

**The role of infection/inflammation, the
TNF family of cytokines and myeloid
cells in perinatal hypoxia-ischaemia
brain injury**

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DECLARATION

I, Eridan Rocha Ferreira, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Synergy between materno-foetal infection and hypoxic-ischemic insult around the time of birth is a known contributing factor to perinatal brain damage. This is a common precursor to cerebral palsy and other neurological deficits, affecting 2 to 5 per 1000 live births. Endotoxin up-regulates several molecules, including the TNF cluster of pro-inflammatory cytokines. Our group has explored the role of this cluster and shown that its deletion abolishes LPS sensitization to neonatal hypoxic-ischemic insult.

In this study we wanted to first investigate the effects of LPS-mediated sensitization across multiple wild type strains (C57BL/5, 129SVJ, BALB/c, CD1 and FVB) in order to then further characterize the TNF cluster, by studying the individual effects of TNF α , LT α and LT β members of this cluster, using either global gene deletion, or peripheral myeloid/macrophage-specific deletion of the floxed TNF α allele with MLys::Cre (MLys+). Additionally, we decided to also look at the acquired cellular immune system, using the athymic nude mouse model of T cell deficiency (nu).

At P7, littermates for each of the wild type strains, wild-type and homozygous knock-out offspring of heterozygous animals listed above underwent hypoxic-ischemic insult, consisting of unilateral carotid occlusion followed 2 hours recovery before being placed in a hypoxic chamber for 30min with continuous 8% oxygen exposure. 12 hours prior, animals received a single intraperitoneal injection of 0.6 μ g/g LPS or saline as a control. 1/3 of animals in the wild type strains group underwent hypoxia-ischemia alone as a control for saline treatment.

LPS pre-treatment resulted in substantial increase inflammation, neuronal injury and infarct in all wild type strains, as well as in the phenotypically wild type littermates of the homozygous mutant animals. Mice lacking both copies of the LT α gene revealed a clear reduction in LPS-mediated sensitization. In reverse, global deletion of LT β had a detrimental effect, with significant increase in brain damage. Global deletion of TNF α showed a trend towards greater damage, but deletion just in MLys+ macrophages was strongly protective, pointing to a dual role for the TNF α gene depending on in which cell-type it is expressed. Finally, nude animals (nu/nu) demonstrated a complete lack of

LPS-mediated sensitization to subsequent hypoxic-ischemic insult, suggesting that LPS sensitization may require T cell function.

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ABC	Avidin-biotin conjugates
α M	AlphaM-integrin
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole
ART	Assisted reproductive technology
BBB	Blood-brain barrier
BGT	Basal ganglia thalamus
BP	Base pair
BW	Body weight
CMV	Cytomegalovirus
cNOS	Constitutive nitric oxide synthase
CNS	Central nervous system
COX	Cytochrome oxidase
CP	Cerebral palsy
CROC	Carotid occlusion
CSF	Cerebrospinal fluid
CTX	Dorsoparietal cortex
DAB	Diaminobenzidine
ddH ₂ O	Double distilled water
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
EAA	Excitotoxic amino acid
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
EtOH	Ethanol
EXC	External capsule
FA	Formaldehyde
GFAP	Glial fibrillary acidic protein
HI	Hypoxia-ischemia
HIE	Hypoxic-ischemic encephalopathy
HIP	Hippocampus
IgG	Immunoglobulin G
IGR	Intrauterine growth restriction
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IUGR	Intrauterine growth restriction
KO	Knockout
LPS	Lipopolysaccharide
LT α	Lymphotoxin alpha
LT β	Lymphotoxin beta
MAP	Mitogen-activated protein

MHC	Major Histocompatibility complex
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response protein 88
NAC	N-Acetylcysteine
NE	Neonatal encephalopathy
NF-kB	Nuclear factor kappa B
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
OCT	Optimal cutting temperature compound
O/N	Overnight
PARP	Poly (ADP-ribose) polymerase
PB	Phosphate buffer
PB/BSA	Bovine serum albumin in 0.1M PB
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PVL	Periventricular leukomalacia
PYR	Pyriiform cortex
Rag1	Recombination activating gene
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SEM	Standard error of the mean
SGA	Small for gestational age
STR	Striatum
TdT	Terminal deoxynucleotidyl transferase
THL	Thalamus
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNF α	Tumour necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
WM	White matter
WMI	White matter injury
WS	Watershed
WT	Wild type

CHAPTER 1: GENERAL INTRODUCTION

William Little, an English Orthopaedic Surgeon, was the first to identify and describe a pattern of disabilities characterized by deformities of the human frame now known as spastic diplegia, a form of Cerebral Palsy (CP). Nowadays, cerebral palsy is applied as an umbrella term that designates a group of permanent non-progressive, non-contagious disorders affecting movement and posture. These disturbances are generally attributed to catalytic events around the time of birth or to abnormal brain development. Furthermore, the human developing brain is highly susceptible to various prenatal, perinatal and early postnatal stresses. Neonatal brain injury is not only a major contributor to infant morbidity and mortality but is also one of the leading causes of cerebral palsy and other neurological disabilities, such as cognitive impairment, epilepsy and autism (Bax et al., 2005).

Epidemiology

Perinatal brain damage remains one of the main precursors to cerebral palsy. For the last 20 years, cerebral palsy has had a continual incidence of 2-5 in 1000 live term births (Vannucci and Vannucci, 1997; Robertson et al., 2007), whereas in low birth weight (<1500 grams) premature newborn infants, the incidence is significantly increased to 60%. When asphyxia is involved, there is an increased fatality of around 25-50% for hypoxic-ischaemic babies still within the neonatal period, mostly due to multiple organ failure (Liu et al., 2006). Of the remaining survivors, up to 25% will display lifelong neurological sequelae in the form of cerebral palsy or other neurological deficits, with an additional 25-50% either/also suffering from mental retardation, cognitive impairment, epilepsy, schizophrenia and autism (Vannucci and Vannucci, 1997; Lindström et al., 2008; Meyer et al., 2009; Haynes et al., 2011).

Lindström et al., 2006, conducted a population based study in Sweden, where moderate neonatal encephalopathy had a prevalence of 0.06%. Of the surviving infants, 30% developed moderate cerebral palsy. 71% of the children not affected by cerebral palsy demonstrated cognitive impairment and 18% had hearing impairment. Furthermore, only 8% of individuals diagnosed with neonatal encephalopathy but without cerebral palsy had no impairment when tested at 15-19 years of age. Prevalence data from the United States indicate that 8000 babies are born every year with cerebral palsy (Winter et al., 2002). In Germany, approximately 1000 children suffer from perinatal brain damage annually, with a considerable proportion of these children later developing cerebral palsy as well (Bumke and Foerster, 1936).

Clinical manifestation and recognition

Perinatal brain injury is typically diagnosed based on signs of encephalopathy, which evolve from lethargy to hyperexcitability to stupor during the first three days of life (Sarnat and Sarnat, 1976). Difficulties with initiating and maintaining respiration, subnormal level of consciousness, seizures and depression of tone and reflexes characterise those affected (Nelson and Leviton, 1991).

However, in cases of prematurity and low birth weight, perinatal brain injury often eludes diagnosis, as obvious signs of injury are not present, possibly due to misinterpretation by attribution of symptoms as consequential to developmental immaturity (Mercuri et al., 2003). Furthermore, subclinical symptoms will also lead to a delay in diagnosis of cerebral palsy, cognitive impairment as well as other complex behavioral disorders which are only noticeable at a later stage in childhood (Mercuri et al., 2004).

Outcomes of perinatal brain damage

Cerebral palsy

Cerebral palsy is the most common disability in childhood. This condition is a manifestation of injuries to the brain parts responsible for controlling body movement and persists through life. As aforementioned, insults leading to cerebral palsy may occur in the antenatal, perinatal and early postnatal periods of life. There is significant evidence from clinical cases that relate severity of neonatal encephalopathy to long-term neurodisabilities. Overall, cerebral palsy in its mild form accounts for only 2% death risk (Collins and Panneth, 2002), whereas a more severe form of cerebral palsy the risk of mortality increases to 85% (see table 1.1). Multiple organ dysfunction is considered the major cause of death in infant sufferers (Cowan et al., 2003).

Severity of CP	Characteristics
Mild	No increased risk of motor or cognitive impairment
Moderate	Significant motor deficits, fine motor disability, memory impairment, visual or visuomotor dysfunction, increased hyperactivity and delayed school readiness
Severe	High death risk (up to 85%); Increased risk of mental retardation

Table 1.1: Types of cerebral palsy. **Abbreviations:** CP – cerebral palsy.

Furthermore, depending on the type of insult and which areas of the brain are affected, cerebral palsy will present itself in different forms. These subtypes can be classified as being spastic (80% of cases), dyskinetic (10%), or ataxic (10%) (Paneth, 2008), and will have different onsets, thus affecting the sufferer differently (Volpe, 2009) (see table 1.2). Spastic cerebral palsy is associated with damage to the brain cortico-motor and underlying white matter areas, and is characterized by hypertonia, i.e. decreased range of muscular motility, and may affect many areas of the body, and in some cases, even affect speech and

continence (Blair and Stanley, 1997). In cases of dyskinetic/dystonic, normally the basal ganglia area of the brain is damaged and sufferers experience uncontrolled muscle contraction, which can be either sustained or intermittent. This type of cerebral palsy can affect all four limbs, thus making difficult maintaining an upright position. Additionally, speech may be impaired due to difficulty in breathing and controlling the tongue (Hou et al., 2006). Ataxia cerebral palsy occurs when the cerebellar part of the brain is affected and is associated with difficulty in coordinating muscle function and balance, with possible additional poor spatial awareness (Cheney, 1997).

Subtypes of CP	Characteristics	Onset
Quadriplegia/dyskinetic	Involvement of all 4 limbs	Perinatal HI
Hemiplegia	One side of the body – more commonly arm than leg	Term infants, stroke-like vascular incident
Spastic	Both sides – more arms than legs	Preterm or growth restricted infants

Table 1.2: Subtypes/classification of cerebral palsy. **Abbreviations:** CP – cerebral palsy and HI – hypoxia-ischaemia.

