chapter and previous published results that the liver can respond to brain injury by producing APPs and that the APR can vary, regarding the molecular cues, with the type of brain injury and age.

While the hepatic APR was only significantly induced after the i.c. injection of 100ng IL-1 β , cerebral inflammation at P14 was greatest after the i.c. injection of 1ng IL-1 β , which was characterised by increased BBB permeability and neutrophil recruitment. In fact, no BBB permeability to IgG and very few neutrophils were found in the brains injected with 100ng IL-1 β . The injection of increasing concentrations of IL-1 β (1ng, 10ng and 100ng) in P14 brains confirmed the dose-dependent effect on BBB disruption and neutrophil recruitment to the brain parenchyma, but was also positively correlated with expression of IL-1 β and ICAM-1. This suggests that while a high dose of IL-1 β in the brain induced the expression of inflammatory mediators in the brain that are important for neutrophil recruitment, it was not followed by an increase in neutrophil recruitment. This implies that another mechanism may be counteracting the molecular inflammatory response in the brain and, consequently, signalling to the periphery.

An inverse relationship between the number of neutrophils in the brain parenchyma and the magnitude of the APR in the liver was observed, which contradicts many previous findings. The APR in the liver has been correlated with the most severe cases of ischemic stroke and with traumatic brain injury (Di Napoli et al., 2005; Smith et al., 2006; Villapol, 2016). Previous studies have also shown that stereotaxic injection of TNF or IL-1 β in adult rats not only induced the APR in the liver, but increased the recruitment of leukocytes to the brain (Campbell et al., 2003, 2005). Furthermore, Campbell et al. (2003)

have observed that i.c. injection of IL-1 β induced the production and release of CXCL-1 by the liver and the recruitment of neutrophils to the brain in a dose-dependent manner. Cerebral hypoxia-ischemia in P7 rats increased CXCL-1 expression in the liver and recruitment of neutrophils to the affected brain hemisphere (Bonestroo et al., 2013). The differences between the study of Bonestroo et al. (2013) and the results in this chapter may rely on the different type of brain injury and species used. When analysing brain sections of each individual age, it was observed that the number of neutrophils in the brain parenchyma decreased at P7, P14 and P21 but increased at 8 weeks from 1ng IL-1 β to 100ng IL-1 β injections in the brain (Figure 8). Although this was not statistically significant, except at P14, this observation suggests that the observed inverse relationship between the severity of brain injury and the magnitude of the APR in the liver is a specific developmental process. This hypothesis is also supported by previous results showing a positive relationship between the magnitude of the APR and the recruitment of neutrophils to the brain after i.c. injection of IL-1 β in adult rats (Campbell et al., 2003). The differences in the brain neutrophil recruitment profile observed in the P14 animals versus adult animals may be dependent on the magnitude of the APR after the injury. It was very noticeable that the APR to an i.c. injection 100ng IL-1 β was much higher at P14 than in adult. This may be, in part, due to the increased basal levels of inflammatory mediators at the early time points and the increased number of basal neutrophils within the P14 naïve liver. Thus, it seems that the liver may be primed at the time of the inflammation in the brain, and this 'head start' in the activation process may result in the increased magnitude of the APR at P14. As a consequence, it seems likely that the peripheral response then overwhelms the inflammatory signals generated locally in the brain and thus neutrophils are redirected to the liver and possibly to other peripheral organs. In the adult, the APR is smaller and thus does not out compete the local injury

signals in the brain that recruit neutrophils (albeit a small number at acute time points). This hypothesis is supported by the observations made after the peripheral injection of IL-1 β .

The inverse relationship between the recruitment of neutrophils to the brain and the magnitude of the APR following focal inflammation to the brain at P14 suggests that activation of the APR in the liver after i.c. injection of 100ng IL-1 β in the brain may protect the brain from the potentially damaging consequences of neutrophil recruitment. Indeed, by injecting 100ng IL-1 β i.v. immediately after stereotaxic injection of 1ng IL-1 β in the striatum of P14 mice, the liver response was activated (increased neutrophils recruitment and expression of inflammatory cytokines and chemokines) and, consequently, eliminated almost all neutrophils from the brain without inducing the expression of IL-1 β , ICAM-1, CXCL-2 and CXCL-5 in the brain. It is known that neutrophils are major players during acute inflammation and these leukocytes are quickly recruited to the injury site in order to resolve the damage. However, excessive activation of neutrophils may result in tissue damage (Kolaczowska and Kubes, 2013). Therefore, it is possible that tissue damage may be controlled through redistribution of neutrophils throughout the body by activating liver APR after inflammation in the brain. This naturally happened when 100ng IL-1 β and not 1ng IL-1 β was injected in the brain, indicating that signalling from the brain to the periphery increases with the magnitude of brain inflammation and it is essential for brain protection. However, it still remains unclear what are the specific mechanisms that underline signalling from the brain to the liver after acute brain injury. Endothelial cells from the BBB may contribute to the signalling pathways. As discussed in the introduction to this thesis, these cells have an important role in immune communication between brain and peripheral immune system as they are able to receive signals from one side and produce and secrete inflammatory

