# Central and Systemic Inflammation in Developmental Brain Injury

*Peripheral to central immune communication in perinatal brain injury*

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*Cover illustration - From stem cell to neuron: Neural progenitor cells at various stages of maturation in the developing hippocampus.*

Printed by Ineko AB, Gothenburg 2014

ISBN 978-91-628-9250-0

*For my dear Grandparents, John and Doris Leahy*

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# ABSTRACT

Brain injury occurring during the perinatal period is an important cause of mortality and morbidity with potentially life long consequences. Both preterm and asphyxiated full term infants are at high risk of developing such injuries, and intrauterine infection has been identified as an independent risk factor. Whilst the primary causes of perinatal brain injury may be diverse and often elude diagnosis, inflammation is a common feature. We have analysed various aspects of inflammation in perinatal models of sterile and infectious insult. Our particular interests have been: initiation of central inflammation, central nervous system (CNS) recruitment of peripheral immune cells, and inflammation-induced disruption of CNS homeostasis and physiological processes.

We demonstrate constitutive expression of all Toll-like receptors (TLRs), a sub-family of pathogen recognition receptors, in the neonatal CNS and active regulation of *TLRs 1, 2, 5, 7 & 8* following, sterile, hypoxic-ischemic (HI) brain injury. We provide evidence of diminished lesion size in TLR2-KO mice, a result strongly implicating TLR2 as an important mediator of lesion development following HI. Additionally, we display active and prolonged recruitment of peripheral immune cells to the injured regions of the CNS following HI, a process that occurs in distinct "waves" and continues for up to two weeks. Interestingly, such recruitment was absent in a model of infectious insult, as initiated by peripheral administration of lipopolysaccharide (LPS). Here, numerous signs of enhanced central inflammation were observed. We detected acute increases in microglial proliferation and total number of microglia, changes coupled to regulation of several inflammation associated genes in the hippocampus. This increased hippocampal inflammatory profile was present for at least two weeks after administration of LPS and occurred in parallel to decreases of neuronal commitment among hippocampal progenitor cells.

Together these results indicate involvement of the TLRs in rapid initiation of inflammation following HI and display active and prolonged participation of peripheral immune cells this inflammatory response. Additionally, we demonstrate that inflammation initiated outside the CNS is sufficient to upregulate cerebral inflammatory responses and transiently disrupt developmental microgliogenesis and neurogenesis.

**Keywords:** Immune-brain communication, perinatal brain injury, leukocyte migration, TLRs

**ISBN: 978-91-628-9250-0**

# LIST OF ORIGINAL ARTICLES

This thesis is based on the following original studies:

I. Stridh, L., Smith, P.L., Naylor, A.S., Wang, X. & Mallard, C. Regulation of toll-like receptor 1 and -2 in neonatal mice brains after hypoxia-ischemia. *Journal of neuroinflammation* 8, 45 (2011).

II. Smith, P.L., Hagberg, H., Naylor, A.S. & Mallard, C. Neonatal Peripheral Immune Challenge Activates Microglia and Inhibits Neurogenesis in the Developing Murine Hippocampus. *Developmental neuroscience* (2014).

III. Smith, P.L., Hellström Erkenstam, N., Mottahedin, A., Ek, C.J., Hagberg, H. & Mallard, C. Prolonged accumulation of peripheral myeloid cells in a murine model of neonatal hypoxic-ischemic brain injury. *Manuscript*.

# ARTICLES NOT INCLUDED IN THIS THESIS

1. Hellström Erkenstam, N., Fleiss, B., Smith, P.L., Wang, W., Boström, M., Gressens, P., Hagberg, H., Brown, K., Sävman, K., Mallard, C. Characterization of Galectin-3 in respect to M1 and M2 populations in the brain after neonatal hypoxia-ischemia. *Manuscript.*
2. Ek C.J., D'Angelo B, Baburamani A, Lehner, C., Leverin, A,. Smith, P.L., Nilsson, H., Svedin, P,. Hagberg, H. & Mallard, C. Brain barrier properties and cerebral blood flow in neonatal mice exposed to cerebral hypoxia-ischemia. *Submitted: Under review.*
3. Wilhelmsson, U., Faiz, M., de Pablo, Y., Sjoqvist, M., Andersson, D., Widestrand, A., Potokar, M., Stenovec, M., Smith, P.L., Shinjyo, N., Pekny, T., Zorec, R., Stahlberg, A., Pekna, M., Sahlgren, C., Pekny, M. Astrocytes negatively regulate neurogenesis through the Jagged1-mediated Notch pathway. *Stem cells* 30, 2320-2329 (2012).
4. Widestrand, A., Faijerson, J., Wilhelmsson, U., Smith, P.L., Li, L., Sihlbom, C., Eriksson, P.S., Pekny, M. Increased neurogenesis and astrogenesis from neural progenitor cells grafted in the hippocampus of GFAP-/- Vim-/- mice. *Stem cells* 25, 2619-2627 (2007).
5. Bogestal, Y.R., Barnum, S.R., Smith, P.L., Mattisson, V., Pekny, M., Pekna, M. Signaling through C5aR is not involved in basal neurogenesis. *J Neurosci Res* 85, 2892-2897 (2007).

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine Diphosphate |
| ATP | Adenosine Triphosphate |
| $NAD^+$ | Aldehyde Dehydrogenase |
| ANOVA | Analysis of Variance |
| AIF | Apoptosis Inducing Factor |
| APAF-1 | Apoptotic Protease Activating Factor-1 |
| ASD | Autism Spectrum Disorder |
| BBB | Blood-Brain-Barrier |
| BrdU | Bromodeoxyuridine |
| CNS | Central Nervous System |
| CBF | Cerebral Blood Flow |
| CP | Cerebral Palsy |
| CLSM | Confocal Scanning Laser Microscope |
| DAB | Diaminobenzidine |
| DCX | Doublecortin |
| EGFP | Enhanced Green Fluorescent Protein |
| EAA | Excitatory Amino Acid |
| FSC | Forward Scatter |
| Fc | Fragment, crystalizable |
| GA | Gestational Age |
| GD | Gestational Day |
| GW | Gestational Week |
| GFAP | Glial Fibrilary Acidic Protein |
| gp130 | Glycoprotein 130 |
| GFP | Green Fluorescent Protein |
| Hes1 | Hairy and enhancer of split-1 |
| HDAC | Histone Deacetylase |
| HI | Hypoxic-Ischemic |
| HIE | Hypoxic-Ischemic Encephalopathy |
| Pi | inorganic Phosphate |
| IFN | Interferon |
| IRF | Interferon-Regulatory Factor |
| IL | Interleukin |

| | |
|---|---|
| Iba1 | Ionized calcium-binding adaptor molecule 1 |
| JAK | Janus tyrosine kinase |
| ki | Knock-in |
| KO | Knockout |
| LED | Light-Emitting Diode |
| LPS | Lipopolysaccharide |
| Ly6C | Lymphocyte antigen 6 complex, locus C |
| GR1 | Lymphocyte antigen 6 complex, locus G |
| Lyz2 | Lysozyme 2 |
| MRI | Magnetic Resonance Imaging |
| MRS | Magnetic Resonance Spectroscopy |
| MACS | Magnetic-activated cell sorting |
| Mash1 | Mammalian archeate-schute complex |
| MiDM | Microglia-derived macrophage |
| MAPII | Microtubule-associated protein 2 |
| MCAO | Middle Cerebral Artery Occlusion |
| MDM | Monocyte-derived macrophage |
| MAL | MyD88-adaptor-Like protein |
| MyD88 | Myeloid Differentiation Factor 88 |
| NK | Natural Killer (cell) |
| NPC | Neural Progenitor Cell |
| NeuN | Neuronal Nuclei (protein) |
| NOS | Nitric oxide synthase |
| NO | Nitrous oxide |
| NMDA | N-methyl-D-aspartate |
| NICD | Notch Intracellular Domain |
| NF-κB | Nuclear Factor-κB |
| Olig2 | Oligodendrocyte transcription factor |
| PFA | Paraformaldehyde |
| PVN | Paraventricular nucleus |
| PAMP | Pathogen Associated Molecular Pattern |
| PRR | Pathogen Recognition Receptor |
| PCr | Phosphocreatine |
| PhH3 | Phosphohistone-H3 |
| PMT | Photomultiplier tube |
| PARP-1 | Poly-ADP-ribose polymerase |

| | |
|---|---|
| PSEN1 | Presenillin 1 |
| ROS | Reactive Oxygen Species |
| RT-qPCR | Real-Time Polymerase Chain Reaction |
| RFP | Red Fluorescent Protein |
| RNA | Ribose Nucleic Acid |
| Runx-1 | Runt-related transcription factor 1 |
| SSC | Side Scatter |
| STAT | Signal transducer and activator of transcription |
| SIRPα | Signal-regulatory protein-α |
| Sirt1 | Sirtuin 1 |
| SD | Standard Deviation |
| SEM | Standard Error of the Mean |
| SARM | Sterile-α and armadillo-motif-containing protein |
| SIM | Structured Illumination Microscopy |
| SGZ | Sub-granular zone |
| SN | Substantia Nigra |
| SVZ | Sub-ventricular zone |
| TICAM | TIR-domain-containing adaptor molecule |
| TIRAP | TIR-domain-containing adaptor protein |
| TRIF | TIR-domain-containing adaptor protein inducing IFNβ |
| TIR | Toll/Interleukin-1 Receptor domain |
| TLR | Toll-like receptor |
| TRAM | TRIF-related adaptor molecule |
| TBS | Tris bufferred Saline |
| TNF-α | Tumour Necrosis Factor-α |
| WT | Wild-Type |

# INTRODUCTION

## Central nervous system development

Human central nervous system (CNS) development is a continuum that begins during early gestation and persists far into postnatal life. One of the earliest identifiable events of brain development occurs 18 days into the 266-288 day (40 week) gestational period as the ectodermal cells overlaying the notochord differentiate into neuroepithelial stem cells (DeSesso et al., 1999). As development proceeds, this small population of cells multiplies and differentiates giving rise to the neurons, astrocytes, and oligodendrocytes from which nearly the entirety of the adult brain and spinal cord will be formed (DeSesso et al., 1999). These complex processes of cell genesis, maturation and organization continue well into postnatal life (Giedd et al., 1999); in humans neurogenesis peaks between gd (gestational day) 60 and 90 (Clancy et al., 2007) and continues through early postnatal development (Sanai et al., 2011) into adulthood, albeit in a limited fashion (Eriksson et al., 1998); gliogenesis occurs through the later stages of gestation and early neonatal life (Roessmann and Gambetti, 1986); synaptogenesis begins as early as GW 8 (Molliver et al., 1973) with peak synaptic density, roughly 40% higher than present in adults, observed 8 months postnatally (Huttenlocher et al., 1982). While these processes are ongoing, microglia, a fourth and ontogenetically distinct cell type, invade the CNS; animal studies indicate that these cells arise from primitive mesodermal progenitors of the embryonic yolk sac and colonise the brain during early development (Alliot et al., 1999, Ginhoux et al., 2010). In humans primitive microglia can be observed as early as GW 4.5 (Verney et al., 2010) although well differentiated microglia are not observed until GW 35 (Esiri et al., 1991, Rezaie et al., 2005, Verney et al., 2010). The process of myelination follows after neurogenesis and concurrent to axonal arborisation, and therefore begins relatively late in gestation: Myelin is first detected in the brainstem at GW 29 (Inder and Huppi, 2000) and continues to accumulate into the third decade of life (Giedd et al., 1999).