Cognitive impairment

Observational studies of children with both severe and moderate neonatal encephalopathy have demonstrated visible memory impairment, visual-motor or -perceptive dysfunction and hyperactivity, the later not being limited to the presence of mental retardation. Marlow et al., 2005, studied 65 children having suffered neonatal encephalopathy; they were followed up to the age of 7 and assessed for neurocognitive and behavioral outcomes. In 50% of severe neonatal encephalopathy cases, affected children had severe cognitive impairment (IQ<55). Despite 75% of observed children not having cerebral palsy, cognitive scores were still low for the severe group, but in the moderate group the difference in scores was relatively small in comparison to classmates. Moreover, both groups showed a higher need for special educational needs than unaffected

children (Marlow et al., 2005). A Swedish population-based cohort study following children born suffering from moderate encephalopathy also showed that children born at term with moderate neonatal encephalopathy had a high rate of cognitive impairment, which occurred with or without the presence of cerebral palsy (Lindström et al., 2008). A review by van Handel et al., has shown that in terms of academic achievement, children with mild encephalopathy fared well, whereas sufferers with moderate encephalopathy had lower scores than children with mild encephalopathy, still these results were within normal range. However, children with severe encephalopathy consistently demonstrated lower IQ and performed worse at school, particularly in subjects such as arithmetic/mathematics and reading and spelling, when compared to age-matched peers (van Handel et al., 2007).

Autism

Badawi et al. performed a population-based case-control study using 276 term newborn infants with identified moderate or severe neonatal encephalopathy, and another 564 term infants were randomly selected as controls. This study revealed a correlation between neonatal encephalopathy and autism. From the neonatal encephalopathy group, around 13% died by the age of 5 years. Of the remaining survivors, 12 (5%) were diagnosed with an autism spectrum disorder. Of these, 10 sufferers met the full criteria for autism, 1 had Asperger syndrome and one had pervasive developmental disorder (Badawi et al., 2006).

Causes and risk factors of cerebral palsy

Birth asphyxia was considered to be the main cause of neonatal encephalopathy and cerebral palsy; however, epidemiological studies have now consistently shown the origin of cerebral palsy as being multifactorial. Even though some cases still have unidentified origins (Bax et al., 2005), other universally accepted causes include infection/inflammation during pregnancy and labor, genetic metabolic defects, such as homozygous mutation of the gene encoding for

glutamate decarboxylase-1 enzyme (McHale et al., 1999; Lynex et al., 2004), as well as maternal exposure to toxins such as methyl mercury (Harada, 1978). Furthermore, these factors have been shown to act either alone or in a synergistic manner (Blair and Stanley, 1993; Badawi et al., 1998; Hagberg et al., 2002). Nonetheless, hypoxia-ischaemia remains the most debilitating factor preceding cerebral palsy, with significant morbidity and a high mortality rate of 30% (Vannucci, 1990; Vannucci and Vannucci, 1997; Nelson et al., 1998; Vannucci and Hagberg, 2004). Despite advances in neonatal care, neonatal encephalopathy still has an incidence of 2-4 per 1000 live births (Peebles and Wyatt, 2002), and as a result, this condition has long-lasting devastating consequences not only to sufferers, but also to carers, as well as an immense cost to society, making understanding of the mechanisms involved imperative, as a means to help improve management of mothers and babies and reduce the risk of long-term brain damage.

A population-based cohort study of neonatal encephalopathy cases revealed that 69% originated from antepartum risk factors alone, 25% had antepartum and intrapartum origin, 4% intrapartum hypoxia alone, with a further 2% being contributed to unidentifiable factors (Badawi et al., 1998). The majority of antepartum risks associated with cerebral palsy include maternal risks such as maternal neurological disorder, infertility treatment, maternal thyroid disease, preeclampsia and intrauterine growth restriction (IUGR) (Wu et al., 2004). Some cases have demonstrated both ante and intrapartum factors, whereas only a minor number of incidents are considered to be the result of intrapartum risks alone such as maternal fever, emergency caesarean, placental abruption and uterine rupture, however, the later was shown to increase the risk of neonatal encephalopathy fourfold (Nelson, 1988; Badawi et al., 1998).

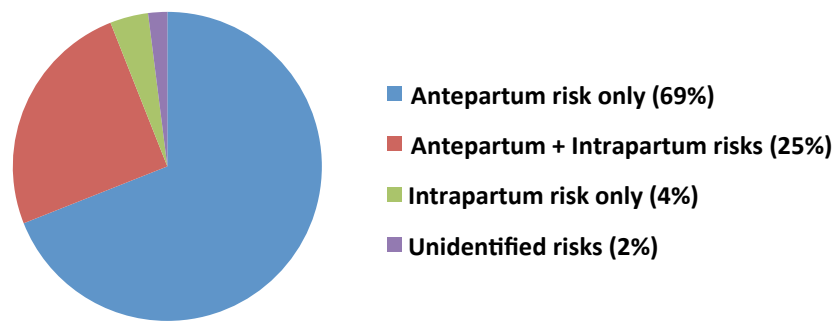


Figure 1.1: Diagram representation of the aetiology of cerebral palsy (adapted from Badawi et al., 1998).

Low Birth weight

Approximately 1.2% of all live born infants have low birth weight. Studies on the prevalence of cerebral palsy have consistently shown that low birth weight infants (<2500 grams) are at a risk of developing cerebral palsy. This risk increases exponentially in very small babies (<1500 grams). Colver et al. investigated epidemiological changes in cerebral palsy in England over a period of 30 years (1964-1993). His findings demonstrated a reduction in perinatal mortality throughout the examined period, and babies born with a weight above 2500 grams demonstrated no significant increase in perinatal brain damage and cerebral palsy. However, infants with low birth weight (<2500 grams) the most severe forms of cerebral palsy were present (Colver et al., 2000). Similarly, Pharoah et al. looked at cases of cerebral palsy covering Scotland and 6 counties of England. This study found that the prevalence of cerebral palsy was of 1.1 per 1000 live births where the birth weight was ≥ 2500 grams. This prevalence increased to 78.1 per 1000 live births in babies with ≤ 1000 grams (Pharoah et al., 1998). Additionally, 25-50% of low birth weight survivors will also exhibit cognitive and behavioral impediments affecting school performance (Skranes et al., 1997).

Prematurity

Advancements in neonatal care have led to an increase in preterm infant survival; however, this does not translate to reduction in morbidity (D'Angio et

al., 2002; Marlow, 2005). Additionally, the cost of care for preterm children over the first 10 years of life is 443 times greater than that of a term infant also suffering from perinatal brain damage (Petrou, 2005). Greenwood et al. conducted a population-based study of two health districts in England. Of the 235 cases of cerebral palsy identified, 31% were born at ≤ 32 weeks, 15% were born between 33 and 36 weeks, and 54% ≥ 37 weeks of gestation. Compilation of these findings with population data revealed that cerebral palsy occurred in 42 per 1000 live births at ≤ 32 weeks, 6 per 1000 live births in 33-36 weeks and only 1 per 1000 births after 37 weeks of gestation (Greenwood et al., 2005).

Multiple pregnancies and assisted pregnancies

Multiple births have demonstrated to increase the probability of acquiring cerebral palsy. A study performed in Sweden revealed that 2.9% of babies born are twins and incidence of cerebral palsy amongst the survivors is of 1.3%. In case of triplets (0.11% of babies), this incidence increases to 4.5%. This leads to 4.33 per 10,000 infants suffering from cerebral palsy being attributed to multiple pregnancies (Pharoah and Cooke, 1996). A commonly accepted cause for this is the fact that children born from multiple pregnancies are often preterm and/or with low birth weight. A considerable percentage of multiple pregnancies are result of assisted reproductive technology (ART) treatments. Infertility treatments increase the likelihood of prematurity in birth or multiple pregnancies (Bergh et al., 1999).

Intrauterine growth restriction (IGR)

Intrauterine growth restriction is associated with an increased risk of perinatal brain damaged resulting in mortality and morbidity. This risk is further augmented in cases of prematurity at birth. A Swedish population study revealed an incidence of 1.6% infants born small for gestational age (SGA). Despite some SGA babies being simply genetically small but healthy, a good proportion of cases are a result of either malformation or hypoxic-ischaemic insult (Wennergren,

1992). In western societies, these HI events are attributed to uteroplacental insufficiency associated with maternal conditions such as chronic hypertension, diabetes mellitus, preeclampsia and infections, as well as maternal substance abuse (Bernstein and Divon, 1997). Furthermore, several studies have shown a correlation between IGR and mild neurological deficits associated with poor school performance (Harvey et al., 1982; Ley et al., 1996).

Infection/Inflammation

Feto-placental infections are highly associated with prematurity in birth, as studies have shown genital tract infections such as bacterial vaginosis, syphilis, and gonorrhoea as increasing the risk of preterm birth (Mc Gregor and French, 1997). Another infection-associated cause of prematurity is the intrauterine infection chorioamnionitis (Gomez et al., 1997). TORCH – toxoplasmosis, rubella, cytomegalovirus (CMV) or herpes simplex virus are also associated with 4-10000 cases of children suffering neurological brain damage (Rudd and Peckam, 1988). Additionally, maternal pyrexia has been known to also cause cerebral palsy. As infectious agents have become increasingly associated with perinatal brain damage and subsequent cerebral palsy, it is believed that these infectious agents act through up-regulation in the production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF α , which can be detected both in the blood and brain of the fetus, thus leading to inflammation-mediated brain damage (Yoon et al., 1997; Hagberg and Mallard, 2000).

Maternal conditions

Mothers with hypothyroidism during pregnancy (Klein and Mitchell, 2002), intellectual disability, or seizures have been linked to a slightly higher risk of having a child with cerebral palsy and/or mental retardation. Maternal poor lifestyle, including smoking and alcohol intake has also demonstrated a correlation with fetal growth restriction, perinatal brain damage, cerebral palsy and/or cognitive impairment (Hagberg and Mallard, 2000).

Prematurity and perinatal brain damage

There is an increasing body of evidence suggesting that preterm labor may be a sign of antenatal insults such as malformation, intrauterine growth restriction and intrauterine infection. The latter has gained recent wide acceptance as genital tract infections such as bacterial vaginosis and gonorrhoea have shown to increase the risk of preterm birth (McGregor and French, 1997). The presence of chorioamnionitis has also demonstrated a strong correlation with preterm birth (Gomez et al., 1997). Premature infants are prone to white matter injury (WMI) with devastating consequences to their neurobiological development, leading to subsequent chronic neurological disorders such as cerebral palsy, cognitive impairment, epilepsy and neurosensory impairments. Studies characterizing the vulnerability of oligodendrocyte precursor cells to hypoxic-ischaemic insult have shown that injury to these cells, with subsequent hypomyelination form the basis of white matter injury (Levine et al., 2001; Volpe, 2009).