mediators to the other side. Unpublished results from our laboratory suggest that brain-derived extracellular vesicles, which can contain RNA and many proteins, may play a role in this communication. Peripheral injection of extracellular vesicles, which were isolated from the blood of adult rodents that received an i.c. injection of IL-1 β , into circulation of naïve rodents was shown to produce both sickness behaviour and a hepatic APR. Moreover, extracellular vesicles derived from brain endothelial cells (*in vitro*) stimulated with IL-1 β were able to induce an APR when peripherally injected into naïve animals, and to exacerbate brain injury in a rodent model of traumatic brain injury. As discussed in the introduction, signalling to the periphery may also occur through the neuroependymal cells covering the ventricular surfaces and, consequently, the blood-csf barrier in the choroid plexus. Both structures are in close proximity to the injection site in our mouse model and, therefore, may play a role in the communication between the brain and periphery.

The results in this chapter showed, for the first time, a protective role of the APR in the periphery to focal inflammation during development. Moreover, they suggest that experimental activation of peripheral immune response may be used to reduce neonatal brain injury in opposition to what it normally happens in adults. Indeed, many anti-inflammatory therapies/drugs have been shown to be beneficial for disorders, such as acute stroke and traumatic brain injury, with an inflammatory component (reviewed in Bergold, 2016 and Smith et al., 2015). Also, Campbell et al. (2007) have shown that peripheral administration of etanercept, a competitive TNF inhibitor, reduced the recruitment of neutrophils to an IL-1 β -induced focal inflammatory lesion in the brain via inhibition of the hepatic APR. The authors have shown that injection of 1000ng IL-1 β in the striatum of adult rats upregulated many inflammatory mediators, including TNF, IL-1 β , CXCL-1, CXCL-2, CXCL-10, CCL-2, and ICAM-1, in the liver 2 hours after focal

inflammation in the brain when compared to saline controls. Moreover, pre-treatment with etanercept was shown to reduce the hepatic expression of many of the previous inflammatory mediators and to decreased the number of neutrophils recruited to the liver and blood 2, 8 and 24 hours after IL-1 β injection in the brain and, consequently, to reduce the number of neutrophils in the brain 8 and 24 hours after i.c. injection of IL-1 β (Campbell et al., 2007b). On the other hand, other studies have shown that peripheral activation may protect the brain from injury, though it seems that this depends of the type of immunogen used to activate the peripheral immune system. For example, i.v. injection of LPS, but not IL-1 β , 2 hours before or after stereotaxic injection of 1000ng IL-1 β in the striatum of adult rats was shown to reduce the recruitment of neutrophils to the brain (Davis et al., 2005). However, there was no changes in the number of neutrophils in the liver, which suggests that brain protection against focal inflammation in adult rats by LPS may result from a different mechanism from the one proposed in this chapter, the redirection of neutrophils. IL-1 β i.v. injection did not protect the brain from neutrophil recruitment in adult rats and this may result from the developmental stage of the animal and from the fact that the authors used rats instead of mice. It would be very interesting to examine the effect of i.v. injection of different immunogens before or after the i.c. injection of 1ng IL-1 β at P14 and at other time points during development in order to discover if brain protection against neutrophil recruitment and BBB disruption varies with age, type of immunogen and time when the peripheral immune activation is performed in mice.

The use of experimental activation of the APR for brain protection observed in this chapter is also supported by other publications using different models of brain injury. In P7 rats, i.v. injection of LPS 24 hours before hypoxia-ischemia was shown to reduce brain injury. However, increased brain injury was observed when LPS was injected 6 or

72 hours before hypoxia-ischemia, highlighting the importance of the time intervals between stimuli (Eklind et al., 2005). In adult rats, i.v. injection of LPS 3 days prior to focal cerebral ischemia was able to reduce lesion size (Furuya et al., 2005; Tasaki et al., 1997), and IL-1 β and TNF administration prior ischemia were also able to reduce injury in the brain (Nawashiro et al., 1997; Ohtsuki et al., 1996). A major difference between most of these studies and the results in this chapter is the fact that they activate peripheral immune system prior brain injury and, therefore, may not be as clinically relevant as injecting inflammatory mediators after brain damage, as was performed here. Therefore, it will be important to further study the effects of experimental activation of the APR after inflammation in the brain not only during development, but also in adults in order to confirm the age-dependent effects of the hepatic immune response to brain damage. Moreover, it is important to examine the long-term effects of peripheral immune activation after focal inflammation on structural and functional integrity of the brain, besides neutrophil recruitment and BBB disruption.