## Developmental origins of neurological morbidity

Injury to the perinatal brain is a leading cause of mortality and neurological morbidity in the newborn with potentially life-long consequences (Marlow et al., 2005, Miller et al., 2005, Degos et al., 2010, Perez et al., 2013). Ultimately, long-term outcome is determined not only by the type and severity of primary pathology but also by ensuing effects on the processes of cerebral development and maturation (Vannucci and Hagberg, 2004). A wide variety of CNS disorders can be traced back to disturbances of foetal and neonatal life. Indeed, strong associations have been displayed between such disturbances and numerous early onset cognitive, attentional, behavioural and motor disorders including; cerebral palsy (CP) (Volpe, 2009), autism (Atladottir et al., 2010, Johnson et al., 2010) and schizophrenia (Boksa, 2008, Fatemi and Folsom, 2009). Interestingly, a developmental component of the adult onset neurodegenerative disorders Alzheimer's (Zawia and Basha, 2005) and Parkinson's (Gardener et al., 2010) has been proposed, although the inherent complications of lifelong longitudinal studies with limited availability of detailed perinatal health records makes these data suggestive rather than conclusive.

## Perinatal brain injury

Both preterm and asphyxiated term infants are at high risk for the development of perinatally-acquired brain injury. Epidemiological investigations into the aetiology of CP have identified intrapartum complications such as asphyxia and trauma, and perinatal infection, as important risk factors for term infants. When considering both term and preterm births, prematurity, low birth weight (Johnston and Hoon, 2006) and intrauterine infection/inflammation (Dammann and Leviton, 1997), are uncovered as additional risk factors. Recent data from the ongoing Swedish CP study (2003-2006) indicates overall prevalence of cerebral palsy at approximately 2 per 1000 live births, with prevalence highest among extremely preterm neonates (< 28 GW, 71.4 per 1000) and decreasing through very preterm (28-31 GW, 39.6 per 1000) and moderately preterm (32-36 GW, 6.4 per 1000) to term (>36 GW 1.41 per 100). Despite the lower prevalence of CP among term infants, the much greater frequency of term births makes this group by far the highest contributor to the overall number of perinatally acquired CP cases (Himmelmann and Uvebrant, 2014). Interestingly, the most common risk factors for perinatally acquired CP differ in preterm and term born infants; HIE represents a more important contributory factor in term infants whilst prenatal ex-

posure to infectious agents is more common in preterms (Himmelmann and Uvebrant, 2014).

The initial processes of brain injury following cerebral hypoxia-ischemia are relatively well defined and follow a course of cerebral energy failure causing inhibition of cellular functions ultimately leading to cell death through a combination of direct and indirect effects (Fatemi et al., 2009, Johnston et al., 2011). The presence of infection in preterm is hypothesised to cause CP both through direct white matter insult and through initiation of preterm labour (an independent risk factor for CP development) (Himmelmann and Uvebrant, 2014). Long-term studies assessing pre-adolescent children (mean age 11.2 years) have also revealed enhanced risk of intellectual, verbal, and motor deficits in children who sustained neonatal HIE without major disability (Perez et al., 2013). In the case of mood disorders, prenatal exposure to infectious agents seems most prevalent: admission of pregnant mothers to hospital with bacterial or viral infection confers significantly higher risk for the development of autism spectrum disorders (ASDs) amongst offspring (Atladottir et al., 2010), and children of mothers exposed to influenza during pregnancy appear to be at greater risk of developing schizophrenia (Boksa, 2008).

Although the primary pathologies underlying brain injury in preterm and term infants may differ, inflammation is a common feature. Indeed, elevated levels of the proinflammatory cytokine IL-6 and chemokine IL-8 are detected in the cerebrospinal fluid (CSF) of asphyxiated infants and these levels correlate with neurological outcome (Savman et al., 1998). Similarly, chorioamnionitis (intrauterine infection) is an important cause of preterm birth (Dammann and Leviton, 1997, Goldenberg et al., 2000) and has been reported as an independent risk factor for: white matter injury, intraventricular haemorrhage, and subsequent cerebral palsy in preterm infants (Yoon et al., 2000, Hagberg et al., 2002b, Berger et al., 2009, Leviton et al., 2010).

## Hypoxic-ischemic encephalopathy

The presence of brain injury in term neonates is often detected through presentation of symptoms of neonatal encephalopathy (NE) (Shevell, 2004). This is a clinically defined syndrome of disturbed neurologic function characterized by delayed onset of respiration, reduced consciousness, altered tone and reflexes, and possible seizures as observed during the first week of life (Nelson and Leviton, 1991). Where strong evidence indicates intrapartum asphyxia as the underlying cause of NE the syn-

drome may be further classified as hypoxic-ischemic encephalopathy (HIE) (MacLennan, 2000, Shevell, 2004, Pin et al., 2009). Diagnosis of HIE in preterm neonates may presents more of a problem as clinical signs of injury are often subtle or absent (du Plessis and Volpe, 2002).

### *Cerebral energy failure in neonatal HI*

The CNS injury which underlies the clinical manifestation of HIE results primarily from impaired cerebral blood flow and reduced oxygen delivery to the brain (Cotten and Shankaran, 2010). This injury should not be considered a single pathological event, but rather an evolving array of pathophysiologic responses, the earliest of which have been characterised both clinically and experimentally. Magnetic resonance imaging (MRI) studies conducted on full term neonates with global cerebral hypoxic-ischemic injury display progressive lesion development during the first four days of life: small lesions are first detected via diffusion weighted MRI in the putamen and thalami with injury later evolving to include more extensive areas of the brain (Takeoka et al., 2002). Likewise assessment of cerebral energy metabolism by magnetic resonance spectroscopy (MRS), which permits measurement of intracellular pH and concentration of phosphorous metabolites including: adenosine triphosphate [ATP], phosphocreatine [PCr] and inorganic phosphate [Pi], in asphyxiated newborn infants indicates normal metabolism on the first day of life with abnormalities developing over the following days (Wyatt et al., 1989). Whilst practicalities prevent MRS based assessment in acutely injured infants, studies on newborn lambs have shown an acute pattern of metabolic dysfunction similar to that observed in older infants with decreased [PCr] and increased [Pi] (overall reduction in [PCr/Pi], and decreased [ATP] and intracellular pH (Hope et al., 1987). Notably, acute changes in pH and phosphorous metabolite concentrations may be normalised within roughly one hour of the hypoxic-ischemic episode (Hope et al., 1987, Hope et al., 1988) whilst the later changes of phosphorous metabolites evolve over a longer time period (Wyatt et al., 1989). The consensus on such data is that hypoxic-ischemic brain injury leads to a rapid yet transient disruption of cerebral energy metabolism, termed "primary energy failure", which initiates a cascade of events leading to a delayed metabolic disruption, referred to as "secondary energy failure" (Wyatt et al., 1989, Shalak and Perlman, 2004, Cotten and Shankaran, 2010, Allen and Brandon, 2011).

### *Mechanisms of hypoxic-ischemic brain injury*

The decreased availability of cerebral ATP following HI ultimately inhibits mechanisms acting to maintain cellular homeostasis, particularly the sodium/potassium (Na/K) pump and mechanisms which maintain low intracellular calcium, resulting in initiation of excitotoxicity and cell death (Choi, 1988, McDonald and Johnston, 1990, Delivoria-Papadopoulos and Mishra, 1998, Johnston, 2001, 2005, Fatemi et al., 2009, Allen and Brandon, 2011, Hagberg et al., 2014).

Two distinct components of excitatory amino acid (EAA) mediated neurotoxicity have been proposed: Primarily, acute disruptions of cellular energy inhibit the Na/K pump leading to $Na^+$ influx followed by passive $Cl^-$ and $H_2O$ influx, which collectively cause cell oedema (Choi, 1988); massive neuronal depolarization occurs in response to increased intracellular accumulation of $Na^+$ and glutamate is released from neuronal synapses. The second component involves inhibition of glutamate reuptake and excessive stimulation of the ionotropic and metabotropic glutamate receptors. Under normal conditions glutamate present in the synaptic cleft is rapidly cleared via energy dependent glutamate transporters present on astrocytes. Inside astrocytes this glutamate is converted to glutamine before being shuttled back to the presynaptic neuron to be recycled. Inhibition of the energy dependent uptake processes leads to glutamate accumulation in the extracellular space (Magistretti et al., 1999, Johnston, 2005), a phenomenon which has been observed in HI (Hagberg et al., 1987, Puka-Sundvall et al., 1997). High extracellular glutamate concentration enhances stimulation of glutamate, particularly the N-methyl-D-aspartate (NMDA), receptors; this combined with energy depletion mediated membrane depolarization precipitates sustained opening of the NMDA receptor ion channel which floods cells with $Ca^{2+}$ (McDonald and Johnston, 1990).

At high intracellular concentrations calcium becomes toxic initiating numerous mechanisms that mediate cell death. $Ca^{2+}$ sensitive proteases and lipases become activated and degrade structural and membrane components of the cell liberating arachidonic acid and xanthine, respectively substrates for oxygen and superoxide free radical production (Choi, 1988, McDonald and Johnston, 1990, Delivoria-Papadopoulos and Mishra, 1998). In the case of severe hypoxic-ischemic insult total mitochondrial failure may occur; triggering rapid cell death through necrosis, a process characterised by cell swelling, disruption of cytoplasmic organelles, loss of membrane integrity and cell lysis (Gilland et al.,

1998a, Gilland et al., 1998b, Johnston et al., 2001, Shalak and Perlman, 2004, Hagberg et al., 2014). Milder occurrences of HI are more commonly associated with apoptosis, the process of programmed cell death. The pathways leading to apoptosis can be categorised as either intrinsic or extrinsic. One activator of the intrinsic cell death pathway is oxidative stress, which encourages the transfer of factors including cytochrome *c* (Perez-Pinzon et al., 1999) and apoptosis inducing factor (AIF) (Cregan et al., 2004) from the mitochondria to the cytosol. In the cytoplasm, cytochrome *c* interacts with APAF-1, ADP, and pro-caspase-9 forming the apoptosome; subsequent cleavage of caspase-9 and proteolytic activation of caspase-3 ultimately initiates cell death through apoptotic DNA fragmentation (Hagberg, 2004, Johnston et al., 2011). Following transference to the cytosol AIF subsequently migrates to the nucleus where it initiates cell death in a caspase independent manner potentially through interaction with the DNA repair enzyme poly-ADP-ribose polymerase (PARP) 1 (Hagberg, 2004, Johnston et al., 2011). Additionally, high cytosolic concentrations of $Ca^{2+}$ may directly activate caspase-3 through effects on calpain. Apoptosis as triggered through the extrinsic cell death pathway involves the cell surface associated Fas death receptor and subsequent activation of caspase-8 and caspase-2 (Johnston et al., 2011).

## Neuroinflammation

Inflammation, although not necessarily a causative factor, is a common feature of diverse central nervous system pathologies and is increasingly considered to play a contributory role in the processes of pathogenesis and where appropriate, repair (Degos et al., 2010, van Noort and Amor, 2011, Hagberg et al., 2012). In the context of neonatal hypoxic-ischemic injury, inflammation, along with excitotoxicity and apoptosis, is thought to contribute to delayed cell death (Inder and Volpe, 2000) and involvement of both the innate and adaptive arms of the immune system have been documented (McRae et al., 1995, Hudome et al., 1997, Bona et al., 1999, Hedtjärn et al., 2004, Nijboer et al., 2008, Jin et al., 2009, Winerdal et al., 2012, Albertsson et al., 2014). As this thesis is primarily concerned with innate immunity, the contribution of adaptive immunity will not be further discussed.