Patterns of preterm brain injury

Clinical studies, together with several experimental animal research models into the pathophysiology of perinatal brain injury have concluded that neurologic dysfunction resultant from perinatal injury depends on the developmental stage. The varying pattern of injury in neonatal encephalopathy is related to maturation dependent susceptibility to adverse stimuli (Cowan et al., 2003; Thibeault-Eybalin et al., 2009). The type, severity, and duration of insult also play an important role in disease outcome. Therefore, basic mechanisms of normal cerebral development and homeostasis form the background for the interpretation of subsequent injury (Ferriero, 2004). Advances in magnetic resonance imaging (MRI) have revealed two main patterns of brain injury, one of which being the watershed cerebral infarction.

Watershed

Premature infants are at a high risk of developing brain injury most predominantly associated with watershed (WS) damage and white matter injury (WMI) (Voipe, 2001; Rezaie and Dean, 2002). White matter injury is a known contributing factor to spastic cerebral palsy and reduction in cognitive and learning capacities (Volpe, 2009). This pattern of injury was first described in 1867 by Rudolph Virchow as congenital encephalomyelitis. Virchow's post-mortem examination of the neonatal brain revealed macroscopic areas of soft and pale degenerated tissue in the periventricular brain region. Post-mortem microscopic examinations by Banker and Larroche (1962), revealed microscopic lesions of coagulative necrosis within areas of liquefaction and surrounded by highly vascularized periphery which were then termed periventricular leukomalacia (PVL). These lesions were particularly present in the subcallosal white matter superior fronto-occipital and longitudinal fascicule and the external and internal sagittal strata of the lateral ventricles' temporal and occipital horns. To a lesser extent, the corona radiate also appeared affected. The particular localization of these focal necrotic lesions at the end zones of the anterior, middle and posterior cerebral arteries provide strong evidence of hypoxia-ischaemia being causal to white matter injury (Banker and Larroche, 1962; Reuck, 1971; Takashima, 1982).

Ultrasound, and more recently, MRI scans of the brains of preterm infants have markedly improved understanding and visualization of periventricular leukomalacia, what has now become known as the predominant form of white matter injury of these patterns of brain injury. These advances in imaging have demonstrated not only microscopic lesions consisting of focal (Volpe, 2001), but also diffuse areas of coagulated necrotic tissue containing axons, oligodendrocytes and astrocytes, within the areas of liquefaction and highly vascularized periphery. Thinning of the corpus callosum is also a characteristic of white matter brain injury (Leviton and Gilles, 1996; Volpe, 2001; Hagberg et al.,

2002; Volpe, 2012). Furthermore, magnetic resonance techniques have shown that diffuse white matter injury are in fact more predominant in preterm infants. Another study following 119 infants born between 22 and 30 weeks, revealed an even greater presence of diffuse white matter injury, where 80% of these infants were affected (Dyet et al, 2006). From a clinical perspective, diffuse white matter injury is thought to be causal to cognitive and behavioral impairments commonly seen in premature birth, whereas focal, or cystic periventricular leukomalacia is highly associated with spastic diplegia, as a result of disruption to the corticospinal tracts within the posterior limb of the external capsule (Volpe, 2001; Marlow et al., 2005). Similarly, another review comprising 12 studies assessing 272 infants with periventricular leukomalacia demonstrated through ultrasound scan showed that 58% of these infants developed severe motor and cognitive impairments. In contrast, only 2.6% of the 655 infants with normal head ultrasound scan developed cerebral palsy (Holling and Leviton, 1999).

Periventricular leukomalacia has two main pathological features; focal periventricular necrosis and diffuse cerebral white matter injury. Focal periventricular necrosis is the result of focal necrotic lesions occurring mainly in the areas supplied by deep penetration arteries. These sites of coagulative necrosis, as well as loss of cellular elements, are visible in the first 6-12 hours following hypoxia-ischaemia. Cranial ultrasonography 1-3 weeks post-insult reveals tissue dissolution and cavity formations ($\geq 2-3$ mm). Studies involving larger number of infants surviving the postnatal period have shown a more diffuse cerebral white matter injury pattern. These lesion patterns are harder to be identified through ultrasonography as they rarely undergo cystic changes, and consist instead of acute glia damage (Hope et al., 1988; Dambaska et al., 1989; Paneth et al., 1990).

Epidemiological studies have identified risk factors other than cerebral hypoxia-ischaemia, which also contribute to the development of periventricular

leukomalacia. Hypocarbica, a decrease of CO₂ in blood, caused by mechanical ventilation management, has shown a strong association with periventricular leukomalacia, possibly due to hypocarbica mediated vasoconstriction, resulting in reduced cerebral blood flow (Saliba and Marret, 2001). Intrauterine infection has a strong correlation with white matter injury, and its dual role as both inducer of preterm labor and perinatal brain injury has been increasingly studied in recent years (Yoon et al., 1996; Damman and Leviton, 1997; Rezaie and Dean, 2002; Kendall and Peebles, 2005).

Other hallmarks of periventricular leukomalacia include maternal intrauterine infection resulting in inflammation, cytokine release and excitotoxicity. Neuropathological, epidemiological and experimental studies by Gilles and co-workers were the first to establish the connection between maternal/fetal infection and periventricular leukomalacia (Gilles et al., 1976; Gilles et al., 1977; Leviton and Gilles, 1984). Further studies have shown increase in incidence of periventricular leukomalacia and/or development of cerebral palsy in the presence of maternal/placental/fetal infection (Zupan et al., 1996; O'Shea et al., 1998), rise in levels of pro-inflammatory cytokine interleukin-6 (IL-6) in cord blood (Yeon et al., 1990), and increase in levels of interleukin-1 (IL-1) and IL-6 in amniotic fluid (Yoo et al., 1997a). Furthermore, both IL-6 and tumor necrosis factor-alpha (TNF α) have been shown within areas of periventricular leukomalacia damage (Deguchi et al., 1996; Deguchi et al., 1997; Yoon et al., 1997b).

Animal studies using hypoxia-ischaemia, infection and excitotoxic insults alone, or in combination, have been able to successfully reproduce white matter injury, thus allowing further understanding the neuropathology of this disease (Volpe, 2009). During this developmental stage (\leq 32 weeks), the subcortical white matter of the immature brain is still unmyelinated and predominantly populated by oligodendrocyte precursor cells. These are highly susceptible to exogenous

free radicals due to a lack of antioxidants such as superoxide dismutase (Volpe, 2001; Deng, 2010; Hristova et al., 2010; Kinney and Volpe, 2012). Subplate neurons are also affected in the presence of hypoxia-ischaemia. These neurons appear only transiently during development of the brain cerebral cortex and are vital in the formation of connections between the visual cortex and the thalamus (Kanold et al., 2003). Despite the immature cerebral cortex being highly vascularized, and thus more resistant to hypoxia-ischaemia (Ferriero, 2004), grey matter regions like basal ganglia, cerebral cortex and thalamus may also occasionally be directly affected by the hypoxic-ischaemic insult, or secondarily affected due to the presence of white matter lesion, as cystic periventricular leukomalacia can lead to subsequent significant reduction in cortical grey matter volume at term (Inder et al., 1999).

Neonatal encephalopathy

Hypoxia-ischaemia-induced severe brain damage in term infants can lead to neonatal encephalopathy within 12-36 hours, this is known as hypoxia-ischaemia encephalopathy (HIE). Neonatal encephalopathy occurs in 1-6 per 1000 live term births, with 20% of mortality. Of the survivors, 25% will develop some form of permanent neurological disorder, and is therefore a major predictor of neurodevelopmental impairment (Volpe 2001). Hypoxia-ischaemia encephalopathy and term asphyxia were thought to be the only causes of neonatal encephalopathy; however, some cases of neonatal encephalopathy have no demonstrable hypoxia-ischaemia or asphyxia in their aetiology (Wu et al., 2004). Studies into the risk factors of neonatal encephalopathy have revealed that other cases associated with antepartum events include maternal hypotension, assisted reproductive treatments or thyroid disease. Intrapartum risks such as maternal fever, abruption of the placenta, prolapsed of the cord, forceps delivery and breech extraction are also present (Badawi et al., 1998). Combined antepartum/intrapartum risks are also known causes of neonatal encephalopathy, and finally, postnatal complications such as sepsis, respiratory

failure and shock occur in less than 10% of neonatal encephalopathy cases (Cowan et al., 2003).

Development and manifestation

Infants with neonatal encephalopathy exhibit in the first hours of life a reduced level of consciousness. This is generally accompanied by periodic breathing with apnea or bradycardia. Nonetheless, in cases where injury is not severe, cranial nerve response may be spared, with intact papillary responses and spontaneous eye movement. It is not uncommon for a transient recovery in levels of alertness within the first week of life (Shah and Perlman, 2009); however, this is not associated with signs of neurological function improvement. Furthermore, jitteriness, episodes of refractory seizures alongside with apnea and shrill cry may be seen during this period. Symptoms persisting for the first months include hypotonia and weakness in the face and proximal limbs, as well as exaggeration of Moro's and muscle-stretch reflexes. These symptoms are more easily identifiable as associated with neonatal encephalopathy, as term infants have higher muscle tone than preterm babies, thus making a proper assessment difficult (Miller et al., 2004). In cases of severe injury, respiratory arrest and other signs of brain dysfunction may precede the initial depression in the level of consciousness.

Seizures occurring due to neonatal encephalopathy represent a sign of malformation, reversible metabolic disorders and structural injury. Neonatal seizure may be subtle in nature with only horizontal tonic deviation of the eye, or sustained blinking or eye opening. Stronger manifestations include oro-lingual movements such as tongue or lip smacking and sucking, rowing or bicycling movements of the extremities, or recurrent apnea. Neurological examinations permitting recognition of seizures include the use of amplitude-integrated electroencephalography to assess brain-wave activity (Rennie et al., 2004). MRI allows distinction between neonatal seizures resulting from metabolic disorders

such as congenital deficiency of sulphite oxidase and from that resultant from hypoxia-ischaemia (Barkovich, 1997). MRI can also be used in conjunction to regular laboratory test to identify other risk factors such as infection, physical traumas (skull fracture), hypoglycaemia, hypocalcemia, hyponatremia, hypoxemia and acidosis (Ferriero, 2004). Another method of diagnosing genetic disorders is lumbar puncture, which can detect pediatric neurotransmitter diseases and glucose-transport defects (Hyland K, 2003). Although difficult to treat, neonatal seizures do not always result in reduced neurodevelopmental disability (Painter et al., 1999).