5.4 Conclusion

The data in this chapter showed, for the first time, a protective role of the APR in the liver to inflammation in the brain during the "window of susceptibility" in mice. Activation of the peripheral immune response after the injection of a low dose of IL-1 β in the brain was able to deplete neutrophils from the brain. It was also shown that the magnitude of the hepatic immune response decreases throughout postnatal development and that the immune environment at the different ages may contribute to this observation. Furthermore, an inverse relationship between the severity of the cellular brain response and the magnitude of the APR in the liver in the early postnatal period was observed. All

these results indicate that young mice behave very differently from an adult mouse following proinflammatory challenges. Importantly, this suggests that in neonates peripherally administered anti-inflammatory agents may not have the neuroprotective role predicted from work on adult animals, and that, in fact, activation of peripheral immune response may be a therapy option for neonatal brain injuries.

CHAPTER 6

GENERAL DISCUSSION

The principal findings of this thesis are as follows:

- That there is a "window of susceptibility" in mouse brain to acute inflammation at P14 induced by the injection of a low dose of IL-1 β in the striatum during postnatal development.
- That this "window of susceptibility" is correlated with the presence of a Iba-1⁺ perivascular cell population (IPCs), which may be manipulated by the use of clodronate filled liposomes.
- That experimental activation of the APR in the liver can be used as protective mechanism against an excessive inflammatory response in the brain during the susceptible period of postnatal development.

Overall, the data in this thesis have demonstrated the importance of the interplay between the brain and peripheral immune responses in defining the magnitude of the acute inflammatory response in the brain to focal inflammation during postnatal development in mice.

Injection of a low dose of IL-1 β (1ng IL-1 β) in the left striatum was shown to promote the acute recruitment of neutrophils and BBB permeability to endogenous IgG in an age-dependent manner. Mice at P14, and not at P7 and P21, were most susceptible to this inflammation. This observation supports a number of previous findings showing that the susceptibility of the brain to immune activation (either central or peripheral) varies according to the stage of development of humans and rodents. In fact, human epidemiologic studies have shown a strong positive correlation between

infection/inflammation at specific developmental ages and the development of neurodevelopmental disorders, such as autism, schizophrenia and cerebral palsy. Autism and schizophrenia were associated with infection/inflammation during the first and second trimester, while cerebral palsy was shown to have the last trimester and early postnatal days as vulnerable time points to infection/inflammation (Atladóttir et al., 2010; Brown et al., 2004; Dubowitz et al., 1985; Mednick et al., 1988). In the case of an IL-1 β challenge in the brain, it seems that P14 mice, which are approximately equivalent to human newborns, are more vulnerable. Rodent studies have also shown that immune activation by peripheral injection of poly(I:C) or LPS at different time points during embryonic development resulted in distinct behavioural, neuropathological and/or inflammatory (levels of cytokines in the brain) outcomes in the offspring (Fortier et al., 2007; Meyer et al., 2006, 2008). At postnatal level, very few studies have examined the variability in the brain response to immune activation induced not only by viral and bacterial components but also by specific cytokines. The results in this thesis are the first showing variability in the acute brain response to IL-1 β during postnatal development in mice. They also support previous findings in rats, where the same dose of IL-1 β was injected in the striatum and, consequently, induced an acute susceptibility (increased recruitment of neutrophils and BBB permeability) in the brain of P21 and not of P0 and adult animals. However, the major challenge has been to discover the cellular and/or molecular mechanisms behind these observations. In this thesis, data has been generated with the aim of providing a better understanding of possible molecular and cellular events that may explain the brain susceptibility to low dose of IL-1 β in the brain. At P7, the increased basal levels of both pro- and anti-inflammatory mediators, but the relatively small number and immaturity of microglia in naïve brains when compared to older mice may explain, in part, why the brain did not respond to the inflammatory challenge and,

consequently, promote neutrophil recruitment and BBB permeability. On the other hand, P21 brains expressed very low levels of inflammatory mediators at baseline, but had more mature and higher number of microglia, which may rapidly respond to inflammatory challenges. After the injection of a low dose of IL-1 β , P21 brains were able to highly express many pro- and anti- inflammatory mediators creating a balanced immune response, which may prevent an excessive recruitment of neutrophils and, consequently, brain damage (Figure 6.1). It is possible that the small number of neutrophils found in the brain at this time point may be contributing to inflammation resolution and not to further injury. At P14, no specific molecular and cellular mechanism was detected in brain parenchyma that could explain the more marked recruitment of neutrophils and BBB permeability. There was also no significant difference in the APR in the liver that might have accounted for the recruitment of neutrophils and increased BBB permeability. However, Iba-1⁺ IPCs were found in naïve P14 brains (Figure 6.1). This population, which appear to be derived from the blood, may facilitate the passage of neutrophils to the brain parenchyma after inflammation at P14. This hypothesis is supported by a previous study demonstrating by multiphoton intravital microscopy that adult perivascular macrophages are important for neutrophil migration into mouse ear skin infected with *Staphylococcus aureus* (Abtin et al., 2014). Although the contribution of Iba-1⁺ IPCs to the "window of susceptibility" was not experimentally proved in this thesis, the systemic injection of liposomes filled with clodronate was shown to significantly reduced the recruitment of this cell population to the perivascular space at P14. Therefore, this approach may be used in the future to discover the role of these cells after focal inflammation in the brain at P14.