### *CNS immune specialization*

From an evolutionary perspective the occurrence of inflammation in the CNS is unfavourable for several reasons. Anatomically the CNS is encased in bone and inflammation induced swelling may lead to dangerous

levels of pressure on nervous tissue (Callahan and Ransohoff, 2004). Additionally, the activity- and experience-driven nature of CNS neuronal circuitry development, coupled with a limited capacity for regeneration (Hua and Smith, 2004, Schafer et al., 2012) leaves the CNS particularly vulnerable to the ravages of inflammation: to this end, physiological central immunity is relatively downregulated when compared to that of the periphery, a characteristic once attributed to its relative isolation or "immune privilege" (Carson et al., 2006). Recent research however, reveals extensive but tightly regulated peripheral to central immune signalling (Carson et al., 2006); the blood-brain barrier (BBB) regulates solute and ion influx, whilst astrocytes, microglia, and neurons all contribute to the CNS immune suppressive environment (Carson et al., 2006, Gao and Hong, 2008). Additionally, ingression of peripheral immune cells is actively limited under physiological conditions (Hickey, 1999, Callahan and Ransohoff, 2004). This CNS immune privilege is however far from all-encompassing, being rapidly degraded under pathological conditions with both central and peripheral immune stimulation leading to CNS inflammation and active attraction of peripheral leukocytes (Vallieres and Rivest, 1997, Turrin et al., 2001, Eklind et al., 2006, Galea et al., 2007, Bland et al., 2010, Schwarz and Bilbo, 2011, Hagberg et al., 2012).

### *PRRs, PAMPs & DAMPs*

The rapid onset of inflammation following sterile or infectious CNS insult occurs partly through activation of the innate immune system via stimulation of pathogen recognition receptors (PRRs). These "danger sensors" are stimulated by the presence of pathogen-associated molecular patterns (PAMPs) on microbes, such as lipopolysaccharide (LPS), bacterial DNA, and double stranded RNA (Uematsu & Akira 2006); and endogenous molecules expressed or released upon tissue injury, which are commonly referred to as damage-associated molecular patterns (DAMPs) (Miyake and Yamasaki, 2012). Of the PRRs the Toll-like receptor (TLR) subfamily has been most widely characterised in the brain and has been implicated in recognition of both PAMPs and DAMPs following ischemia in the adult brain (Cao et al., 2007, Caso et al., 2007, Lehnardt et al., 2007, Tang et al., 2007, Ziegler et al., 2007). In total 13 TLRs have been identified in the human and mouse, TLRs 1-10 are present in humans and all but TLR 10 are present in mice (Mallard, 2012). Presence of the majority of these receptors has been convincingly displayed in both the human and mouse brain, or cells derived thereof (Bsibsi et al., 2002, Olson and Miller, 2004,

Jack et al., 2005, Mishra et al., 2006). Microglia possess the widest repertoire of TLRs with members 1-9 constitutively expressed in mice (Olson and Miller, 2004, Mishra et al., 2006) and in humans (Bsibsi et al., 2002, Jack et al., 2005). Astrocytes also appear to be endowed with several TLRs although discrepancies exist between studies (Bsibsi et al., 2002, Jack et al., 2005).

The TLRs are transmembrane receptors consisting of an extracellular, transmembrane, and intracellular domain. TLRs 1, 2, 4, and 5 are located on the outer cell membrane, while TLRs 3, 7, 8, and 9 are localised on the membranes of endosomes and lysosomes within the cell (Mallard, 2012). The subcellular compartments to which these receptors are localised gives some indication of their function; TLRs 3, 7, 8 and 9 recognize viral PAMPs, most commonly nucleic acids released from pathogens undergoing degradation within lysosomes or endosomes (Blasius and Beutler, 2010).

Signalling through TLRs involves a group of adaptor proteins which share a common Toll/interleukin-1 (IL-1) receptor (TIR) domain, these adaptors bind specific protein kinases activating transcription factors such as nuclear factor-κB (NF-κB) and members of the interferon (IF-N)-regulatory factor (IRF) family, which results in the transcription of an array of immune response genes including numerous cytokines and chemokines (O'Neill et al., 2003, Uematsu and Akira, 2006, O'Neill and Bowie, 2007). To date this family includes five adaptor molecules; MyD88, MAL (also known as TIRAP), TRIF (also known as TICAM1), TRAM (also known as TICAM2), and SARM (O'Neill and Bowie, 2007). Central to the TLR signalling process is the TIR domain which is found on the intracellular, or intra-endosomal, domain of each TLR receptor and each of the adaptors. Upon stimulation TLRs form hetero- or homodimers (Ozinsky et al., 2000, Mallard, 2012) likely through interaction of the two receptor's TIR domains with the resultant conformational change enabling recruitment of the TIR domain containing adaptor proteins (O'Neill et al., 2003).

### *CNS inflammatory cells: The Microglia*

Microglia are the primary immune competent and phagocytic cells in the brain and constitute 12-15% of the CNS cellular population (Kreutzberg, 1996, Kim and de Vellis, 2005, Block et al., 2007, Gao and Hong, 2008). Analysis of microglial distribution has revealed a variation of approximately five-fold between specific regions, with more microglia present

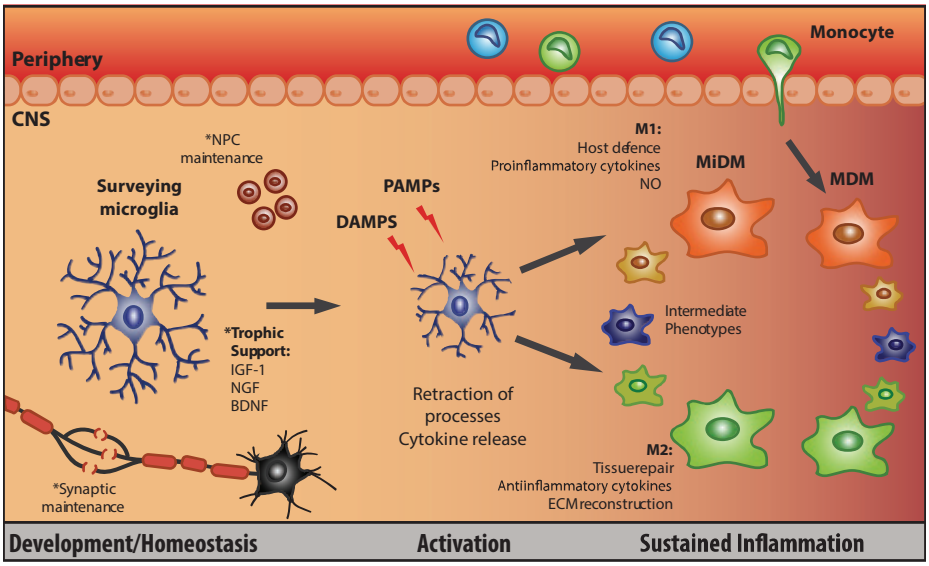


**Fig.1.** ***Roles of microglia and macrophages in the intact and inflamed CNS:*** Under physiological conditions microglia exist in a "surveying" state, constantly remodelling their processes and sampling the CNS parenchyma. Physiological roles include providing trophic support, removing excess synapses, and clearance of apoptotic neurons from the neurogenic niches. In response to pathological stimuli their processes retract and numerous cytokines and chemokines are upregulated. Under conditions of severe or prolonged inflammation, these cells adopt an amoeboid macrophage phenotype and monocyte derived macrophages from the periphery may be actively recruited from the periphery to participate in the ongoing inflammatory response. Macrophages may exhibit pro-, anti-, or and intermediate- inflammatory phenotypes.

in the grey matter than white, and particular enrichment observed in the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra (SN) (Lawson et al., 1990). In contrast to the brain's astro- and oligodendroglial populations, microglia are of myeloid origin, being derived from a subset of primitive macrophages that invade the CNS during embryogenesis (Ginhoux, Greter et al. 2010). These amoeboid microglial precursors proliferate extensively through the late embryonic to early neonatal period giving rise to microglia which gradually develop numerous fine, highly motile processes: a characteristic of mature surveying microglia (Alliot, Godin et al. 1999, Davalos, Grutzendler et al. 2005, Nimmerjahn, Kirchhoff et al. 2005). In rodents postnatal proliferative potential declines rapidly, all but ceasing by the end of the second postnatal week (Alliot, Godin et al. 1999). Microglial turnover is extremely limited in juvenile and adult animals, and replacement by peripheral monocytes is almost non-existent under physiological conditions (Ransohoff 2011, Hagberg, Gressens et al. 2012). Collectively these observations highlight another

interesting property of microglia, namely that they are long-lived, potentially surviving throughout the lifespan of the organism (Ransohoff 2011).

Although well characterised, and often considered, in the context of their immune functions during CNS pathology (Block et al., 2007, Amor et al., 2010, Ransohoff and Cardona, 2010), microglia are increasingly understood as cells with a wide repertoire of developmentally and physiologically important functions (Hanisch and Kettenmann, 2007, Pont-Lezica et al., 2011, Kettenmann et al., 2013, Miyamoto et al., 2013). In addition to their role as potential effector or sentinel cells of the CNS (Amor et al., 2010), they are actively involved in CNS development and maintenance of CNS homeostasis. Developmentally, microglia have been shown to phagocytose neural precursor cells in the cortical proliferative zones as cortical neurogenesis nears completion (Cunningham et al., 2013) and partake in activity dependent synaptic refinement (Paolicelli et al., 2011, Schafer et al., 2012); both of these functions extend into later life with microglia observed to interact with synaptic boutons in an activity driven fashion in juvenile mice (Tremblay et al., 2010) and actively survey the adult hippocampal stem cell niche, where they phagocytose apoptotic newborn cells (Sierra et al., 2010).

In response to pathological alterations of the CNS microenvironment, microglia rapidly adopt an upregulated or activated phenotype. Expression of cell-surface antigens and synthesis of both cytokines and chemokines are altered (Hanisch, 2002) and simultaneous characteristic alterations in cell morphology occur; microglial processes retract and thicken as each cell transitions towards an amoeboid macrophage morphology (Kreutzberg 1996, Davalos, Grutzendler et al. 2005, Nimmerjahn, Kirchhoff et al. 2005). Two key signalling mechanisms govern the reactivity of microglia: The first of these is related to the presentation of factors that are not usually present such as bacterial or viral PAMPs, or as is the case with DAMPs; factors not commonly present at critical concentrations or in specific conformations, for example intracellular components or protein aggregates (Nakamura, 2002, Block et al., 2007, Hanisch and Kettenmann, 2007). The presentation of such factors would be recognised by microglia-expressed specific PRRs, such as the Toll-like receptors, resulting in microglial reactivity being controlled by receptor signalling (Olson and Miller, 2004, Hanisch and Kettenmann, 2007). The second involves constituent tonic inhibition of microglial activity through ligand-receptor pairs including; CD200-CD200R (Hoek et al., 2000), CX3CL1- CX-

3CR1 (Cardona et al., 2006) and SIRPα-CD47 (Chavarria and Cardenas, 2013), all of these ligands have been detected on neurons illustrating direct neuron-immune signalling. This latter pathway provides a mechanism for microglia to respond to loss of neuronal integrity in response to an unrecognised threat (Hanisch and Kettenmann, 2007).