Neonatal encephalopathy score		
Variable	Score=0	Score=1
Feeding	Normal	Gavage, gastronomy or intolerance of oral feeding
Alertness	Alert	Irritable, poor response or comatose
Muscle tone	Normal	Hypotonia or hypertonia
Respiratory status	Normal	Respiratory distress
Reflexes	Normal	Absent, hyporreflexia or hyperreflexia
Seizures	None	Suspected to confirmed clinical seizure

Table 1.3: Assessment of neonatal encephalopathy (adapted from Miller et al., 2004).

Patterns of neonatal encephalopathy brain injury

Basal Ganglia Thalamus (BGT)

Despite hypoxic-ischaemic encephalopathy not being the main cause of term perinatal brain damage, it is still the major cause of morbidity in infants born after 37 weeks of gestation. Pathological assessment of term babies suffering from hypoxic-ischaemic insult as well as MRI scans have revealed deep gray

matter injury, with the hippocampal, lateral geniculate nuclei, putamen, ventrolateral thalami and dorsal mesencephalon and peri-rolandic cerebral cortex areas particularly affected (Barkovich et al., 1995; Folkerth, 2005).

Hypoxia results most commonly in selective neuronal necrosis, as neurons are more sensitive to hypoxia-ischaemia insult at term in comparison to preterm (Terrie and Volpe, 2002). Also, white matter susceptibility is greatly decreased in term infants, as mature myelin basic protein-expressing oligodendrocytes are more resistant, due partially to the now presence of antioxidant enzymes and proteins (Deng, 2010). As a result, basal ganglia thalamus (BGT) most commonly shows damage in affected term babies. Population specific neurons in the deep grey nuclei and periolandic cortex are more likely injured via a failure of mitochondrial oxidative phosphorylation. The BGT pattern of injury involves the central grey nuclei, the cortex around the grey sulcus and the hippocampal pyramidal cell layers, particularly the CA1-3 hippocampal regions (Berger et al., 1992; Johnston et al., 2001; Volpe, 2012).

Mitochondrial swelling leads to cytoplasmic vacuolation in the neurons within 5-30 minutes after the onset of hypoxic-ischaemic insult (Brown and Brierley, 1973; Kim, 1975). Neurons most frequently affected by hypoxia-ischaemia encephalopathy include neurons present in the CA1 region of the hippocampus, deeper layer of the cerebral cortex, putamen, thalamus and cerebellar Purkinje cells (Rivkin, 1997). Neuronal cell death is most prominent in the cerebral cortex and in the depths of the sulci watershed brain regions. The cerebral cortex and subcortical white matter of the parasagittal and superomedial are also frequently affected. When BGT is injured there is initial neuronal loss, gliosis and hypermyelination. This is apparent from 8 months after term birth and is categorized by marbled appearance of the basal ganglia (Inder and Volpe, 2000). Focal and multifocal necrosis is also present in neonatal encephalopathy as a result of interruption of blood flow from one or multiple blood vessels.

Fetal sheep studies where only hypoxia insult alone was investigated have shown different selective patterns of injury depending on type and severity of insult. Fetal sheep exposed to a single 30 minutes exposure to ischaemic insult had strong neuronal cell loss in both parasagittal cortex and hippocampal brain regions (Williams et al., 1992). Another study revealed severe neuronal loss in the striatum with only a reduced loss of neurons within the cortex, when fetal sheep were exposed to 3 repeated events of 10 minutes ischaemia at 1 hour and 5 hours intervals (Mallard et al., 1993). Repeated occlusion of the umbilical cord also resulted in predominant striatum damage (Mallard et al., 1995a). However, single 10 minutes occlusion resulted in hippocampal rather than striatal neuronal damage (Mallard et al., 1992).

More recently, a large number of studies using different animal models of hypoxia-ischaemia have been able to reproduce a predominant gray matter infarction with substantial neuronal loss seen in human infants (Johnston et al., 2005). Neonatal models of rat hypoxia-ischaemia have demonstrated similar injury to what is seen in MRI of infants in the basal ganglia and cerebral cortex, where unilateral carotid occlusion followed by transient hypoxia results in predominant injury to the ipsilateral brain hemisphere and reduced injury to the contralateral side (Rice et al., 1981; Nakajima et al., 2000; Kendall et al., 2011a). In the mouse model of hypoxia-ischaemia, P7 mice had visible ipsilateral dorsal cortex, hippocampus, striatum and thalamus brain regions severely affected (Kendall et al., 2011a).

Hypoxic-ischaemic insult

Despite universal acknowledgement that hypoxia-ischaemia (HI) insult during the neonatal period is not the only cause of cerebral palsy and other neurological debilities; it still remains a major cause of perinatal brain damage, and the main precursor to these neurological impairments, with an incidence of 2 per 1000 live

births as well as a mortality rate of 30% (Nelson, 1988; Vannucci, 1990; Vannucci and Vannucci, 1997; Vannucci and Hagberb, 2004).

The neuropathological characteristics of hypoxia-ischaemia encephalopathy are determined by type, duration and severity of insult, as well as gestational age and metabolic status of the infant (Terrie and Volpe, 2000). There are three main different onsets of hypoxia-ischaemia. Maternal origin is associated with impaired or inadequate perfusion of maternal placenta. Placental abruption, cord prolapse and uterine rupture are also associated with hypoxia-ischaemia (Peebles and Wyatt, 2002). Additionally, ischaemic insults may originate in the fetus itself through impaired fetal oxygenation/perfusion (fetomaternal haemorrhage, fetal thrombosis) (Higgins and Shankaran, 2009; Vincer et al., 2006). Intrauterine asphyxia is the most common antecedent to neonatal hypoxia ischaemia encephalopathy (Hill and Volpe, 1981). Furthermore, neonatal patterns of injury also differ depending on maturation, as the developmental stage of preterm children differs from that of term babies, which in turn gives rise to different selective vulnerability of the different cell populations (Cowan et al., 2003). It is important to note that prognosis for both infants and children suffering from movement debilities after hypoxic-ischaemic insult is not only different between preterm and term birth, but it is also different from that of adults (Jiménez et al., 1997). In the case of neonatal hypoxia-ischaemia, there is generally a longer delay before the development of the disorder with a higher possibility of generalization (Vannucci, 1993), whereas in the adult the reduction in mobility will appear from the onset of injury and as strength improves the disorder has a tendency to stay localized. This difference in response demonstrates again a relation between age-related changes in neuroplasticity or differences in the brain's metabolic response to injury (Janvas and Aminoff, 1998).

Oxygen is a fundamental requirement for human survival as it is essential to maintain respiration, and allows regulation of cellular energetic, growth and differentiation processes. The human central nervous system (CNS) is a complex structure, which requires a constant supply of blood and oxygen in order to function. This high oxygen demand allows the brain to maintain homeostasis (King and Parer, 2000) with only a minimal capability for anaerobic metabolism (Hossman, 1994). In the event of absence or reduced oxygen availability the fetus is able to maintain a temporary degree of homeostasis through either reduction of non-obligatory energy consumption, including in cases of severe insult, suppression of neuronal activity (avoiding neuronal depolarisation) through mediation of inhibitory neuromodulators, such as adenosine (Hunter et al., 2003; Hossmann, 2006). Secondly, cells can maintain anaerobic metabolism in order to maintain high-end production of metabolites, albeit only for a short time, as theoretically, anaerobic glycolysis produces lactate and only 2 molecules of ATP, whereas aerobic glycolysis produces 38 ATP molecules. This reduced ATP production eventually leads to rapid consumption of glucose reserves, followed by severe metabolic acidosis caused by lactic acid accumulation (Locatelli et al., 2008), leading vascular tone and cardiac contractility, in an attempt to reduce blood acidity. In fact, a study by Graham et al., showed that neurological mortality and morbidity of term infants with cord pH<7.0 was 25% (Graham et al., 2008). Under conditions where both oxygen and tissue blood flow are reduced, depletion of high energy metabolites occurs rapidly and with greater consequences. However, due to the speed with which the insult is developing, this may actually represent less metabolic acidosis as there is reduced glucose availability for metabolism to lactate (Gunn and Bennet, 2009).

If this period of reduced oxygen and glucose availability is sustained for a prolonged period of time, then injury to the cells of the developing brain starts occurring. Both clinical and experimental studies of cell death following hypoxia-ischaemia have originated the seminal concept that brain cell death does not

necessarily occur during the hypoxic-ischaemic insult but rather, as an evolutionary event that precipitates a cascade of biochemical processes, which in turn lead to delayed neuronal cell death days to weeks after hypoxic-ischaemic event. Experimental studies using animal models such as the immature rodent, piglet and fetal sheep attempting to replicate hypoxia-ischaemia encephalopathy have shown the presence of a primary energy failure phase occurring during the hypoxic-ischaemic insult, followed by a recovery phase, known as “latent phase”, followed by a secondary energy failure (Williams et al., 1991; Lorek et al., 1994; Blumberg et al., 1997). These findings are similar to various human cases where a secondary energy failure occurs after transient recovery and within 6 to 15 hours after birth. This secondary phase is directly associated with neurodevelopmental impairments seen during childhood (Roth et al., 1997; Azzopardi et al., 1989). Additionally, experimental studies in the fetal sheep have shown that a single short insult leading to reduced or no neural injury may lead to increased vulnerability to later insults repeated in a similar window frame, leading to intrauterine death or exacerbation of striatal brain damage (Mallard et al., 1993; Mallard et al., 1995b).