As stated above, no significant acute inflammatory response in the liver was detected after the injection of a low dose of IL-1 β in the brain during the first three weeks after

birth. This was also observed in adult mice. However, a cellular and molecular inflammatory response in the liver was detected when a more severe focal inflammation was induced by injecting 100ng IL-1 β in the brain (Figure 6.2). The APR in the liver was characterised by the upregulation of many pro-inflammatory mediators and the recruitment of large numbers of neutrophils. This was observed either in young and adult mice. Previous findings have also shown an APR to high doses of IL-1 β and TNF in the brain of adults rats (1000ng TNF or IL-1 β ; (Campbell et al., 2003, 2005)), stroke in adult mice (Chapman et al., 2009) and cerebral hypoxia-ischemia in P7 rats (Bonestroo et al., 2013). An interesting result in this thesis was that the magnitude of the APR decreased throughout the development. This has not been reported before as most of published studies are focus on a specific time point during development. This observation may be explained, in part, by the immune environment in naïve animals during postnatal development. The expression of many inflammatory mediators and the number of neutrophils in naïve liver were highest in younger mice and decreased with age (Figure 6.2). Surprisingly and for the first time, it was observed that the activation of the APR in the liver of young mice did not contribute to the inflammatory response in the brain; all previous studies in humans and rodents have correlated the APR with increased inflammatory response and/or injury in the brain (Acalovschi et al., 2003; Campbell et al., 2003; Smith et al., 2004, 2006; Vila et al., 2000). Indeed, the increased recruitment of neutrophils and BBB permeability observed in brains of P14 mice after injection of a low dose of IL-1 β completely disappeared after injection of a higher dose of IL-1 β in the brain and, consequently, a significant hepatic APR (Figure 6.2). Furthermore, injection of increasing concentrations of IL-1 β in the brain at P14 induced the APR in a dose-dependent manner, but decreased the number of neutrophils recruited to the brain and permeability of the BBB (Figure 6.3). It seems that the excessive APR observed early in

the early postnatal period may account for the absence of neutrophils in the brain after i.c. injection of a high dose of IL-1 β . These results were not expected as previous findings, in adult or neonate rodents, have shown that activation of the APR in the liver to inflammatory damage or other type of injuries in the brain was followed by the recruitment of neutrophils to the brain (Bonestroo et al., 2013; Campbell et al., 2003, 2005; Wilcockson et al., 2002). Campbell et al. (2003) have discovered that injection of IL-1 β in the brain of adult rats induced the APR in the liver (increased levels of CXCL-1 and number of neutrophils in the liver and blood) and recruitment of neutrophils to the brain in a dose-dependent manner. The differences between the data in this thesis and these previous results may rely on the fact that Campbell et al. (2003) used adult rats and, therefore, suggests that not only the specie but the developmental stage of the animal may account for the age-related differences. Bonestroo et al. (2013) have also correlated the upregulation of CXCL-1 in the liver and the recruitment of neutrophils to the brain after cerebral hypoxia-ischemia in P7 rats. In this case, the opposite findings may be explained by the different type of brain injury used. In fact, cerebral hypoxia-ischemia is a very complex and distinct type of injury from focal inflammation, which was used in this thesis. Considering the results from Campbell et al. (2003), and in light of the data from this thesis, the inverse relationship between the magnitude of the hepatic APR and the recruitment of neutrophils to the brain at P14 may be a development-specific mechanism to inflammation in the brain induced by the injection of IL-1 β . It remains unknown if this observation is specific of IL-1 β and, therefore, future experiments should study the relationship between brain and hepatic inflammatory responses in different brain inflammatory contexts. Focal inflammation by i.c. injection of TNF or IL-6 should be selected as these cytokines have been strongly upregulated in brains of many rodent models and human patients of neurodevelopmental disorders (Arrode-Brusés et al., 2012;

Cai et al., 2000; Kadhim et al., 2001, 2003; Li et al., 2009; Patterson, 2009; Rousset et al., 2006; Vargas et al., 2005; Yoon et al., 1997). These experiments, together with the results presented in the thesis, will allow us to differentiate cytokine-specific mechanisms of brain and liver inflammatory responses. However, it is also important to study brain and liver immune responses and their interaction in more clinically relevant rodent models of brain injury occurring early in postnatal life, such as traumatic brain injury and cerebral hypoxia-ischemia.