The potential changes in microglial functionality induced by signalling through these mechanisms are diverse: depending on context microglia may participate in cytotoxic responses, immune regulation, and injury resolution (Chhor et al., 2013); although it is important to bear in mind the full diversity microglial activation phenotypes may not be reflected by altered morphology or expression of limited panels of cell surface antigens (Perry et al., 2010). Much of the present conceptual understanding of microglial activation has been built upon *in vitro* studies of monocytes activated to adopt macrophage phenotypes through exogenous application of "prototypical" factors such as LPS or Interleukin (IL)-4 adopting classical (M1) cytotoxic or alternative (M2) anti-inflammatory phenotypes respectively (Gordon and Taylor, 2005, Mosser and Edwards, 2008, Chhor et al., 2013). Importantly, microglia display a high degree of phenotypic plasticity and may exhibit numerous functionally distinct phenotypes which lie at any point on the spectrum between surveying, or M1 and M2 activated (Mosser and Edwards, 2008, Perry et al., 2010).

### *Immune to brain communication: Leukocyte Trafficking*

As previously mentioned, CNS immune privilege is greatly undermined under inflammatory conditions and circulating leukocytes, including monocyte derived macrophages (MDMs), neutrophils, mast cells, and NK cells may be actively recruited to participate in CNS inflammatory responses (Bona et al., 1995, McRae et al., 1995, Hudome et al., 1997, Bona et al., 1999, Nijboer et al., 2008, Jin et al., 2009). Accurate identification of MDMs in the CNS has traditionally proved difficult due to their similarities with microglia derived macrophages (MiDMs) (Perry et al., 1985, Sedgwick et al., 1991) (Kreutzberg, 1996). This problem is further confounded by the presence of functionally distinct blood monocyte subsets with both "resident" and "inflammatory" subtypes distinguishable based on Ly6C expression (Geissmann et al., 2010). Leukocyte attraction is mediated by expression of chemokines, a family of small, structurally similar proteins best known for their roles in leukocyte trafficking (Callahan & Ransohoff 2004). The chemokine family comprises four subfamilies (C, CC, CXC, & CXXXC), each categorised by the number of cysteine resi-

dues or number of amino acids located between the first two cysteine residues. The effects of chemokine molecules are mediated by corresponding families of chemokine receptors named CR, CCR, CXCR and CXXXCR (Callahan and Ransohoff, 2004). Indeed, in the context of neonatal HI enhanced expression of numerous chemoattractant molecules including CCL2 & CCL7, CCR1 & CCR5, and CXCL1 have been observed (Hedtjärn et al., 2004). These ligand groups are respectively known for their roles in emigration of Ly6C$^{hi}$ "inflammatory" monocytes from the bone marrow, recruitment of monocytes into inflamed tissue (Shi & Pamer 2011) and neutrophil recruitment (Hedtjärn et al 2004).

## Neurogenesis

The majority of neurons residing within the adult central nervous system are developmentally generated, post-mitotic, and terminally differentiated. As such, these neurons represent a stable population with, almost, no turnover. As development proceeds, neurogenic potential becomes gradually restricted to the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus where it is maintained throughout adult life (Eriksson et al., 1998, Gage et al., 1998, Curtis et al., 2007). Such continuous addition of new neurons suggests that the adult hippocampal network is an architecturally dynamic structure comprised of a heterogeneous population of neurons at various stages of maturation, a property likely essential to the correct function of the hippocampal network. Indeed, factors known to have positive effects on neurogenesis, such as enriched environment and physical exercise, improve certain aspects of learning and memory (Fabel et al., 2009, van Praag, 2009). Conversely aging, stress and inflammation are negative for both memory and neurogenesis (Warner-Schmidt and Duman, 2006, Jessberger and Gage, 2008, Schoenfeld and Gould, 2011).

### *Inflammatory control of adult neurogenesis*

Inflammation occurring in the germinal regions of the adult CNS can negatively regulate the differentiation and survival of newly born neurons (Ekdahl et al., 2003, Monje et al., 2003). Practically, these inflammatory mediated effects likely result from cross talk between several known inflammatory and neurogenic pathways. In response to stimulation, microglia can become activated and produce a number of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and INF-γ (Monje et al., 2003, Leem et al., 2011). Of these, both TNF-α and IL-6 are sufficient to reduce *in vitro* neurogenesis, whilst addition of a neutralizing anti-IL-6 antibody

to conditioned media from activated microglia is sufficient to restore neurogenesis to control levels (Monje et al., 2003). These results implicate IL-6 as a key player in inflammation induced regulation of neurogenesis. Mechanistically IL-6 is known to signal via the IL-6R co-receptor gp130 (Nakashima and Taga, 2002, Chojnacki et al., 2003) activating the Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway and leading to nuclear signal propagation. Signalling via this pathway can directly affect gliogenesis in two ways: STAT3 can bind the *GFAP* promoter leading to enhanced transcription in neural precursor cells (Nakashima and Taga, 2002), and signalling via gp130 can stimulate the notch1 pathway (Chojnacki et al., 2003) leading to increased Hes1 expression and antagonism of the proneural *Mash1*, thereby pushing neural progenitor cells (NPCs) towards a glial fate (Ishibashi et al., 1994, Ishibashi et al., 1995, Castella et al., 1999, Nakamura et al., 2000). Interestingly, in cultured NPCs *Mash1* can also be regulated in a redox sensitive fashion by the class III $NAD^+$ dependent histone deacetylase (HDAC) Sirt1; increased oxidative stress occurring within the intracellular environment can cause Sirt1 upregulation whilst simultaneously initiating formation of a Sirt1-Hes1 complex which binds to, and deacetylates, histones at the *Mash1* promoter. This leads to repression of *Mash1*, inhibiting neurogenesis and enhancing gliogenesis (Prozorovski et al., 2008). Sirt1 expression has also been positively correlated with hippocampal cell proliferation, an observation partially explained by the ability of Sirt1 to regulate transcription of *presenilin1* (*PSEN1*), a part of the PSEN1/γ-secretase complex required for ligand induced cleavage of the notch intracellular domain (NICD) (Torres et al., 2011). Additionally, both TLR2 and TLR4 are present on NPCs of the adult SVZ and SGZ and have been shown to play important roles in the regulation of NPC self-renewal and cell fate decision, providing a direct link between CNS immunity and adult brain function (Rolls et al., 2007).

# AIMS

The general aim of this thesis has been to investigate aspects of the cerebral inflammatory response following perinatal injury of both sterile and infectious origin. Our particular focus has been peripheral to central immune interaction.

Specifically, we aimed to investigate:

- The expression and regulation of the Toll-like receptors in the neonatal brain both under physiological conditions and in response to experimental hypoxic-ischemic brain injury.

- Acute and chronic effects of LPS-mediated peripheral immune stimulation on microgliogenesis, inflammation associated gene expression, and the ongoing processes of neurogenesis in the developing hippocampus.

- To investigate recruitment of macrophages and neutrophils into the inflamed central nervous system in response to hypoxic-ischemic brain injury.

# METHOLOGICAL CONSIDERATIONS

## Mouse models for the study of human pathology

Mice represent an important model system in modern biomedical research. Of primary importance is their genetic, anatomical and physiological similarity to humans; similarities which permit valuable extrapolation of results between species. Secondary considerations include their small size, short generation time and accelerated life spans; factors collectively making them time and cost effective models and reducing ethical considerations by comparison to larger mammalian models or non-human primates. Disadvantages of mouse models include gross anatomical differences of brain structure; unlike humans their brains are lissencephalic as opposed to gyrencephalic (Hagberg et al., 1997, Hagberg et al., 2002a). Additionally, their small size prevents procedures such as catheterisation which would allow repeated blood sampling and recording of basic physiological parameters such as mean arterial pressure (MAP) and heart rate (HR). Notably, many of the qualities that make mice so readily useful for experimental research also make them particularly amenable to genetic manipulation.

### *Colony maintenance*

Today's research commonly utilises mouse strains that have been inbred (brother x sister and/or parent offspring) for a minimum of 20 generations. This process creates mice with a high degree of genetic similarity which standardises responses to experimental manipulation and hence reduces the number of animals required for the study biological phenomenon.

By definition, a substrain arises when 20 generations of separation occur between a parental colony and subcolony: Following routines designed to limit such genetic drift is essential for maintaining reproducible results through generations and between laboratories. At the simplest level substrain emergence can be avoided by replacing subcolony breeding pairs with mice from the parental colony every 5-10 generations. Where transgenic mice are involved, and repeated purchase of founders is prohibitively expensive, a more relevant strategy is to periodically backcross the genetically modified mice from the sub-colony onto their background strain. Backcrossing involves selectively breeding offspring which exhibit the desired mutation with a member of their background strain originat-

ing from the parental colony for 10 generations (Hedrich and Bullock, 2004, Crawley, 2007).

### *Strains used and specific considerations*

The research presented herein has utilised five strains of inbred mice:

1-2. Wild type mice of the C57BL/6J and C57BL/6 strains.
3-4. TLR1 (Takeuchi et al., 2002) (Oriental Bioservice, Tokyo, Japan) and TLR2 (Wooten et al., 2002) (B6.129-Tlr2t$^{m1Kir}$/J, Jackson Laboratory, US) knock-out (KO) mice, both on C57BL/6J background, in which TLR1 and TLR2 respectively have been functionally inactivated by genetic targeting.
5. *Lys*-EGFP-*ki* (Faust et al., 2000) (Lyz2t$^{miGraf}$, obtained directly from Dr. Tomas Graf, Autonomous University of Barcelona, Spain) reporter mice on a mixed 129x1/SvJ x 129S1/Sv genetic background, which express the jellyfish derived (Morise et al., 1974, Prasher et al., 1992, Chalfie et al., 1994) enhanced green fluorescent protein (EGFP) (Cormack et al., 1996) under control of the *Lyz2* gene promoter region allowing visualization of the active *Lyz2* promoter in live and post-mortem tissue.

In paper I we used both TLR1 and TLR2 deficient mice. These animals were purchased specifically for these experiments and were not used beyond generation 3. Control C57BL/6J mice were purchased directly from the supplier.

The targeting vector used to create the *Lys*-EGFP-*ki* mouse was designed in such a way as to ensure that the *Lys* gene would no longer be transcribed or translated. Therefore homozygous *Lys*-EGFP-*ki* mice should be considered as *LysM* functional knockouts. These mice display no obvious phenotype (Faust et al., 2000) and display no significant differences in proportion of monocytes, neutrophils or lymphocytes in the blood, bone marrow and spleen when compared to C57BL/6 mice (Mawhinney et al., 2012). *Lys*-EGFP-*ki mice* display EGFP expression in neutrophils, monocytes and macrophages (Faust et al., 2000) and have been successfully employed to differentiate between microglia derived macrophages and monocyte derived macrophages in the injured CNS (Kim et al., 2009, Mawhinney et al., 2012, Thawer et al., 2013).