Fetal models of hypoxic-ischaemic encephalopathy have demonstrated the fetus ability to survive without injury mild to moderate reductions in oxygen availability, from values above 20mmHg down to 10-12mmHg (Giusssani et al., 1994; Jensen et al., 1999). During late gestation, the fetal sheep goes through an initial phase of moderate bradycardia followed by tachycardia and increased blood pressure, sometimes accompanied by increase in lactate circulation. These events are followed by rapid vasoconstriction within the periphery, thus reducing blood flow to peripheral organs in favor of the heart, brain and adrenal gland (Jensen et al., 1999). This redirected increase in blood flow to the brain enables restoration of oxygen delivery and extraction, and transfer to lower frequency EEG states resulting in 20% reduction in oxygen consumption whilst maintaining normal brain oxygen consumption (Lee et al., 2009). In cases where hypoxic

insult is maintained, the sheep fetus can potentially adapt indefinitely to these conditions. Normalization of heart rate and blood pressure are achieved as well as return of normal sleep cycle. However, this requires maintenance of blood flow redistribution, which will result in reduced somatic growth (Richardson and Bocking, 1998; Danielson et al., 2005).

Mechanisms of hypoxic-ischaemic brain injury

Hypoxia-ischaemia is not a single event leading to immediate neuronal cell death, but rather an evolving process where a cascade of biochemical events lead to delayed cell death hours after insult. In fact, hypoxia-ischaemia-mediated cell death can evolve for days, possibly even weeks after resuscitation (McKinstry et al., 2002). This continuous progression of hypoxia-ischaemia injury is described below:

Acute energy breakdown and cell depolarization

In the presence of cerebral asphyxia, there is a decrease in cerebral oxidative metabolism leading to diminished cardiac output. This is followed by reduction in high-energy phosphate metabolism, instigating various injury mechanisms such as cerebral lactic acidosis and cell membrane ionic transport failure. As the Na^+/K^+ pumps stop functioning, accumulation of Na^+ , Ca^{2+} and Cl^- within the cell occurs. This calcium overload causes activation of lipases, proteases and endonucleases leading to the destruction of the cellular skeleton (Jensen et al., 2003). Rat models of neonatal hypoxia-ischaemia have shown that this reduction in ATP followed by failure of Na^+/K^+ pumps and cell depolarization is the initial step occurring during hypoxic-ischaemic damage which will lead to cytoplasmic accumulation of calcium and severe cell swelling, causing necrosis and eventual activation of multiple cascading events leading to further cell death at a later stage (Hausman et al., 2007; Brillault et al., 2008; Fatemi et al., 2009).

Glutamate release

This change in neuronal membrane voltage leads to depolarization and excessive pre-synaptic release of the excitatory neurotransmitter glutamate. This neurotransmitter is used in most synapses within the brain and is removed by perisynaptic glial glutamate reuptake pumps. However, severe hypoxia-ischaemia models have demonstrated raised levels of glutamate, as well as other neurotransmitters in the brain and cerebrospinal fluid (CSF) (Johnston et al., 2002). Anaerobic metabolism of glucose is required to provide the energy required for the perisynaptic glial uptake pumps to remove glutamate (Magistretti et al., 1999). As ischaemia reduces glucose availability, reuptake of glutamate is severely depleted leading to an overactivation of the receptors. This excitotoxicity is present in multiple brain regions, including the periolandic cerebral cortex, thalamus and putamen (Sie et al., 2000; Johnston et al., 2001). Neurons and oligodendrocyte progenitor cells are among the cells that express glutamate receptors, including N-methyl-D-aspartate (NMDA), which functions as a transmembrane calcium channel. Other glutamate receptors include α -amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) and kainate receptors (Biagas, 1990; Ferriero, 2004; Volpe, 2012), which during membrane depolarization open voltage-dependent calcium channels (Choi, 1988). Additionally, glutamate also activates metabotropic receptors, known regulators of intracellular G-protein signal cascades (Jensen et al., 2003). Overstimulation of these receptors leads to a substantial increase of calcium influx into neurons causing mitochondrial dysfunction (Kristian and Siesjo, 1996; Johnston et al., 2002). The relevance of NMDA and AMPA receptors in hypoxic-ischaemic injury has been further evidenced in studies where blockage of these receptors led to significant reduction in brain injury following hypoxic-ischaemic injury in the neonatal rat (Hagberg et al., 1994).

Inhibition of protein synthesis

Acute disruption in energy metabolism can hinder protein synthesis. Animal studies looking at monkey brain resuscitation after 1 hour of global ischaemia showed that protein synthesis is not only reduced during the ischaemic insult, but also during the early post-ischaemic phase in both vulnerable and non-vulnerable brain regions (Kleihues et al., 1975). Non-vulnerable brain regions are capable of returning to pre-ischaemic levels of protein synthesis, however, in vulnerable brain regions this inhibition remains (Bodsch et al., 1985). Biochemical studies together with electron microscopy have shown that continuous post-ischaemic inhibition of protein synthesis is associated with disaggregation of polyribosomes from the mRNA molecule. This occurs in the post-ischaemic period, as during the ischaemic phase disaggregation is not possible due to energy metabolism breakdown affecting all stages of protein synthesis (Kleyhues et al., 1975). Therefore, during the early post-ischaemic period, reestablished energy metabolism is only capable of reinitiating the elongation and termination phases of protein synthesis, thus leading to disaggregation of polyribosomes and continuation of protein synthesis inhibition (Hossmann et al., 1992). Prolonged inhibition is therefore not only an early indicator of cell damage, but it may also be one of its contributors.

Secondary/delayed neuronal cell death

When short in duration, "primary energy failure phase" is rapidly compensated during the latent phase by cerebroprotective mechanisms, which allow redistribution and increase of brain, heart and adrenal glands mediated cardiac output (Vannucci, 1990; Jensen and Berger, 1991; Gunn et al., 1998). However, when reduction in blood gas exchange is prolonged, or following cerebral reperfusion and successful resuscitation, a secondary injury phase may occur (8-24 hours later), with a substantial increase in depletion of cellular energy reserves (ATP) associated with a rise in lactate, pH fluctuation and increase in oxidative stress (Hope et al., 1984; Penrice et al., 1997). These are followed by

epileptogenic activity, which can be supervised through EEG. Several different animal studies have demonstrated not only this biphasic evolution in injury, but also that it is during the second failure phase that the majority of cellular death occurs (Williams et al., 1991; Lorek et al., 1994; Blumberg et al., 1997), together with abnormal neurodevelopmental outcome and suboptimal head growth (Roth et al., 1992; Kendall et al., 2011a). This is likely to be a result of the presence of oxygen radicals, nitric oxide, inflammatory response and excitatory amino acids such as glutamate.

Oxygen radicals

Whereas production and release of free radicals has been shown to occur during the hypoxic-ischaemic event, it is in fact during the reperfusion period that most of these oxidative stress markers are generated. As previously mentioned, it is known that the brain has a high requirement for aerobic respiration, which signifies a higher rate of mitochondrial respiratory activity, thus potentiating the risk of free radical release from this organelle. Additional sources of reactive oxygen species include nitric oxide synthase (NOS), several steps in the metabolism of arachidonic acid, as well as compromise in pathways involving xanthine and superoxide dismutase. Furthermore, hypoxia-ischemia-mediated decrease in intracellular pH may alter binding of metals, such as iron, thus increasing this molecule's catalytic activity in the Haber-Weiss reaction (Kehrer, 2000). Brain lipids are highly enriched in polyunsaturated fatty acids (PUFAs); also many brain regions contain a high concentration of iron (e.g. striatum). This causes the brain to be highly susceptible to lipid peroxidation, destruction of cellular membrane, as well as DNA damage, degradation of protein structure and tissue deterioration (Davies and Goldberg, 1987; Helfaer et al., 1994; Shah et al., 2014).

In correlation to these findings, a neonatal rat model of perinatal hypoxia-ischaemia has demonstrated that use of allopurinol, a xanthine oxidase inhibitor

prevented severe neuronal cell loss, an indication of the significance of oxygen radicals in the development of secondary/delayed neuronal cell loss (Palmer et al., 1993). Additionally, a study looking at permanent focal ischemia in rats has shown an immediate transient increase in iron levels within the hypoxic tissue. Furthermore, this same study has also shown that iron chelation resulted in reduction of ischemic-mediated damage (Millerot-Serruot et al., 2008).

Nitric oxide

Nitric oxide (NO) is synthesised within the brain from arginine, NADPH and oxygen by NO synthase (NOs). This production is initiated by excessive glutamate release which causes coupling and activation of the NMDA receptor, allowing calcium uptake into the cells of the brain, including the thalamus and basal ganglia regions. Excessive intracellular calcium causes activation of NOs, which then produces NO, water and citrulline. Oxidative stress leads to an excessive production of NO within different brain regions. NO then combines with superoxide radicals to produce peroxynitrite (Beckman, 1991), which is quickly decomposed to form NO_2^+ , nitrogen dioxide and hydroxyl radicals. This results in mitochondrial dysfunction and permeabilization, accompanied by failure of oxidative phosphorylation (Bal-Price and Brown, 2001; Rousset et al., 2012). This NO-induced neuronal toxicity has been demonstrated in neonatal rodent models of hypoxia-ischaemia where both inhibition of NOs 1.5h before insult in a rat, as well as neuronal NOs (nNOs) deficient mice demonstrated a highly neuroprotective effect, particularly in the hippocampal and cortex brain regions (Hamada et al., 1994; Ferriero et al., 1996). Furthermore, nNOs and inducible NOs (iNOs) inhibition also improves long-term outcomes in the neonatal rat model of hypoxia-ischaemia (van den Tweel et al., 2002).

Epileptogenic activity

A study by Gluckmant et al. has shown that development of epileptic seizures occurs within hours after hypoxic-ischaemic insult, and is associated with aggravation of brain injury. Mature sheep fetuses showed epileptogenic activity 8 hours post 30 minutes cerebral ischaemia, with a peak 10 hours after insult. These seizures were completely inhibited by using a glutamate antagonist (MK-801), and this was associated with visible reduction in brain damage (Williams et al., 1991; Tan et al., 1992). Therefore, it is suggested that excessive release of glutamate as well as imbalance between inhibitory and excitatory neurotransmitters after cerebral reperfusion may cause neurons to fire in an abnormal manner leading to impairment of energy metabolism and subsequent lead to delayed/secondary neuronal cell loss (Jensen et al., 2003).

Necrosis

Necrosis form of cell death following severe hypoxic-ischaemic insult occurs as a result of extensive damage caused to organelles such as mitochondria. In this process, loss of membrane integrity combined with extravasation of cytoplasmic contents into the extracellular matrix (Jensen et al., 2003).