Another paradoxical result obtained in this thesis was that increasing doses of IL-1 β in the brain at P14 induced the expression of endogenous IL-1 β and ICAM-1 in the brain, but decreased the recruitment of neutrophils and BBB permeability (Figure 6.3). It is known that both IL-1 β and ICAM-1 are essential for neutrophil recruitment and migration across vessels (Allen et al., 2012; Bernardes-Silva et al., 2001; Kolaczowska and Kubes, 2013). However, it seems that in these experiments the role of both inflammatory mediators as potential players for immune cell recruitment were somehow interrupted. It is possible that the liver may play an important role in this process. In fact, the last experiments in this thesis were able to show that the activation of the hepatic APR directly contributed to the absence of neutrophils and increased BBB permeability after inflammation in the brain (Figure 6.4). Experimental activation of the APR immediately after the injection of a low dose of IL-1 β in the brain, which was shown to promote extensive recruitment of neutrophils and BBB permeability without inducing hepatic inflammatory response, protected the brain from neutrophil recruitment and increased BBB permeability. These results definitively showed that the acute phase in the liver can protect the brain from extensive neutrophil recruitment and BBB permeability after inflammation in the brain induced by the i.c. injection of IL-1 β . However, this may only occur when the inflammation in the brain is substantial enough to activate the APR that

would then counteract the molecular inflammatory response in the brain by redirecting the neutrophils from the brain. Neutrophils may be relocated to the liver as more neutrophils were found there after APR, but they may also migrate to other organs and this, therefore, needs to be investigated. In addition, future experiments should also include the inhibition of the APR after the injection of a high dose of IL-1 β in the brain in order to mimic the brain scenario when a low dose of IL-1 β is injected into the brain and no APR is detected. This would further support the current hypothesis that activation of the hepatic APR protects and can be used to protect the brain against extensive neutrophil recruitment after focal inflammation in P14 mice. Finally, it is also important to discover the major molecular players that directly contribute to the protective role of the hepatic acute phase in order to create a more target-specific strategy for peripheral immune activation and, therefore, avoid possible side effects from a general inflammation generated by peripheral injection of IL-1 β .

The data in this thesis not only support the current view that susceptibility of the brain to infection/inflammation varies with age, but also suggests that the peripheral immune environment may define the injury outcome. All experiments in this thesis were focused on the acute inflammatory response (4 hours) and, therefore, more experiments need to be performed to study the long term effects, including molecular, neurochemical and morphologic changes in the brain and behavioural deficits, of focal inflammation and subsequent hepatic APR during postnatal development in mice. These experiments are going to be essential to discover if the brain susceptibility to a low dose of IL-1 β observed in this thesis corresponds to a more profound brain pathology and behavioural changes later in life in opposition to the injection of a higher dose of IL-1 β or experimental activation of the APR. Regardless of the lack of long term experiments in this thesis, the data presented here strongly suggest that the APR is an important player in the aetiology

of brain dysfunction during development. It is, therefore, necessary to give more attention to the APR not only in rodent models but also when analysing human tissues for different types of brain injury occurring either during embryonic development or early postnatal period.