## Comparing milestones of rodent and human CNS development

Comparing CNS maturational age between species is widely accepted as challenging (Hagberg et al., 1997, Hagberg et al., 2002a). Some of the earliest work addressing this issue was based on measurement of post-mortem brain weights and comparison of the temporal occurrence of the "brain growth spurt", a phenomenon which occurs at term in humans but is delayed into the early postnatal period in rats (Dobbing and Sands, 1979). In adapting the Levine model of adult anoxic-ischemic brain injury (Levine, 1960) to model neonatal HIE (Rice et al., 1981), Rice and colleagues selected the P7 rat pup as "the germinal matrix was small and cortical layering was complete in all 6 layers, making the brain of the P7 rat pup most similar to the 34- to 35-week human infant, with the P10 rat approximating the human infant at term" (Hagberg et al., 2002a). As data from human and rodent neonates has accumulated, more comprehensive comparisons of various developmental milestones have been facilitated; in the early 90's Romijn *et al* reviewed human and rat data on numerical synapse formation, GAD and ChAT enzyme activity and developmental pattern of cortical electrical activity concluding that a rat P12-13 rat brain is approximately at the same stage of development as a term human (Romijn et al., 1991). Hagberg *et al* later summarised available data on growth/proliferation, presence of the periventricular germinal matrix, neurochemical and metabolic data, EEG pattern, synapse formation and patency of the blood-brain barrier concluding that the brain of a P7-14 rat corresponds to that of a term human (Hagberg et al., 1997). A recent and comprehensive review by Semple and co-workers taking into account factors including; oligodendrocyte maturation, immune system development, establishment of the BBB, peak gliogenesis, brain growth spurt, and axonal and dendritic density equates a 23-32 week human preterm brain with a P1-3 rodent, and a 36-40 week term human brain with the P7-10 rodent.

With the now burgeoning information on developmental events in different species, integration starts to present an issue. An ongoing collaborative project from the University of Central Arkansas and Cornell University attempts to address this by incorporating available data into a predictive model of 271 developmental events in 9 species (www.translatingtime.org) (Workman et al., 2013). Using this tool to translate the age of mice used in this thesis, based on comparative whole brain myelination, we obtain the following results: P5 mouse = P6 rat = GW 30

(preterm) human; P9 mouse = P10 rat = GW 40 (term) human.

In summary, translating brain maturity across species is difficult, whilst a general consensus exists over approximate timings, discrepancy over exact figures remains (Dobbing and Sands, 1979, Romijn et al., 1991, Hagberg et al., 1997, Hagberg et al., 2002a, Semple et al., 2013, Workman et al., 2013) and may well be borne of differential characteristics of individual developmental processes between species. Our decision to use P5 and P9 mice to model preterm and term injury respectively is well supported by the available literature.

## Injury Models

### *P5 LPS (Paper II)*

LPS was administered at a dose of 1mg/kg *i.p.* (intraperitoneal) to P5 mice with the aim of modelling aspects of neonatal infection in preterm infants. At P5 the general level of CNS maturity in the mouse is broadly comparable to that of a preterm human and the processes of developmental hippocampal neurogenesis (Bayer, 1980) and microglial precursor proliferation are highly active (Alliot et al., 1999). In experimental systems *i.p.* administration of LPS, an outer membrane component of gram-negative bacteria, induces systemic inflammation and leads to increased expression of proinflammatory cytokines, enhanced microglial activation and inhibited neurogenesis in the CNS (Vallieres and Rivest, 1997, Turrin et al., 2001, Monje et al., 2003, Eklind et al., 2006, Wu et al., 2007, Fujioka and Akema, 2010). We selected the dose 1mg/kg (1mg/kg) and administration route (*i.p.*) based on studies displaying LPS mediated effects on hippocampal neurogenesis in adult animals (Monje et al., 2003, Wu et al., 2007, Fujioka and Akema, 2010).

### *Experimental HI (Papers I and III)*

The experimental model of neonatal HI used in papers I and III originates from the Levine adult rat anoxia-ischemia model (Levine, 1960), which combined unilateral carotid artery ligation with hypoxia to produce conditions of combined "anoxic-anoxia" (oxygen deprivation) and ischemic-anoxia (deficiencies in oxygen, glucose and other substrates) (Levine, 1960). In searching for an appropriate model of neonatal HIE Rice *et al* adapted this model to the P7 rat, creating the Rice-Vannucci model (Rice et al., 1981), which has since been adapted for mice (Ditelberg et al., 1996) and with minor variations of age and technique remains one of the most widely used systems for studying HI (Yager and Ashwal,

2009).

The procedure follows a general protocol of anaesthesia followed by permanent unilateral common carotid artery occlusion and exposure to a variable degree of hypoxia for a varying length of time; in our experiments we have used 10% $O_2$ for 50 minutes respectively.

In rats hypoxemia and hypocapnia are observed during hypoxia (Vannucci et al., 1995) and cerebral blood flow (CBF) is reduced 40-60% of control rate in the ipsilateral hemisphere, an effect that is normalised immediately upon return to normoxic conditions (Vannucci et al., 1988). Similarly data from our lab displays greatly decreased CBF in the ipsilateral hemisphere of P9 C57BL/6 mice during hypoxia, with values returning to their physiological range between 2-6 hours after following to normoxic conditions (Ek et al., 2014). Histopathological examination reveals reproducible damage to the ipsilateral cerebral cortex, striatum, hippocampus and white matter (Rice et al., 1981, Silverstein et al., 1986, Towfighi et al., 1991, Bona et al., 1995, Vannucci and Hagberg, 2004), which is rarely observed in the contralateral hemisphere and is not present in pups subjected only to hypoxia (Towfighi et al., 1995, Vannucci and Hagberg, 2004). Thus this model displays similar neuropathological lesions to those commonly observed following severe asphyxia in human term neonates. However, other brain regions, such as the brain stem and cerebellum that may be affected in clinical HI are poorly modelled in this rodent system (Hagberg et al., 1997, Volpe, 2008). Other disadvantages of this model are the lack of multi organ involvement, as observed in cases of severe clinical asphyxia (Hagberg et al., 1997) and the inherent variability of lesion size (Grafe, 1994, Hagberg et al., 1997), which results in greater numbers of animals being required for experiments; this effect is counteracted to some degree by the predominantly unilateral nature of the lesion, which depending on experimental context, allows use of the contralateral hemisphere as an internal control.

## Histology (Papers I-III)

### *Histological preparations*

Good histological preparations result from four main processes: Fixation, embedding, sectioning and staining. There are numerous possible variations, and permutations, of these steps which should be carefully considered and correctly applied for specific applications.

Fixation prevents tissue autolysis and preserves morphologic and molecular characteristics. It is therefore desirable for fixation to occur either

before, or immediately after, removal of tissue from the organism. Here fixation has been performed with either 4% paraformaldehyde (PFA) or Histofix, the latter being a commercially available formulation of 6% paraformaldehyde. Exposure of tissue to paraformaldehyde leads to protein "cross-linking", the net result of which is preserved cellular and ultimately tissue structure. Here, PFA has been applied via transcardial perfusion of the terminally anaesthetized animal. A process proceeded by a brief flush with isotonic saline which clears residual blood cells from the vasculature. Following dissection, brains were post-fixed for a further 24 hours to facilitate more complete cross-linking.

Paraffin embedding and sectioning were performed in paper I, and cryo-embedding and sectioning in papers I-III. Paraffin embedding has the advantage of facilitating collection of extremely fine sections, commonly 7μm, and greatly preserving gross anatomical structure. The paraffin embedding process involves a standardised process of sequential dehydration through an increasingly concentrated series of alcohols followed by xylene clearance and finally paraffin infiltration. Cryoembedding methodologies generally preserve antigenicity to a greater degree than paraffin processing but at the cost of gross tissue morphology; this is to some degree counteracted by cutting thicker sections, typically 20-60μm depending on age of animal. When preparing tissue for cryosectioning the greatest potential problem is formation of large ice crystals. This process leads to abnormal vacuolisation of tissue, potentially rendering it useless for further analysis, the so-called "Swiss-cheese" effect. Ice crystal formation is facilitated by slow freezing and slow thawing; rapid freezing leads to the formation of smaller crystals, which do not disrupt cellular membranes and tissue structure, slow thawing facilitates the aggregation of smaller crystals into larger crystals: this effect becomes significant above -30$^0$C and especially problematic above -20$^0$C. We combated these issues in several ways, the first of which is through fixation, which has previously been mentioned. Following fixation, tissue is submersed in 30% sucrose for a minimum of 24 hours, a process which facilitates tissue water displacement and sucrose infiltration. Samples are then frozen on liquid nitrogen, to ensure rapid freezing, and cut on a Leica CM3050 S cryostat (Leica, Sweden) at ≈ -22$^0$C to avoid thawing artefacts. Once cut, sections are transferred to a solution of 25% ethylene glycol and 25% glycerine in 0.1M phosphate buffer (a cryoprotectant solution) and stored at -20$^0$C until immunohistochemistry. (Asahina et al., 1970, Rosene et al., 1986, Johnstone and Turner, 1997).

### *Immunohistochemistry*

Immunohistochemistry is widely employed in both biological research and diagnostic histopathology. In essence the procedure relies on detection of an endogenous antigen through application of an exogenously derived (primary) antibody, which may be unconjugated or directly conjugated to a label which allows visualisation. In the case of unconjugated primary antibodies, a secondary conjugated antibody that reacts to the primary is required for successful visualisation. Antibodies may be conjugated to enzymes or fluorescent molecules; enzyme based labels, such as horseradish peroxidase, can be activated in the presence of hydrogen peroxide causing oxidisation of a substrate (for example: Diaminobenzidine or DAB), and visualisation of the bound antigen-antibody complex. Stained tissue can later be analysed on a brightfield microscope. Conjugation of fluorescent molecules allows direct visualisation on microscopes configured for this application. A great advantage of visualisation based on fluorescence is that it allows the detection of multiple antigens simultaneously, a property useful for microscopy and essential for other antibody based applications such as flow cytometry. Primary antibodies may be polyclonal or monoclonal, and it is important to be aware of the specific clonality for correct experimental design. When selecting combinations of fluorescence conjugated antibodies for multi-labelling experiments, one should consider the configuration of the microscope that will be used for downstream analysis and the emission and excitation spectra of the fluorescent labels: a well selected panel of antibodies will guard against overlap of emission spectra and consequent incorrect identification of antigen-antibody complexes.

All immunohistochemical staining procedures used in this thesis follow a general protocol of pretreatment (where required), blocking and application of primary followed by secondary antibodies. Antigen retrieval pretreatment steps are generally considered obligatory for successful immunohistochemical staining of paraffin sections, but potentially dispensable for successful staining of cryosections: Following PFA fixation, the cross-links made between protein molecules may make the targeted antigen unavailable for antibody binding; antigen retrieval steps aim to "unmask" antigens by breaking the cross-links formed during fixation and leaving the antigen available for binding. Antigen retrieval was performed on paraffin- (Paper I) and cryo- (Papers II & III but not I) sections by 10 minute incubation with 10mM sodium citrate buffer (pH6) at 95-100$^{O}$C. Blocking is performed to inhibit assumed non-specific an-

tibody binding due to hydrophobic interactions and reactions between the Fc portions of antibodies and Fc-receptors. We performed this step through 30 minute incubation (room temperature) in TBS, or TBS plus 0.1% triton-x-100, containing 3% normal serum. Here, incubation with normal serum inhibits Fc-FcR interactions while the inclusion of a detergent counteracts unspecific binding through hydrophobic forces. (Johnstone and Turner, 1997, Buchwalow et al., 2011).

## Microscopy

Various microscope systems and stereological analysis techniques have been used in this thesis, below follows information about these systems and their application herein.