Apoptosis

Despite necrotic cell death being the prominent form of cellular degeneration immediately after severe cerebral insults due to rapid loss of mitochondrial function and cessation of ATP production, animal studies have demonstrated that apoptosis is in fact, the predominant mode of cell death during the delayed phase of injury, where release of cytochrome c activates proteases and caspases causing DNA fragmentation (Chung et al., 1998; Northington et al., 2001b; Ferriero, 2004). The apoptotic process is a programmed form of cell death which requires time, energy and transcription and translation of new genes. One of the ways in which apoptotic programmed cell death occurs following hypoxia-ischaemia, is the intrinsic pathway where mitochondrial cytochrome c is released

through the permeability transition pore controlled by the anti-apoptotic Bcl-2 proteins. Cytochrome c forms a complex with Apaf-1 and caspases-9 in the cell cytosol, leading to activation of caspases-9 between 3 to 24 hours after insult, which is then followed by activation of caspase-3 6 to 48 hours after injury (Nakajima et al., 2000; Gill et al., 2002). Caspase-3 is a protease and cleaves critical proteins as well as triggering apoptotic execution by activating downstream caspases and endonucleases (Taylor and Colgan, 1999). Cell fragments are then 'shrink/wrapped' within the contracting plasma membrane becoming apoptotic bodies, which in turn are phagocytosed by surrounding health cells (Tominaga et al., 1993). A rat study of neonatal hypoxia-ischaemia has shown that blockade of caspases provides significant neuroprotection in this particular form of injury (Cheng et al., 1998).

Cytokine stimulation is part of the inflammatory response to hypoxia-ischaemia and is causal to activation of cell death signaling programs (Blomgren et al., 2007). Cell surface receptors including the tumor necrosis factor receptor superfamily (TNFRS) belong to this group of cytokine-responsive receptors (Collette et al., 2003). TNF α is a major extrinsic mediator of apoptosis, where the binding of TNF α to its receptor TNFR1 leads to caspases activation via Fas-associated death domain protein (FADD) and TNF receptor associated death domain (TRADD). Fas death receptor, a TNFRS member regulates apoptosis involving activation of caspases-8 and subsequent caspase-3 activation (Le et al., 2002). Additionally, a neonatal mouse model of caspase-3-deficiency has demonstrated hypoxia-ischaemia-mediated activation of Fas death receptor (Northington et al., 2001a).

Non-caspase-associated cell death is also present in neonatal encephalopathy. The nuclear enzyme poly (ADP-ribose) polymerase (PARP-1) functions by transferring the ADP ribose groups from NAD⁺ to nuclear proteins, thus facilitating DNA repair (Fatemi et al., 2009). This enzyme is over-activated

following adult hypoxic-ischaemic insult and contributes to neuronal death by nuclear translocation of apoptosis inducing factor, causing large-scale DNA fragmentation (Andrabi et al., 2008). A neonatal mouse study of hypoxia-ischaemia, where *Parp-1* gene was deleted showed through histological assessment 10 days after injury that knockout male animals had moderate, but significant protection, however this was gender-related, female knockout mice did not appear to be protected against hypoxic-ischaemic insult (Hagberg et al., 2004).

Autophagy

Autophagy is characterized by degradation of cellular organelles by lysosomes, thus allowing the cell to degrade and recycle its own cytoplasmic contents (Kionsky and Emr, 2000). Adult mouse studies have shown an association between autophagy and hypoxia-ischaemia. Deletion of the autophagy-related protein 7 (*atg7*) gene in mice has resulted in near complete protection in these mice from hypoxic-ischaemic insult (Koike et al., 2008). However, induction of autophagy in neonates has been shown to have some endogenous neuroprotective effect (Carloni et al., 2008). Rapamycin administration prior to hypoxic-ischaemic insult has shown to augment the expression of beclin 1, a Bcl-2-interacting protein required for autophagy. This up-regulation has led to reduction in necrotic cell death and brain damage (Carloni et al., 2008).

Mechanisms of HI injury
<i>Primary Energy Failure</i>
<ul style="list-style-type: none"> • Decline in CBF, O₂ substrates and high-energy phosphate compounds
<ul style="list-style-type: none"> • Initiation of neurotoxic cascade
<ul style="list-style-type: none"> • Reduction of membrane homeostasis leading to calcium influx, mitochondrial dysfunction, brain acidosis, apoptosis and necrosis
<i>Latent phase</i>
<ul style="list-style-type: none"> • Normalization of oxidative metabolism
<i>Secondary Energy Failure</i>
<ul style="list-style-type: none"> • Continuation of neurotoxic cascade
<ul style="list-style-type: none"> • Inflammatory response (microglia)
<ul style="list-style-type: none"> • Caspase activation
<ul style="list-style-type: none"> • Decrease in levels of protein synthesis and growth factors
<ul style="list-style-type: none"> • Continuation of apoptosis and necrosis

Table 1.4: Summary of pathophysiological cascading mechanisms involved in HI injury to the immature brain (adapted from Shankaran, 2012).

Infection/inflammation

Although infection/inflammatory signals can affect the neonatal brain directly, recent research and clinical evidence have demonstrated that prenatal maternal pyrexia and/or intrauterine infections (including sub-clinical), are not only causal to cerebral palsy, but may also triple its risk. Congenital infections are considered to be a result of toxicity to the fetal brain caused by cytokine production as part of the inflammatory response (Peebles and Wyatt, 2002). Maternal to fetal infection are transmitted either across the placenta during fetal development (preterm), or during labor and passage through the birth canal (perinatal). Bacterial infection of both maternal and fetal origin leads to activation of pro-inflammatory cytokines, which play a critical role in inducing preterm labor and neonatal encephalopathy (Duggann et al., 2001). Despite only a small percentage of fetuses having demonstrable infection caused by chorioamnionitis, it is

associated with initiating labor of over a third of preterm births. Furthermore, chorioamnionitis has a risk factor for cystic periventricular leukomalacia, and a fourfold increase risk factor for cerebral palsy (Goldenberg et al., 2000; Wu, 2002).

Lipopolysaccharide

Lipopolysaccharide (LPS) endotoxin is a major component of Gram-negative bacteria outer membrane, and has been used extensively in different animal models as a means of inducing a robust inflammatory response via circulating monocytes/neutrophils and tissue macrophages, triggering secretion of cytokines through a cascading series of mechanisms (Hagberg and Mallard, 2005). The initial inflammatory response to LPS occurs upon its binding with the serum proteins LPS-binding protein (LBP) or sepsins. This complex results in activation of different cell populations by binding to its receptor complex CD14, toll-like receptor 4 (TLR4) and the extracellular adaptor protein MD-2 (Hagberg and Mallard, 2005). The TLR4 receptor has now being recognized as the signal-transducing receptor for LPS (Calvano et al., 2003). Transduction through TLR4 induces an intracellular cascading event, including mitogen-activated protein (MAP) kinase activation, together with translocation of nuclear factor- κ B (NF- κ B), followed by transcription of inflammatory genes, numerous pro-inflammatory cytokines including IL1, IL6 and TNF α , and anti-inflammatory cytokines such as IL10 and TGF β 1. TLR4-mediated LPS signal induction occurs via two distinct signaling cascading pathways, the myeloid differentiation primary response protein 88 (MyD88)-dependent and MyD88-independent pathways. Classical induction of inflammatory cytokines such as TNF α and IL-1, as well as chemokines and cell adhesion molecules such as E-Selectin, P-Selectin and ICAM-1 are thought to occur via the MyD88-dependent pathway following NF- κ B translocation to the cellular nucleus (Lehnhardt et al., 2003; Yamamoto et al., 2003; Hagberg and Mallard, 2005; Wang et al., 2008; Dean et al., 2010; Kendall et al., 2011b), whereas the MyD88-independent pathway is considered to

regulate IFN-inducible genes including IP-10 (Yamamoto et al., 2003; Dean et al., 2010).

Animal studies using LPS exposure study have reproduced focal and diffuse white matter injury, which closely resembles the clinical injury pattern of preterm infants (Hagberg et al., 2002; Mallard et al., 2003; van de Looij et al., 2012). Both LPS administration intraperitoneally in immature animal models and in maternal infection models have consistently induced up-regulation of pro-inflammatory cytokines. In the latter model, LPS crosses the placenta and prompts activation of pro-inflammatory cytokines in the neonatal brain (P8) white matter. Additionally, maternal LPS administration decreases myelin basic protein, alters microglial immunoreactivity and increases glial fibrillary acidic protein (GFAP)-positive astrocytes (Cai et al., 2000). Other animal studies have also been able to recreate patterns of injury more resembling the damage seen on term babes, including high levels of damage to the cortex, hippocampus, striatum and thalamus (Kendal et al., 2011b).

Synergy

Continuous study of the cerebral palsy aetiology has led to the now widely accepted suggestion of a synergistic interaction between infection and inflammation and other types of damage such as trauma, neurodegenerative disease and hypoxic-ischaemic insult as one of the causal to perinatal brain injury (Peebles and Wyatt, 2002). Furthermore, the combined exposure of the neonatal brain to infection and hypoxia-ischaemia dramatically increases the risk of spastic and spastic quadriplegic cerebral palsy (Grether and Nelson, 1997). Additionally, a case study has specifically demonstrated that both chorioamnionitis and hypoxia-ischaemia brain injury had been present in children suffering from cerebral palsy (Wu et al., 2003). Also, increased cytokine expression has been found in cerebrospinal fluid of patients with neonatal encephalopathy, and

therefore, it is possible that these cytokines may have had a role in the aetiology of cerebral palsy (Aly et al., 2006).

Experimental animal models have consistently been used to assist in understanding the mechanisms involved in perinatal brain damage, and subsequently cerebral palsy. Different animal models where LPS is administered either systemically (Eklind et al., 2001; Yang et al., 2004) or intracisternally (Coumans et al., 2003) before unilateral carotid occlusion and hypoxic induction directed an exacerbation of inflammation and tissue loss in comparison to either LPS or hypoxic-ischaemic insult alone (Eklind et al., 2001; Kendall et al., 2011b). Interestingly, both endotoxin sensitization and hypoxia-ischaemia are capable of raising the concentration levels of pro-inflammatory cytokines IL-1, IL-6 and TNF α alone, which have been implicated in numerous studies to be part of the pathway causal to neonatal encephalopathy. Therefore, it is possible that this shared inflammatory mechanism may be partially explanatory to this synergistic effect (Peebles and Wyatt, 2002). However, the mechanism by which this synergistic interaction occurs still remains largely unknown.