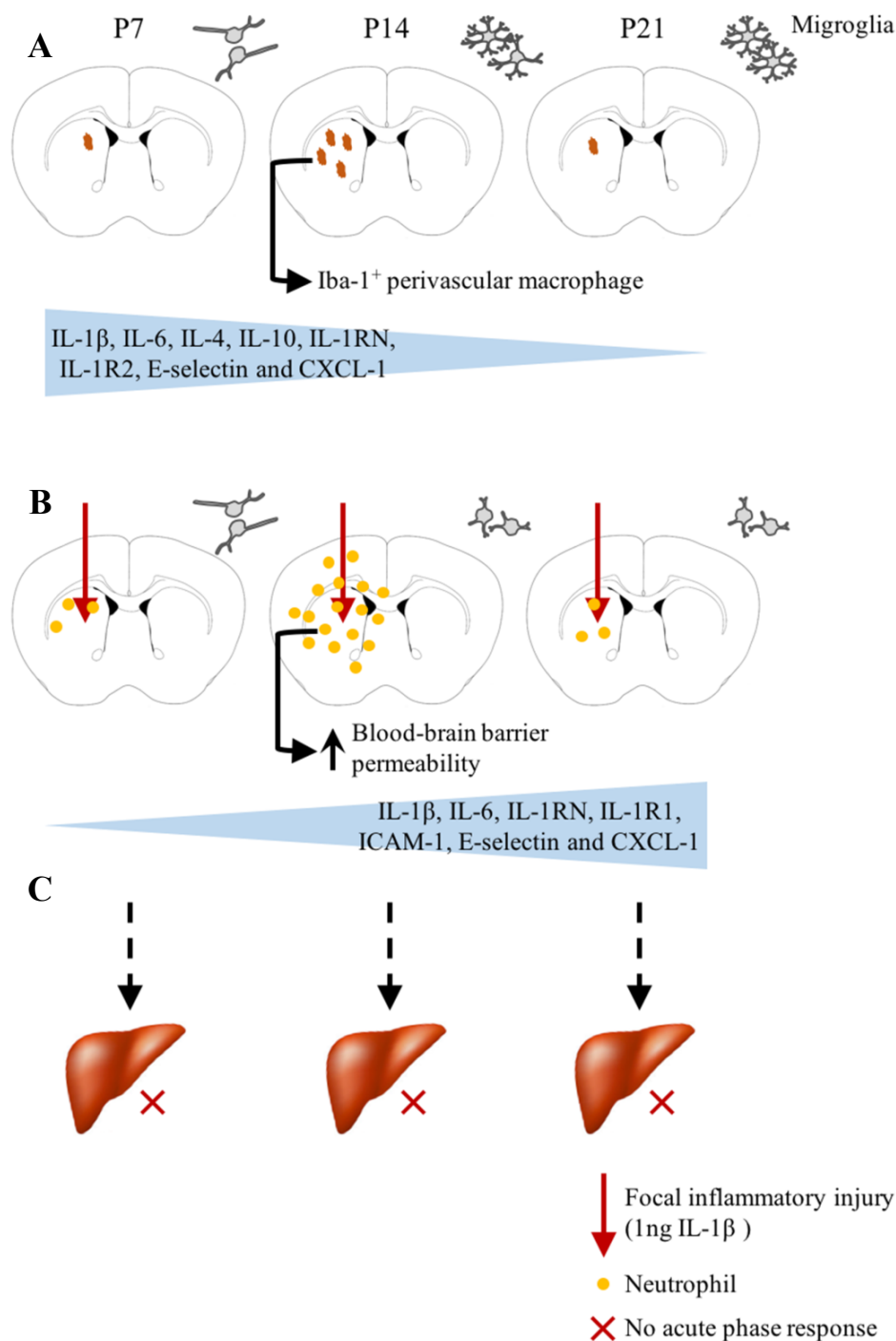


Figure 6.1 Developmental response to a low dose of IL-1 β in the brain. The baseline immune environment in brains of P7, P14 and P21 C57Bl/6 mice was studied (A) before i.c. injection of 1ng IL-1 β (B). A) The expression of inflammatory mediators was highest in the striatum of P7 mice and decreased throughout postnatal development. Microglia were rounded with few thick processes at P7 and developed into a ramified mature morphology with age. Iba-1⁺ IPCs were mainly found in the striatum at P14. B) 4 hours after i.c. injection of 1ng IL-1 β , neutrophil recruitment and BBB permeability to endogenous IgG were highest at P14. Microglia did not change their morphology at P7 but presented an amoeboid activated state at P14 and P21. Many inflammatory mediators were highly upregulated in the ipsilateral striatum of P21 mice. The presence of Iba-1⁺ IPCs was found to be the only event specific of P14 mice. In this model of focal inflammation, no significant APR in the liver was detected (C).