### *Brightfield and epifluorescence*

Brightfield microscopes consist of three main elements: a light source (commonly a halogen lamp), condenser, objective and ocular. The light path runs through source to condenser, specimen, objective and finally ocular lens or detector. This is a trans-illumination technique with stained structures observed as darker areas on a bright background. This technique is most commonly used for visualization of histologically stained tissue or chromogen based immunohistochemical/immunocytochemical preparations (Papers I&II).

Epifluorescence microscopy systems are commonly built on brightfield systems. Their main elements are: lamp (commonly halogen or mercury and increasingly LED), excitation filter, dichroic mirror, objective, emission filter ocular and detector. Key to understanding this type of system is a basic knowledge of the behavior of fluorophores: when these molecules are excited by light of a particular wavelength they emit light of a longer wavelength (referred to as a fluorophore's excitation and emission spectra respectively). This allows the system to be built to both transmit light to the sample, and receive reflected light, via the objective lens. Here, the light path will run from the source to the excitation filter, dichroic mirror, objective specimen, objective, and finally to the ocular and/or detector. The dichroic mirror reflects light of shorter wavelengths whilst transmitting that of longer, which means that the shorter wavelength light from the lamp will be reflected by the dichroic mirror towards the specimen via the objective, whilst returning longer wavelength light will be transmitted through the dichroic mirror to the ocular lens or detector. The presence of emission filters (located between the dichroic mirror and ocular/detector) limits returning light to one particular region of the spec-

trum (usually correlating to the emission spectra of a related group of fluorophores). The use of several filters and sequential imaging therefore facilitates multi-channel fluorescence microscopy. A common filter configuration would be three filters allowing visualization of fluorophores with emission spectra of approximately 405nm, 488nm and 555nm. A major limitation of epifluorescence systems is the undesired detection of light from outside the focal plane. Practically this limits resolution of fine cellular and intracellular structure, a problem which increases in severity with greater magnification. Several systems have been developed to counter this and improve resolution, two of which are discussed below.

### *Confocal laser scanning microscopy (CLSM)*

CSLM builds on the principles of epifluorescence microscopy with several important modifications designed to facilitate acquisition of focused images from selected depths within thick biological specimens, a technique referred to as "optical sectioning". Sampling of multiple optical sections throughout the height (axial- or Z-plane) of a specimen and subsequent computer-based reconstruction, allows generation of high magnification, in focus photomicrographs and three-dimensional (3D) reconstructions of cells and cellular structures (Papers I-III). The concept of the confocal microscope was patented in 1957, essentially describing a standard fluorescence microscope with the addition of a "pinhole" to the light path. Theoretically, this pinhole could eliminate the return of light originating from outside the focal plane to the detector. However, technological solutions to the problems associated with this design (limited light from only a tiny point in the specimen returning to the detector) would not become available until the 1980s.

A modern confocal microscope is in essence a modified epifluorescence system. The ability to perform confocal laser scanning microscopy comes from the presence of laser illumination sources and the scan head. The scan head comprises of the laser inputs, raster scanning mechanism, beam splitters, filter sets and the detectors, which are commonly photomultiplier tubes (PMTs). Typically, a confocal system will have three to four lasers, which may be gas lasers (e.g. Argon 488nm or Helium Neon (HeNe) 543nm and 633 nm), solid-state lasers (e.g. Ti:S 700 – 1000nm), dye lasers (e.g. Rhodamine 6G 580nm) or semiconductor (diode) lasers (e.g. blue 405nm). The point source lasers used by CLSM systems allow high intensity light to be focused on extremely small areas of the specimen, ensuring sufficient light is transmitted via the pinhole to the detec-

tor: although this laser illuminates only a small point in the specimen it still produces a 3D diffraction pattern, meaning that the final resolution is controlled by the pinhole diameter. If the pinhole is set to 1 airy unit (the theoretical pinhole diameter at which the maximum amount of focused light is collected) only the first order of the diffraction pattern is collected by the detector; this gives the highest possible resolution at the cost of signal intensity. In practice, this may be too conservative, especially in the case of low signal-to-noise ratio, and the pinhole diameter may have to be widened and resolution will be sacrificed in favour of signal intensity. Other means to compensate for poor signal-to-noise ratio: include scanning the same Z-plane multiple times and compiling images, increasing the sensitivity of the photo-detectors or increasing the intensity of the laser. An issue with the latter of these options is that it greatly increases the risk of photo bleaching and/or damaging the specimen. (Hibbs, 2004, Pawley, 2006).

### *Structured illumination microscopy (SIM)*

Structured illumination technology represents an alternate and comparatively inexpensive way to improve upon the limitations of epifluorescence microscopy and obtain optical sectioning capabilities. The SIM system is, like the CLSM, built upon a standard epifluorescence base unit but represents a comparatively cost effective and low complexity solution which requires little more than the addition of a relatively small SIM module and suitable control software. The SIM module imposes a movable grid system in the light path, thereby "structuring" the transmitted light. Discrepancies in modulation of fluorescence signal along the structure allow discrimination of light emitted from structures within and outside the focal plane. Comparison of several images acquired with the grid positioned in different planes relative to the sample facilitates computational reconstruction of focused optical sections of the specimen (Langhorst et al., 2009). Although CSLM generally, but not exclusively, provides better quality imaging than SIM, the low complexity of the latter solution combined with its high-throughput capabilities confer advantages in certain applications. We have used this system extensively (Papers II&III) to rapidly generate multi-channel tiled Z-stacks of large brain structures, such as the hippocampus and cortex: a technique that has been particularly useful when employed in conjunction with stereological cell sampling techniques.

## Stereology

Design based stereology is a core technique of this thesis which has been used to quantify neuropathological changes (infarct size, Paper I) and size of various cellular populations (Paper II) based on histological preparations.

### *Paper I*

In paper I we estimated MAPII positive volume in ipsilateral and contralateral hemispheres in accordance with the Cavalieri principle. MAPII-DAB positive area was measured in every 40$^{th}$ section throughout the brain (section thickness = 10μm) on a standard brightfield microscope.

Volume was calculated as:

$$V = \sum\alpha \cdot (1/ssf) \cdot t$$

Where $V$ is total volume (μm$^3$), $\sum\alpha$ is the sum of the measured areas, $ssf$ is section sampling fraction and $t$ is the section thickness.

### *Paper II*

In Paper II we used design based stereology to estimate absolute number and/or density of numerous cellular populations. Various techniques were applied depending on characteristics of the population quantified.

Quantification of absolute number of BrdU, PhH3 and DCX positive cells was performed through exhaustive counting of single labelled cells in serially cut sections spanning the hippocampus. Here, antibody-antigen complexes were visualised through horseradish peroxidase/DAB chromogen reaction allowing examination and quantification on a standard brightfield microscope. In this application exhaustive counting is both feasible and relevant, as the cells of interest occupy a small anatomical region (the hippocampal sub granular zone) and are present in relatively small numbers. In situations where the cellular population of interest is not uniformly distributed throughout a wide area, unbiased sampling in the lateral plane may be irrelevant and introduce error. Exhaustive counting is therefore appropriate as it eliminates sampling bias.

Absolute number of cells was calculated as:

$$N = \Sigma Q \cdot ssf$$

Where ***N*** represents the absolute cell number of, ***ΣQ*** represents the sum of the counted cells and ***ssf*** is the sampling fraction.

We used the Fractionator technique for quantification of Iba1-DAB positive microglia as these cells are uniformly spread throughout the region of interest. This technique facilitates unbiased sampling of a defined two-dimensional region of interest and is used to sample populations considered too large for exhaustive counting.

Absolute cell number was calculated in accordance with the Fractionator methodology:

$$N = \Sigma Q \cdot (1/asf) \cdot (1/ssf)$$

Where ***asf*** represents area sampling factor.

Estimates of cells per unit volume (cell density) were made using a modified Optical Fractionator methodology:

$$N = \Sigma Q \cdot t \cdot (1/asf) \cdot (1/ssf)$$

Analysis of marker co-expression in the hippocampal granular cell layer and subgranular zone was performed on Z-stacks captured on the CLSM system. In this application the use of stacked optical sections is necessary to avoid misidentifying cells as co-expressing the markers of interest when they are located in the same xy-plane but at different levels within the z-plane. Here, a minimum of 100 BrdU positive cells were analysed for co-expression.

Estimates of absolute number of co-expressing cells calculated as:

$$P = N \cdot (ccl/ce)$$

Where ***N*** represents absolute number of BrdU positive cells assessed through exhaustive counting (discussed previously), ***ce*** represents total

number of cells examined for co-immunoreactivity and ***ccl*** represents number of co-labelled cells.

Proliferation of Iba1 positive microglia (Iba1/BrdU co-labelled) was assessed on tiled z-stacks of the entire hippocampal formation as captured on the SIM system. As microglial cells are spread relatively uniformly throughout the region of interest we employed a random sampling technique. 100 Iba1 positive cells were examined for co-expression of BrdU and data were presented as percent of total sampled cells that were co-labelled.

## Flow cytometry

The majority of analyses included in Paper III were performed using flow cytometry. This technique involves individual analysis of cells contained in a single cell suspension as it flows through an optical-electronic detection system. Basic physical characteristics, such as size (forward scatter or FSC), granularity (side scatter or SSC), and fluorescence intensity are registered for each particle passing through the detector. Combined with fluorescence conjugated antibody-mediated labelling of target antigens, this becomes a powerful tool for characterising populations of cells.

As this is an optical based analysis system, many of the components will be familiar from the prior discussion of fluorescence microscopes. A standard configuration for a flow cytometer may include two lasers and two photomultiplier tube (PMT) based detection arrays. These detection arrays may have up to eight PMTs coupled with a series of dichroic mirrors and band-pass filters: this allows light of desired wavelengths to be transmitted to the desired PMT whilst non-desired wavelengths will be directed to the next dichroic mirror to be further split in a similar fashion. These mechanisms facilitate simultaneous collection of data regarding multiple fluorophores, and hence antigens. Flow cytometry is considered a high throughput technique, with information being recorded for many thousands of cells per minute.

## Magnetic activated cell sorting (MACS)

In paper II we used MACS techniques to isolate cells from microdissected hippocampi of P21 mice. Like a flow cytometer a MACS system is a microfluidics device that relies on antibody based technologies. This system, however, has no optical analysis component; being only able to sort cells based on expression of single cell surface antigen. The sorting

method requires creation of a single cell suspension from a living sample or organ of interest and labelling the cells with magnetic bead conjugated antibody. The suspension is then taken up by the MACS system and passed through a magnetic column which facilitates the sorting process.

A major drawback for this system is that it is designed with analysis of large (Human) samples in mind: a situation in which material is abundant. We found this methodology to be poorly specified for isolation of CD11b positive cells from the microdissected P21 mouse hippocampus where starting material is extremely limited: recovery of the isolated target population was greatly limited and highly variable, which pushed our downstream analysis techniques to their absolute limit. Indeed, our attempts to analyse isolated cells from P7 hippocampi failed. A potential solution to such problems would be to pool material from several animals into each sample.

## RT-qPCR

In papers I & II, we analysed gene expression by Real Time quantitative Polymerase Chain Reaction (RT-qPCR). This technique allows quantification of mRNA transcripts isolated from cells or tissue. Depending on quantification strategy this technique provides data indicative of either relative change in gene expression (ΔΔCT-method) or absolute gene expression (standard curve method). The general principle follows a course of mRNA isolation, reverse transcription to cDNA followed by RT-qPCR reaction.