A study by Clancy et al. using histological and functional multivariate analysis from multiple brain regions across different mammalian species has enabled comparison to human early postnatal brain development, thus giving credit to the continual use of the P7 rodent model of hypoxia-ischaemia as a representative of the human neonate at term (Clancy et al., 2001). Carotid occlusion followed by timed continuous hypoxia leads to 90% of animals demonstrating visible brain injury, with 56% also showing infarct (Ride et al., 1981). Observation of the mouse brain neuroanatomy reveals damage to the ipsilateral cerebral cortex, hippocampus, striatum, thalamus and subcortical and periventricular white matter (Roohey et al., 1997). Mild combination of hypoxic-ischaemic insult with LPS bacterial endotoxin has demonstrated a significant increase in perinatal brain damage (Kendall et al., 2011b).

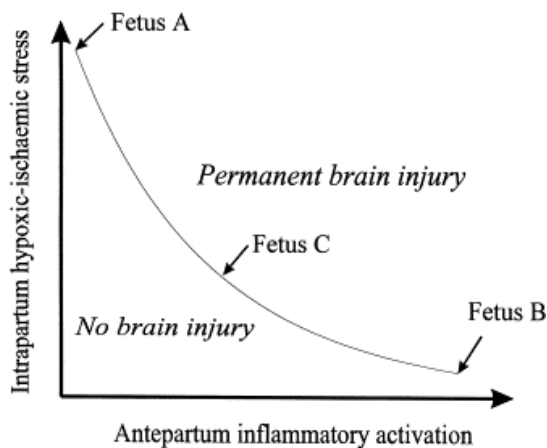


Figure 1.2: Demonstrative graph of the possible relationship between infection and hypoxia-ischaemia. Fetus A can withstand substantial hypoxic-ischaemic stress before sustaining brain damage; fetus B will develop perinatal brain damage following antepartum inflammatory event, despite reduced intrapartum stress; Fetus C is vulnerable to hypoxic-ischaemic stress, however may not develop brain damage if hypoxic-ischaemic stress is not increased, nor risk of infection/inflammation (From Peebles and Wyatt, 2002, reproduced with permission).

Mechanisms of inflammation in the brain

Inflammation is considered both a protective and detrimental response to the aforementioned types of insult, with acute inflammation being an integral part of the immune response. Furthermore, injury to neurons leads to a rapid change in their gene expression with stimulation of astrocytes and microglia activation and aggregation for survival support (Raivich et al., 1999). Neuroglial activation is a graded response accompanied by production of pro-inflammatory cytokines, activation of vascular endothelial cells and recruitment of peripheral cells of the immune system into the damaged brain. This response has been clearly observed in hypoxia-ischaemia, infection, autoimmune disorders, neurodegenerative and cardiovascular diseases (Norenberg, 1994; Kreutzberg, 1996; Raivich et al., 1996). In the immature brain, astrocytes and microglia are involved in regulation of viability, function and differentiation of progenitor cells. However, when activated under pathological conditions, these glial cells also mediate a cytotoxic effect on oligodendrocytes, by production of pro-inflammatory cytokines (Pang et al., 2000).

Blood-brain barrier

The blood-brain barrier (BBB) is an integral part of the neurovascular unit, and is formed by non-fenestrated capillary endothelial cells connected by tight

junctions, The tight junctions are formed at an early stage during fetal brain development (Baburamani et al., 2012). The endothelial cells regulate diffusion of molecules into the CNS by both restricting hydrophilic molecules and allowing diffusion of hydrophobic molecules. Additionally, metabolic products such as glucose can be actively transported across the BBB. The BBB is surrounded by a basal membrane, pericytes and astrocyte foot processes (Ahmad et al., 2012; McAdams and Juul, 2012). Astrocyte cells surrounding the BBB epithelial cells play an important role in providing biochemical support to these cells, ensuring a cellular link to neurons as well as participating in signal transduction pathways, tight junction formation and BBB regulation (McAdams and Juul, 2012). As the BBB is selectively permeable, it plays a significant role in separating circulating blood from the brain and maintaining CNS homeostasis.

Additionally, the neonatal BBB has different properties when compared to the adult, as the developing brain has different requirements. During neonatal development there is an additional ependymal barrier (Fossan et al., 1985), a higher transport influx of glucose (Cornford and Cornford, 1986) and amino acids (Braun et al., 1980; Tuor et al., 1992) as well as reduced entry of many compounds such as drugs and toxins through active efflux (Ek et al., 2010).

However, in the presence of environmental and physiological insults such as hypoxia-ischaemia and inflammation there is an increase in expression of cytokines, chemokines, adhesion molecules and other inflammatory mediators such as NO (Iadecola and Alexander, 2001). This inflammatory response leads to transmigration of leukocytes into the brain parenchyma. Additionally, microglial activation leads to production of pro- and anti-inflammatory cytokines, antigen presentation and release of matrix metalloproteinase (MMP), resulting in BBB breakdown and further infiltration of peripheral immune cells into the brain, and exacerbation of injury (Liu and McCullough, 2013).

Microglia

Microglial cells are the resident macrophages of the CNS, form 10-20% of total glial population and are responsible for the CNS immune response. In the normal brain they are present in a resting state with highly ramified and motile processes. Microglial receptors sense the presence of environmental changes to the brain, inducing rapid activation (Raivich et al., 1999; Raivich, 2005). Morphologically, microglial activation happens through several sequential steps, starting with retraction of microglial processes and increase in cell body size. Further activation into phagocytic microglia and subsequent migration to the injured site is dependent on the extent of damage induced to the brain (Kreutzberg, 1996).

Immediately following ischaemia, microglial cells become activated. This allows them to become phagocytic, produce pro-, anti-inflammatory cytokines and present antigen. Microglia are also capable of releasing matrix metalloproteinases (MMPs), leading to breakdown of the blood-brain barrier (Iadecola and Anrather, 2011). This allows influx of leukocytes into the brain, which further exacerbates inflammation and subsequently, brain damage. Retrospective clinical studies on postmortem neonatal brains have shown that microglial activation and aggregation is a hallmark of hypoxia-ischaemia encephalopathy. These studies have shown a dense infiltration of microglia in the dentate gyrus of the hippocampus which was not seen in infants which had died of other causes such as trauma or sepsis. (Del Bigio and Becker, 1994). TLR4, the main LPS receptor, highly expressed in microglia, and mediate endothelial activation and leukocyte recruitment to the brain (Zhou et al., 2006), thus suggesting that microglia/macrophages activation is a major source of inflammation (Lehnardt et al., 2002; Zhou et al., 2006). This activation is widely considered as highly detrimental to the brain, however, contrasting data also suggests that microglial activation inhibition does not improve outcome to the immature brain (Dean et al, 2010). Furthermore, there is an abundance of

microglia in the periventricular white matter, and prominent parts of the internal and external capsule. These are white matter tracts that are primarily affected areas in many cases of cerebral palsy, where these glial cells are thought to generate free radicals, secrete cytokines and enhance excitotoxicity (Hristova et al., 2010; Hagberg et al., 2012).

Astrocytes

Astrocytes, the predominant glial cells in the CNS, are characteristic star-shaped glial cells that provide structural support and homeostasis to the CNS. In the presence of injury, such as CNS trauma, hypoxia-ischaemia, infection, autoimmune and neurodegenerative disease, astroglia are responsible for the formation of scar tissue surrounding the areas of CNS tissue loss, and in severe cases, inhibit neuronal axonal regeneration (Norenberg, 1996; Raivich, 1999). Astrocytes are thought to be activated within minutes after injury due as a result of pro-inflammatory cytokines and reactive oxygen species being released by injured neurons and glia (Tuttolomondo et al., 2008). Astroglial role in neuronal repair after an ischaemic event include release of superoxide dismutase (SOD) (Swanson et al., 2004) and glutathione (Sims et al., 2004), as well as enhanced extra-synaptic glutamate uptake (Anderson and Swanson, 2000) and maintenance of ion gradients, including potassium (Walz, 2000). Conversely, astrocytes can also increase brain damage through production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF α (Tuttolomondo et al., 2008), which leads to exacerbation of brain ischaemic injury, as rapid increase of these pro-inflammatory cytokines lead to neuronal apoptosis (Stoll et al., 1998), increase of nitric oxide levels and inhibition of neurogenesis (Monje et al., 2003). Chemokines are also secreted by astrocyte, which in turn, attract migration of immune cells into the brain (Kim, 1996).

A clinical study on autopsies performed on hypoxic-ischaemic neonates showed the presence of white matter injury, including periventricular leucomalacia, with

a prevalence of astrogliosis of 15-40% within the white matter (Rezaie and Dean, 2002). Nonetheless, it still remains unknown whether astrogliosis present in the white matter is a direct cause to periventricular leucomalacia, or if this astrocyte activation is a result of injury to neurons and oligodendrocytes (Sen and Levison, 2006). Animal models of hypoxia-ischaemia have also shown astrocyte activation. However, this appears to be secondary to microglial activation, possibly as a response to microglial expression of the pro-inflammatory cytokine IL-1 (Ohno et al., 1995). Astrocytes also produce other pro-inflammatory cytokines (TNF α) and free radicals. An *in vitro* study has demonstrated that the additional presence of astrocytes shifts the mechanism of toxicity in the presence of LPS from NOs and peroxynitrite to a TNF α -dependent mechanism (Li et al., 2008). Conversely, astrocytes may also produce anti-inflammatory cytokines (TGF β) in PVL regions (Meng and Takashima, 1999).