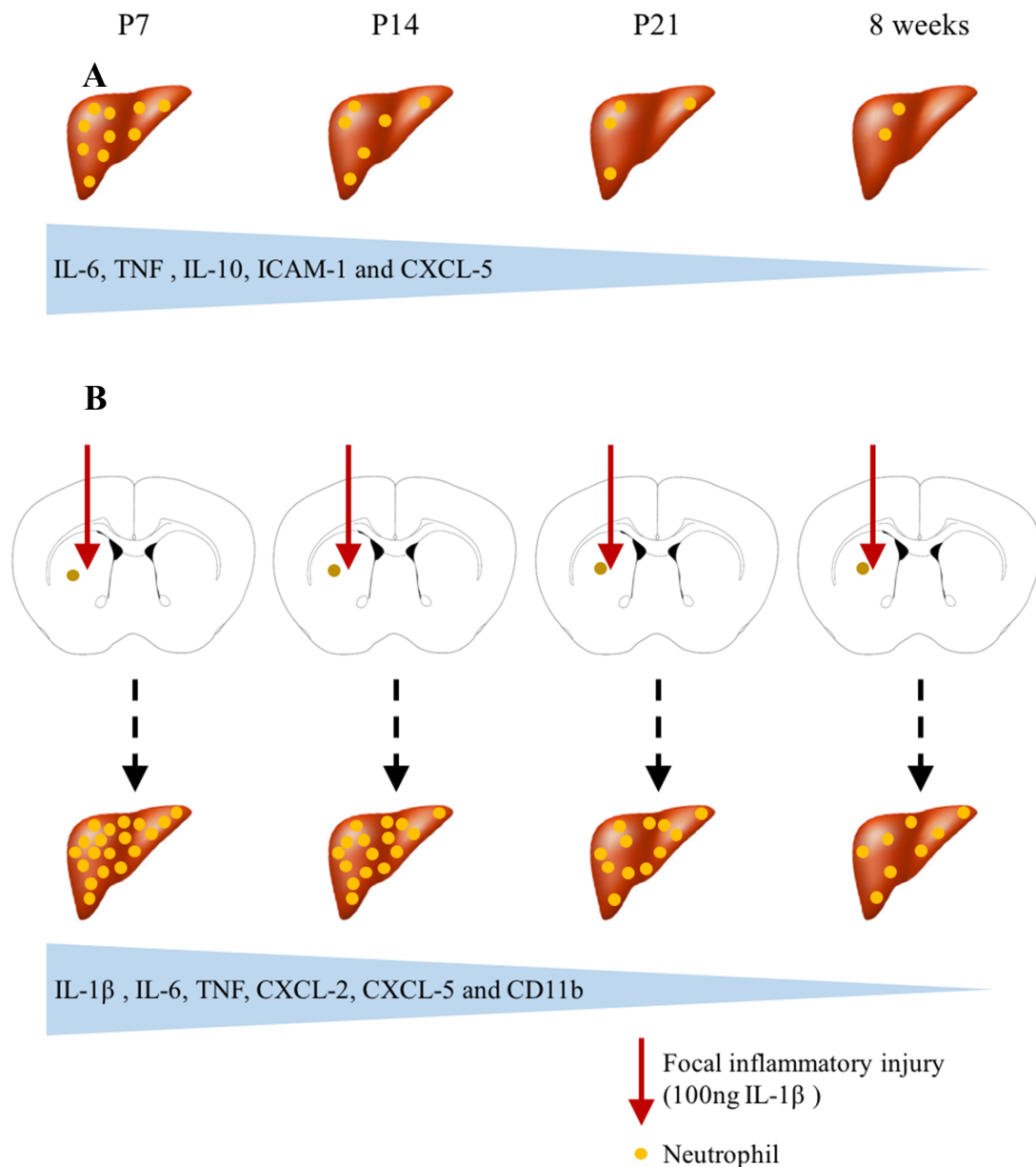


Figure 6.2 The effects of a high dose of IL-1 β in the brain on the inflammatory responses in the brain and liver. Expression levels of inflammatory mediators and the presence of neutrophils in the liver were studied in naïve mice (A) and in mice that received i.c. injection of 100ng IL-1 β (B) at P7, P14, P21 and 8 weeks. Neutrophil recruitment and BBB permeability were also measured in the brain. A) The expression of many inflammatory mediators and the number of neutrophils in naïve livers were highest at P7 and decreased with age. B) After i.c. injection of a high dose of IL-1 β , there was an APR in liver in all ages, but it decreased throughout development. The APR was characterised by the upregulation of many pro-inflammatory mediators and increased recruitment of neutrophil when compared to saline injected mice. Contrary to what was observed after the injection of 1ng IL-1 β in the brain (Figure 6.1), minimal recruitment of neutrophils to the brain and no increased in BBB permeability to IgG was observed at P14 mice after i.c. injection of 100ng IL-1 β .