An important consideration when employing this technique is the correct choice of reference genes, which provide an important control for differences in reverse transcription reaction efficiency. We have found that several genes which are assumed to be constitutively expressed vary greatly with age and experimental manipulation in the early neonatal period. When setting up new assays we normally test a panel of housekeeping genes on samples spanning the full range of ages and treatments we intend to analyse and select the two which exhibit the most stable expression for normalization purposes.

## Statistics

In paper I we used one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison to contrast total infarct volume between genotypes or between brain level. In paper II we have exclusively performed comparisons by Student's t test. In paper III, analysis of

comparisons were made by student's t test between ipsilateral and contralateral hemispheres at each time point and comparisons of changes in composition of the recruited cell population were made by one-way ANOVA followed by Holm-Sidak's multiple comparison tests for selected pairs of time points. Data in paper I were presented as mean ± standard deviation (SD) and data in papers II & III were presented as mean ± standard error of the mean (SEM).

# RESULTS & DISCUSSION

A plethora of neurological disorders have their origins in disturbances of perinatal life. For some of these, particularly those leading to injuries easily detected by neuroimaging, causality, mechanisms of injury, and patterns of damage are gradually becoming well characterised and understood. For others, where cause and outcome seem distant and neuropathology is allusive as with schizophrenia, ASDs and maternal infection, understanding remains poor. Neuroinflammation is increasingly considered an important mediator of outcome following neonatal HI and would appear to provide a link to the less characterised pathologies of neonatal origin. The work presented herein aims to investigate unexplored aspects of the neonatal inflammatory response to both sterile and (simulated) infectious insult.

## TLR expression in the neonatal brain (Paper I)

Toll-like receptors are suggested to sense tissue damage following ischemia in the adult brain and potentially contribute to the rapid onset of inflammation (Cao et al., 2007, Caso et al., 2007, Lehnardt et al., 2007, Tang et al., 2007, Ziegler et al., 2007). Although expression of the majority of the TLRs has been demonstrated in numerous cell types derived from both the human and mouse brain (Bsibsi et al., 2002, Olson and Miller, 2004, Jack et al., 2005, Mishra et al., 2006), limited data has been available regarding their expression and functionality in neonates. We investigated the presence of Toll-like receptors in the neonatal mouse brain under physiological conditions and in response to experimental hypoxia-ischemia. Assessment of mRNA transcript expression with TLR directed $Rt^2$-PCR profiler arrays indicated constitutive expression of *TLRs 1-9* with significant regulation of *TLRs 1, 2, 5, 7* & *8* being observed at either 30 minutes, 6 hours or 24 hours following cessation of hypoxia (Paper I, Table.1.). Our observations of constitutive *TLR* expression mirror several recent studies which have displayed presence of *TLRs 1-9* in both 6-8 week mouse brains (Mishra et al., 2006) and cultured microglia isolated from P1-3 mouse brains (Olson and Miller, 2004). Similarly expression of *TLRs 1-9* have been observed in cultured human microglia (Bsibsi et al., 2002, Jack et al., 2005); Jack and co-workers also demonstrated expression of *TLRs 1-7 & 9, 10* in human astrocytes whilst Bsibsi *et al* found only *TLRs 2 & 3*. Collectively these data provide convincing

evidence of constitutive TLR expression in neural tissue or cells across several time points and in at least two species. Discrepancies of expression in cultured astrocytes may well be related to differential origin of tissue samples and ages of donors: The Bsibsi study utilised post-mortem tissue from individuals aged between 87 and 96 while the Jack study used that from young adults undergoing partial temporal lobe resection for non-tumour/non-infection related intractable epilepsy (Bsibsi et al., 2002, Mishra et al., 2006).

Our observations of *TLR 1, 2, 5, 6 & 8* regulation following neonatal HI are partially supported by studies of focal cerebral ischemia as induced by mid-cerebral arterial occlusion (MCAO) in adult rodents where enhanced expression of *TLR 2* (Lehnardt et al., 2007), *TLR 2 & 4* (Barakat et al., 2014) or *TLR 2, 4 & 9* (Ziegler et al., 2007) have been displayed. The obvious discrepancies here relate to TLRs 4 & 9 and may be indicative of an immature response to cerebral ischemia in the neonate. We observed the most robust changes, in terms of magnitude and temporal consistency, in *TLR1* and *TLR2* expression; *TLR1* was acutely downregulated (30 min, FC = -1.92, $p < 0.01$) and then progressively upregulated through 6 (FC = 2.39, $p < 0.001$) and 24 (FC = 3.36, $p < 0.001$) hours. *TLR2* displayed the second most robust regulation with expression transitioning from baseline at 30 minutes (FC = 1.23, p = *n.s.*) to significantly upregulated at 6 (FC = 1.63, $p < 0.01$) and 24 (FC = 2.27, $p < 0.01$) hours (Paper I).

We next assessed gross anatomical localisation and cell specific expression of TLRs 1 & 2 at 24 hours after HI. General localisation was assessed via DAB staining and widefield microscopy (Paper I, Figure.2.) and cellular localisation was assessed through multi-immunofluorescence staining with markers for neurons (NeuN, HuC/D), astrocytes (GFAP), microglia (Iba1) and oligodendrocytes (Olig2) by confocal microscopy (Paper I, Figure.3 & 4.). TLR1 was upregulated in the ipsilateral hemisphere and predominantly expressed by neurons of the hippocampus, striatum and thalamus (Paper I, Figure.2a, 3.); all known sites of injury in this model (Rice et al., 1981, Silverstein et al., 1986, Towfighi et al., 1991, Bona et al., 1995, Vannucci and Hagberg, 2004). TLR2 by contrast, displayed consistent expression across ipsilateral and contralateral hemispheres with staining found in the hippocampus, sub-cortical white matter, stria terminalis and paraventricular nucleus (PVN) (Paper 1, Figure.2b-d.). Further, TLR2 was found to be expressed by astrocytes and a population of neurons located in PVN of the hypothalamus (Paper I, Figure.4.).

Expression of TLR2 in both neurons (Tang et al., 2007) and astrocytes (Mishra et al., 2006) have been previously documented. Our observations of TLR2 expression in the uninjured stria terminalis, PVN and supraoptic nucleus closely resemble results from in situ hybridisations in adult animals (Laflamme et al., 2001).

***Signalling through TLR2 but not TLR1 potentiates injury in HI***

We next asked whether signalling through TLRs1 & 2 might affect outcome after HI. We subjected P9 TLR1 and TLR2 deficient mice to HI and assessed gross neuropathology 5 days later (Paper I, Figure.5.). Genetic ablation of TLR1 had no effect on injury size, suggesting that while regulated in response to HI, it plays no significant role in the injurious process. Genetic deletion of TLR2 by contrast was significantly neuroprotective, leading to reduced loss of MAPII expression after HI, and hence improved neuropathological outcome (Paper I, Figure.5.), a finding consistent with several studies of adult focal cerebral ischemia (Lehnardt et al., 2007, Tang et al., 2007, Ziegler et al., 2007).

## Central response to peripheral immune stimulation (Paper II)

Microglia are widely accepted to originate from yolk-sac derived myeloid precursors that invade the murine CNS during prenatal development (Ginhoux et al., 2010, Prinz and Mildner, 2011, Ransohoff, 2011). These primitive microglial precursor cells are amoeboid in shape and proliferate extensively through the latter stages of development progressively populating the microglial compartment (Alliot et al., 1999, Zusso et al., 2012). During the first two weeks of postnatal life microglial proliferation is rapidly restricted and microglia gradually assume their mature ramified morphology (Alliot et al., 1999, Zusso et al., 2012). Turnover of microglia in the mature organism is extremely limited (Lawson et al., 1992) and replacement by peripheral monocytes is scarce under physiological conditions (Ajami et al., 2007, Ransohoff, 2011). Our experiments indicate that peripheral immune stimulation with LPS acutely increases microglial number, and density, in the developing hippocampus (Paper II. Figure.1.), results supported by previous studies of peripheral immune stimulation with *E.coli* in neonatal (Bland et al., 2010) and LPS in juvenile and adult rodents (Monje et al., 2003, Wu et al., 2007, Diz-Chaves et al., 2012). These increases in the absolute number of hippocampal microglia were solely due to enhanced proliferation with a total absence of

peripheral myeloid cell recruitment (Paper II, Figure.2.). Of particular interest, analysis of microglial density, a parameter that unlike absolute microglial number takes account of the rapid growth of the hippocampus during the early neonatal period, revealed an interesting phenomenon: LPS administration at P5 prematurely boosts the density of microglia in the hippocampus to the relatively stable levels observed from P21 onwards (regardless of treatment). With these data in mind it is tempting to hypothesise over the existence of a target, or maximum, CNS microglial density. Such a parameter could potentially be controlled through juxtacrine mediated control of microglial proliferation. Indeed, microglia are endowed with the Notch-1 receptor and its ligands Jagged-1 and Delta-1 (Cao et al., 2008) and signalling through this receptor induces expression of the Runx1 (Runt-related) transcription factor (Burns et al., 2005) which has been shown to inhibit amoeboid microglial precursor proliferation and facilitate the transition from amoeboid precursor to ramified microglia (Zusso et al., 2012).

### *Systemic LPS administration alters hippocampal inflammatory status in the neonate (Paper II)*

In response to pathological stimulation microglia assume an upregulated immune phenotype (Hanisch, 2002) which facilitates their participation in processes aimed at maintaining tissue homeostasis. However, under certain circumstances activation may endow microglia with neurotoxic properties (Hanisch and Kettenmann, 2007, Chhor et al., 2013) and the potential to inhibit adult neurogenesis (Ekdahl et al., 2003, Monje et al., 2003). Indeed, cell culture studies have indicated that LPS-activated microglia (M1) reduce neurogenesis whereas Il4-activated microglia (M2) enhance it (Butovsky et al., 2006).

We employed qRT-PCR to investigate hippocampal expression of a panel of eight microglia associated genes known for their pro- or anti-inflammatory properties. Our data displayed regulation of both M1 and M2 associated genes at 48 hours progressing to M1 only at 2 weeks (Paper II, Figure.3.). Further analysis of gene expression in MACS isolated hippocampal microglia 2 weeks after LPS administration proved less conclusive (Paper II, Figure.3.), potentially due to the inherent technical difficulties of isolating and analysing such a small population of cells.

Although these results do not conclusively prove microglial activation in this model, they are strongly suggestive of it. Moreover, they clearly indicate upregulated immune activity in the hippocampus following pe-

ripheral LPS administration.

### *Effects of systemic inflammation on neurogenesis (Paper II)*

The process of neurogenesis is highly plastic and may be influenced by numerous factors including stress, exercise and inflammation (Ekdahl et al., 2003, Butovsky et al., 2006, Rolls et al., 2007, Cacci et al., 2008, Bland et al., 2010, Lucassen et al., 2010). At P5 the hippocampus is relatively immature and both cell proliferation (Paper II, Figure.4.) and developmental neurogenesis (Bayer 1980a) remain highly active. Having observed LPS induced changes in hippocampal immune activity, we asked whether these changes would impact hippocampal neurogenesis. We observed a transient reduction of neuronal commitment amongst hippocampal progenitors entering the synthesis phase of the cell cycle at 16 days after LPS administration (Paper II, Figure.5.). This alteration was manifest solely through reduced numbers of type 3 late stage precursor cells with less mature hippocampal stem/precursor populations unaffected (Paper II, Figure.6.). Interestingly, when we analysed long-term fate of cells born during the P22-P25 period (when decreased neurogenesis and increased hippocampal inflammatory state were detected ) we found no effect on the number of surviving neurons (Paper II, Figure.7.), a result which strongly suggests enhanced survival and potentially enhanced network integration of the fewer neurons born during this period. This hypothesis garners some support from the fact that many more cells are born in the hippocampus than are required, and that a great number of these die long before maturing to granule cells (Biebl et al., 2000, Kempermann et al., 2003).

## Leukocyte trafficking in neonatal HI (Paper III)

The immune privilege displayed by the CNS (Hickey, 1999, Ransohoff et al., 2003, Callahan and Ransohoff, 2004, Carson et al., 2006, Galea et al., 2007, Gao and Hong, 2008) is widely understood to be severely undermined in the context of both central and peripheral inflammation (Vallieres and Rivest, 1997, Turrin et al., 2001, Eklind et al., 2006, Galea et al., 2007, Schwarz and Bilbo, 2011, Hagberg et al., 2012). In rodent models of neonatal HI numerous chemokines are upregulated (Hedtjärn et al., 2004) and accumulation of macrophages (McRae et al., 1995), neutrophils (Bona et al., 1995, Hudome et al., 1997, Nijboer et al., 2008), mast cells (Jin et al., 2009) and NK cells (Bona et al., 1999) have been documented. Under neuroinflammatory conditions macrophages can arise from both activated microglia and circulating monocytes (Ajami

et al., 2011), and are respectively referred to as microglia derived macrophages (MiDMs) and monocyte derived macrophages (MDMs). These two ontogenetically distinct cell populations have proven difficult to differentiate using traditional techniques due to their shared expression of numerous surface markers (Perry et al., 1985, Sedgwick et al., 1991) and presumed morphological similarities (Kreutzberg, 1996). Although such differentiation may sound arbitrary, modern analysis techniques are increasingly demonstrating differential roles of these cellular populations in CNS pathology (Ajami et al., 2011, Yamasaki et al., 2014). By combining the *Lys*-EGFP-*ki* with the modified Rice-Vannucci neonatal HI model we were able to unambiguously identify infiltrating peripheral myeloid cells in the post-HI CNS.

### *MiDMs and MDMs are morphologically distinct*

Although MiDMs and MDMs are generally considered to be morphologically similar we found EGFP positive infiltrating cells to be distinct from Iba1 positive central microglia at 7 days post-hypoxia-ischemia. Peripheral MDMs where generally elongated or rod shaped and displayed low Iba1 immunoreactivity whilst Iba1 immunoreactive MiDMs displayed the characteristic ramified morphology with thickened processes expected of activated central microglia (Kreutzberg, 1996) (Paper III, Figure.2.). Similar observations have recently been made in CCR2-RFP/CX3CR1-GFP mice subjected to experimental autoimmune encephalomyelitis (Yamasaki et al., 2014).

### *Myeloid cell recruitment in the ischemic neonatal brain*

Utilising flow cytometry we were able to quantify myeloid cell recruitment in the post-HI brain. We observed significant presence of EGFP positive cells in the ipsilateral hemisphere at 1, 3, 7 and 14 days, with peak presence at 1 and 7 days, after HI (Paper III, Figure.3g.). Previous studies have addressed the question of myeloid cell accumulation in the CNS following neonatal stroke (Denker et al., 2007); here the authors performed flow cytometry at 24 and 48 hours and classified MDMs and MiDMs based on level of CD45 expression. Interestingly, we observed a much greater response than previously demonstrated, with infiltrating cells constituting 48% of the ipsilateral hemisphere's myeloid cell population at 1 day after HI vs 10% in the neonatal stroke model (Denker et al., 2007). Although likely due to inherent differences of the two neonatal injury models, it is possible that such previous findings may have contributed to an under appreciation of the role of peripheral immune cells

in neonatal brain injury.

As EGFP is expressed in both neutrophils and monocytes in the *Lys*-EGFP-*ki* mouse (Faust et al., 2000) we further characterised the recruited EGFP positive cells based on expression of GR1 and Ly6C by multichannel flow cytometry. This facilitated identification of three distinct myeloid cell populations; Neutrophils ($CD11b^{+}EGFP^{+}GR1^{hi}Ly6C^{int}$) (Hestdal et al., 1991), resident monocytes ($CD11b^{+}EGFP^{+}GR1^{lo/-}Ly6C^{lo/-}$) (Geissmann et al., 2010), and inflammatory monocytes ($CD11b^{+}EGFP^{+}GR1^{lo/-}Ly6C^{int/hi}$) (Geissmann et al., 2010). We found that the inflammatory cells, namely neutrophils and inflammatory monocytes displayed significantly increased presence in the ipsilateral hemisphere at 1, 7 and 14, but not 3, days after HI. Peak accumulation was seen at 1 and 7 days (Paper III, Figure.4e, f.). In contrast to the inflammatory cell types, the resident monocyte subset was significantly present (albeit at much lower levels) in the ipsilateral hemisphere at 3, 7 and 14, but not 1, days after HI (Paper III, Figure.4d.).

Together these data illustrate prolonged presence of inflammatory cells characterised by biphasic accumulation of neutrophils and inflammatory monocytes occurring over a background of stable resident monocyte accumulation. This biphasic response is strongly indicative of occurrence of distinct phases of myeloid cell accumulation and clearance. Our data is partially supported by existing studies in neonatal mice exposed to HI which show peak presence of $CD11b^{+}CD86^{+}$ macrophages (both MiDMs and MDMs) at 1 and 7 days with reduced presence at 3 days (Winerdal et al., 2012). Additionally, data from our own lab has indicated peak chemokine expression at 8 hours post HI with reduced expression at 3 days (Hedtjärn et al., 2004), suggesting reduced central recruitment of peripheral immune cells at this time.

Although highly speculative, the idea of distinct phases of accumulation and clearance also finds some support in the existing literature: macrophages display peak neutrophil engulfment at 3 and 15 days post-ischemia in the adult rat (Weston et al., 2007). It would be interesting to see if this phenomenon extends to the neonatal HI model.

# CONCLUSIONS

In this thesis we investigated various aspects of cerebral inflammation and peripheral to central immune communication in two different models of neonatal brain injury.

We show, for the first time, that the immature brain is endowed with a full complement of Toll-like receptors. Additionally, we find that expression of several members of this family are regulated at gene level in response to neonatal HI. Importantly, we show that TLR2 is an important mediator of lesion development after hypoxia-ischemia.

We also demonstrate significant leukocyte recruitment in to the inflamed CNS following HI. We find that infiltrating macrophages and neutrophils hone to injured regions of the brain where they remain for up to 14 days after injury. Moreover, we find that up to 48% of the brains myeloid cell population may be of peripheral origin at times of peak accumulation. This immune cell recruitment is characterised by a steady influx of resident monocytes and distinct phases of infiltration and clearance of inflammatory neutrophils and macrophages.

Induction of peripheral inflammation through *i.p.* administration of lipopolysaccharide leads to gene-level regulation of several cytokines in the developing CNS, demonstrating peripheral to central immune signal transduction. Although we observed no gross neuropathological changes in this model, we did find changes in the processes of neurogenesis and microglial proliferation in the hippocampus. Microglial proliferation was acutely increased causing microglial cell density to prematurely reach juvenile levels, an effect that occurred without leukocyte contribution. Neurogenesis, by contrast, was transiently decreased: This change was manifest through effects on late stage precursor cells and appeared to be compensated for by enhanced neuronal survival. Although this change did not ultimately lead to a paucity of mature neurons, it may, speculatively, reduce hippocampal plasticity in the face of secondary challenge.

Together our results tentatively hint at mechanisms governing initiation of injury in the developing brain and illustrate peripheral to central immune communication in two different injury models. Further, we show that inflammation without gross neuropathology is sufficient to disrupt developmental microgliogenesis and hippocampal neurogenesis but not initiate recruitment of peripheral myeloid cells.

# ACKNOWLEDGEMENTS

The path leading to the publication of this thesis has, at times, seemed more like an assault course. I am consequently greatly indebted to many wonderful individuals from both the **Department of Physiology** and **Center for Brain Repair and Rehabilitation (CBR)** at the University of Gothenburg for their help, kindness, and support.

My special thanks to:

**Andrew Naylor**, my first supervisor: for being bold enough to help when I most needed it; for long hours in the lab, great discussion, bad jokes, and fun science. I wish we could have made it to the end together.

**Carina Mallard**, main supervisor: for being one of the few people in this world whose door truly is always open. For always having time to discuss results, ideas, and offer support. I will forever aspire to manage my time as effectively as you.

**Henrik Hagberg**, co-supervisor: your dedication, energy, and knowledge are awe-inspiring. Thank you for the invaluable intellectual guidance during these past years.

**Holger Nilsson**, co-supersvisor: for being a reasoned voice in turbulent times and one of the great personalities of the department. Having your input on the thesis has been a great pleasure.

To everyone in the lab: I can't imagine having a better workplace, so many wonderful personalities! Particularly to my office buddies: **Barbara D'Angelo, Kristina Subotka,** and formerly **Ana Baburamani**, for so much good advice and generalised silliness, for somehow making even the worst of days fun. **Joakim Ek**, for your adopted-antipodean humour and pancake Thursdays. **Linnea Stridh Orrhult**, for so much help with the thesis, not sure what I would have done without you. **Amin Mottahedin**, for being on the same page as me and also sharing the "microglia love…" **Syam Nair**, for being one of the good guys. **Anna-Lena Leverin** and **Pernilla Svedin,** thanks for the excellent technical help and

semi-tolerance of my lax attitude towards the use of labcoats... **Xiaoyang Wang, Anna-Maj Albertsson, Xiaoli Zhang, Gabriella Koning, Nina Hellström Erkenstam, Arshed Nazmi**, for making the lab a place that I actively enjoy working in.

From the Physiology Department: Extra special thanks to **Veronika Golubinskaya,** for great friendship and being one of the faces I always look forward to seeing. **Mats Sandberg**, for fun discussions and much inspiration. **Kerstin Ebeförs,** just keep your hands off! **Maria Johansson, Marcus Ullaryd, Peter Micallef, Sansan Hua,** for friendship and oh-so-much help with the FACS.

To my previous labmates who helped me so much when I first moved here; especially **Daniel Andersson,** my oldest friend in Sweden and truly one of the finest people I know; **Maryam Faiz**, gone, but impossible to forget... also **Åsa Widestrand, Yalda Rapheymi Bogestål & Lizhen Li** for the much needed support and great introduction to science.

Special thanks to the former CBR technicians **Rita Grandér, Anne-Marie Alborn** and **Birgit Linder**, such wonderful personalities, endless patience and remarkable kindness.

**Kerstin Hörnberg**, where can I even begin... for putting up with my chaotic disorganisation and my somewhat "taskiga svenska".

**Oskar Bergström** and **Marcus Johansson** thanks for all the computer help, but more importantly for being a welcome distraction from science.

To my very best of friends **Tomas Försman** and **Christer Eriksson,** if I'd never met the two of you I'd probably have made it to work on time a lot more often, but life would be oh-so tedious.

**Kristina Hamilton,** one of my oldest and dearest friends, without you I never would have made it this far. Thank you for giving me a good nudge in the right direction when I needed it.

**To my family**, for endless support and encouragement, for sharing the good and bad times, and for always being there.

**To my dearest Annika,** oh how my life changed since you became a part of it, and how it just keeps getting better! Thank you for your unwavering support throughout these past months. More importantly, thank you for so much joy and for bringing a light to even the darkest of days: Jag älskar dig!

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