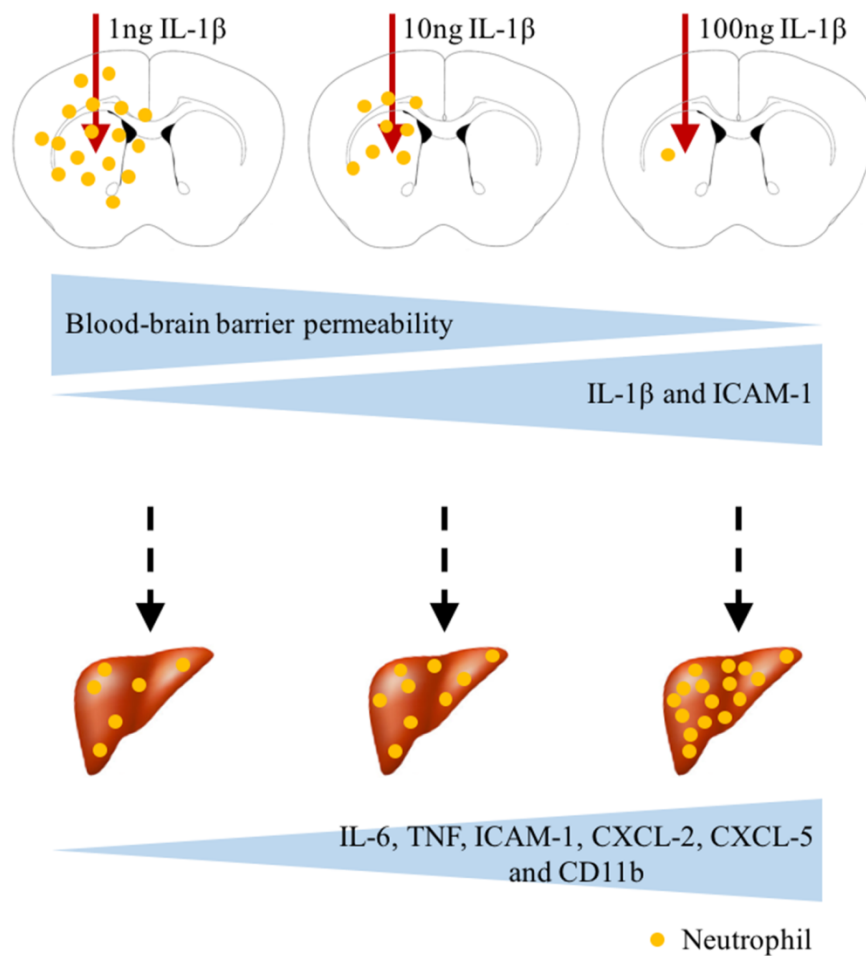


Figure 6.3 The inflammatory response of P14 mice to increasing severity of inflammation in the brain. The brain and liver acute inflammatory responses to increasing doses of IL-1 β injected in the brain was examined in P14 mice. The number of neutrophils recruited to the brain and BBB permeability to endogenous IgG decreased while the expression of IL-1 β and ICAM-1 increased with the severity of the inflammation. The APR in the liver, characterised by the expression of many pro-inflammatory mediators and recruitment of neutrophils, increased in a dose-dependent manner.

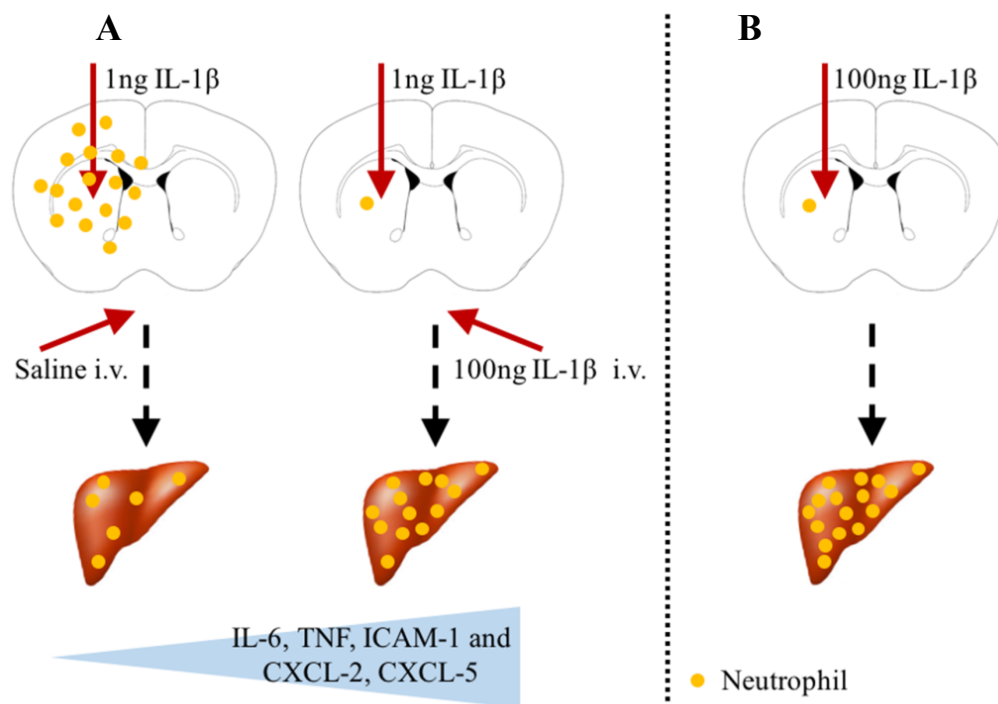


Figure 6.4 Experimental activation of the acute phase response after injection of a low dose of IL-1 β in the brain. The brain and liver acute inflammatory responses were examined in P14 mice that received systemic injection of saline or 100ng IL-1 β immediately after i.c. injection of 1ng IL-1 β (A). Peripheral injection of 100ng IL-1 β induced the APR in the liver and protected the brain from excessive neutrophil recruitment, which was observed when saline was injected in the periphery (A), resembling brains after severe inflammation (100ng IL-1 β) in the brain (B).

FINAL CONCLUSION

The data in this thesis support the current view that susceptibility of the brain to infection/inflammation varies with age and that the peripheral immune environment may define the injury outcome. Activation of the APR in the liver was shown to invert the "window of susceptibility" in a mice model of cerebral inflammation and, therefore, may be a good therapy option. The results from this thesis together with previous findings also suggest that APR contribution to brain injury may vary from young animals to adults, where increasing APR appears to further increase leukocyte recruitment and BBB damage. These evidences highlight the importance of the use of age-dependent therapies and, therefore, it is possible that peripherally administered anti-inflammatory drugs in neonates may not have the neuroprotective role predicted in many published studies using adult animals. Finally, in the absence of a peripheral APR to inflammation in the brain, data in this thesis suggest that the baseline immune environment in the brain may define the brain response and susceptibility to inflammation. Specifically, Iba-1⁺ IPCs in the brain were found at the susceptible time during postnatal development and, consequently, may be a possible cellular target for neuroinflammation resolution.

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