Lymphocytes

It is generally accepted that lymphocyte cells have a detrimental effect in ischaemic brain damage. A study using adult Rag1 $^{-/-}$ mice - deficient in B and T cells - has shown that following middle cerebral artery occlusion, Rag1 $^{-/-}$ mice have reduced brain infarction compared to wild type controls. The same study showed that reconstitution of splenocytes in the Rag1 $^{-/-}$ animals removed the protective effect seen following stroke insult, thus indicating that these peripheral immune cells play a detrimental role mediating post-ischaemic injury (Yilmaz et al., 2006). Studies of lymphocyte infiltration to the neonatal brain have shown that in a P7 rat model of ischaemia, lymphocytes are only present for up to 96 hours in the brain following insult (Benjelloun et al., 1999). This is in concordance with clinical studies of newborns with hypoxic-ischaemic encephalopathy, showing that agranulocytes of newborns are rather undifferentiated, with low surface markers expression levels (Wang and Lu, 2008). However, a study by Hagberg et al. has shown infiltration of CD4 $^{+}$ lymphocytes 7 days after hypoxic-ischaemic insult. Furthermore, this study

showed that these lymphocytes persisted in the area of damage for up to 35 days. However, it is not known if this immune response is exacerbating damage or enhancing repair (Bona et al., 1999). However, there are still few studies investigating the role of lymphocytes in neonatal hypoxia-ischaemia, and how these cells may be involved in a more severe immune inflammatory response following this particular type of insult, or in the synergistic model of infection and hypoxia-ischaemia.

Cytokines

Both pro and anti-inflammatory cytokines and their receptors are present in the brain and cerebrospinal fluid, and act as part of the CNS inflammatory response to adverse stimuli (Huleihel et al., 2004). In fact, it is believed that cytokines work as a final common pathway to injury from a number of varying insults, including the ones previously mentioned.

In neonatal encephalopathy and cerebral palsy cases, the high levels of pro-inflammatory cytokines in the fetal brain can also be originated peripherally, i.e., from other cells of immune lineage such as T lymphocytes and natural killer (NK) cells, or even from the maternal uterus and placenta, following intrauterine infection/inflammation, as the blood-brain barrier can be permeable to cells of the immune system. Elevated levels of IL-6 and TNF α have been found in the amniotic fluid of pregnant women with chorioamnionitis (Greig et al., 1993). Nevertheless, not all cytokines that are present at high levels in the maternal uterus are also upregulated in the fetal brain (Dammann and Levinton, 1997; Nelson et al., 1998; Saliba and Henrot, 2001).

The early-response IL-1, IL-6 and TNF α cytokines are believed to be influential in the progression of injury in the neonatal brain via further stimulating the synthesis of other cytokines and adhesion molecules, and prompting leukocyte infiltration, the latter prompting recruitment of more immune cells to the site of

inflammation, and inducing neuronal injury mediators such as NOs. This continual and progressive stimulus has influencing modulatory effect on glial gene expression, activating microglia and astrocytes. Depending on the extent of this cytokine-mediated cytotoxic inflammatory cellular activation, cell damage and subsequent death occurs (Yoon et al., 1996; Gomez et al., 1998; Raivich et al., 1999; Duggan et al., 2001; Volpe et al., 2001; Huleihel et al 2004). Additionally, IL-1 neurotoxic effect following hypoxic-ischaemic encephalopathy has been clearly shown in a combined model of maternal endotoxin and neonatal hypoxic-ischaemic (P1) encephalopathy study, where there was a clear up-regulation of IL-1, particularly in the areas of brain damage (Girard et al., 2008). Furthermore, IL-1 receptor antagonist administration in 7 day old rats has shown to be neuroprotective following hypoxic-ischaemic insult (Hagberg et al., 1996). Conversely, the anti-inflammatory IL-10 cytokine has presented a neuroprotective effect in both stroke and head trauma injuries, thus suggesting potential therapeutic effects for using anti-inflammatory cytokine modulation in hypoxic-ischaemic encephalopathy (Saliba and Henrot, 2001).

TNF family of pro-inflammatory cytokines

The tumor necrosis factor (TNF) family of cytokines contains over 20 members, however, the actual TNF gene cluster is 12kb long and is located inside the major histocompatibility complex locus. It encodes for only three ligands, tumor necrosis factor alpha (TNF α), lymphotoxin alpha (LT α), and lymphotoxin beta (LT β). These three genes are structurally and functionally highly related (Kuprash et al., 2002; Giles et al., 2011b).

TNF α was discovered due to its anti-tumor properties seen in mice (Carswell et al., 1975) and is the most widely studied TNF cluster member. From an early period, studies have demonstrated a dual role for this cytokine. Use of anti-cachectin/TNF monoclonal antibodies 2 hours prior to bacterial challenge prevented death in a baboon model of sepsis (Tracey et al., 1987). Conversely,

mouse studies have shown an apparent protective effect for TNF α immune response against *Mycobacterium tuberculosis* infection (Flynn et al., 1995; Bean et al., 1999). Although TNF α is produced mainly by activated macrophages, this cytokine has also been shown to be produced by other cells such as neurons, microglia, astrocytes, lymphocytes, dendritic cells and neutrophils (Liu et al., 1994; Grivennikov et al., 2005). TNF α is expressed mainly as a 26-kDa transmembrane protein that can be cleaved and release a 17-kDa soluble form of TNF α cytokine (Idriss and Naismith, 2000). TNF α 's primary role is thought to be regulation of immune cells and it is involved in cellular proliferation and differentiation, necrosis and apoptosis (Locksley et al., 2001). TNF α has been shown to interact with two different receptors: TNF receptor type 1 (TNFR1) and TNFR2. TNFR1 is expressed in most tissues and contains a death-domain region (Tartaria et al., 1993).

Both LT α and LT β production expression is limited to activated lymphocytes, NK cells (Ware et al., 1992). LT α lacks a transmembrane and exists as homotrimer. When bound to LT β , a type II transmembrane protein, they form the heterotrimeric complex LT $\alpha\beta_2$, which signals through LT β receptor (LT β R) (Crowe et al., 1994). The binding of LT $\alpha\beta_2$ to this ligand is essential for the development of Peyer's patches and lymph nodes, organization of the spleen's white pulp (deTogni et al., 1994; Alimzhanov et al., 1997), expression of lymphoid chemokines (Ngo et al., 1999) and recruitment of NK and dendritic cells (Ito et al., 1999).

This gene cluster is a known mediator of septic shock, and up-regulation of its members is a fundamental modulator of severe CNS damage associated with neuronal cell loss (Raivich et al., 1999; Loddick and Rothwell, 1999). These molecules have consistently shown significant detrimental impact on neuronal survival in the presence of neural injury (Raivich et al., 2002). Nelson et al., (2005) has demonstrated that single nucleotide polymorphism in LT α is

associated with development of cerebral palsy. In a mouse model of hypoxia-ischaemia by Kendal et al., deletion of the entire TNF gene cluster abolished the LPS sensitization of neonatal brain to hypoxic-ischaemic insult (Kendal et al., 2011b). The damaging clinical effects seen by the activation of this pro-inflammatory cytokine cluster, combined with the scientific knowledge that in an animal model its deletion removes such cytotoxic inflammatory response further implicates these cytokines in the mechanism of perinatal brain damage.

Neuroprotection

Current clinical therapies – hypothermia

Following initial resuscitation and stabilization, treatment of neonatal encephalopathy is largely supportive, focusing on maintaining ventilation, perfusion and careful fluid management, preventing hypoglycemia or hyperglycemia, and avoidance/treatment of seizures (Shankaran, 2002; Stola and Perlman, 2008). Despite scientific and medical advances, there is still very little else that can be done in terms of therapy, with mild/moderate hypothermia being the only clinically available treatment for infants with hypoxia-ischaemia encephalopathy (Azzopardi et al., 2008).

Mild hypothermia is so far the most successful clinical treatment used in term neonatal encephalopathy, however its success is still moderate (Gluckman et al., 2005). Several experimental studies have shown the efficacy of therapeutic hypothermia when applied 3-4°C below baseline temperature and initiated within few hours – no later than 6 hours – after hypoxic-ischaemic event. However a therapeutic window for the use of hypothermia has not yet been identified, with different studies using varied duration of hypothermia treatment from as little as 3 hours and up to 72 hours, which have shown varying degrees of successful reduction in brain damage (Busto et al., 1987; Colbourne and Corbett, 1994; Thoresen et al., 1996; Johnston et al., 2011). The exact mechanisms by which hypothermia prevents exacerbation of neonatal brain

damage are not yet fully understood (Drury et al., 2010), but it is widely accepted that therapeutic hypothermia reduces brain metabolism, thus inhibiting release of oxygen free radicals and accumulation of cytotoxic free radicals, inhibition of inflammatory cascade, and reduction of necrosis and apoptosis cell death (Shankaran, 2012) (see table 1.5).

Multiple clinical trials, including CoolCap and TOBY looking at death and disability at 18-24 months, have also demonstrated visible reduction in mortality and morbidity in babies treated with hypothermia when compared to normothermic babies (Gluckman et al., 2005; Shankaran et al., 2005; Azzopardi et al., 2009; Simbruner et al., 2010; Jacobs et al., 2011).

Mechanisms of therapeutic hypothermia
• Reduction of brain metabolism , prevention of edema
• Decrease of energy requirement
• Reduction of nitric oxide and cytotoxic amino acid accumulation
• Inhibition of inflammatory cascade
• Suppression of free radicals activity and lipid peroxidation
• Amelioration of secondary energy failure
• Reduction of necrosis and apoptosis
• Amelioration of brain damage

Table 1.5: Summary of mechanisms involved in therapeutic hypothermia (adapted from Shankaran, 2012).

Pre-clinical treatments: future potential

Unfortunately, therapeutic hypothermia is not sufficient to treat babies at risk of cerebral palsy and other neurological deficits, as it only provides a certain degree of protection, with 11% reduction in mortality and morbidity (Robertson et al., 2012), highlighting the need for identification of other therapeutic agents are required that can be used either alone, or in combination with hypothermia. Furthermore, several studies investigate various pharmacological and non-pharmacological agents that, according to their mechanisms of action, could be used at different time points in the hypoxic-ischaemic event.

Primary neuroprotection – preventing damage

There are currently no clinically established interventions that can be given prior to the hypoxic event, or prevention of its neurotoxic cascade, which could assist in preventing or ameliorating subsequent brain damage.

Allopurinol – hypoxia-ischaemia leads to the damaging release of free radicals such as hydroperoxide, hydroxyl radical, peroxyxynitrite and superoxide radical. The enzyme xanthine oxidase is considered a primary source of superoxide, and therefore, its inhibition could potentially provide a degree of neuroprotection. Allopurinol has a free radical scavenger property and is a xanthine oxidase inhibitor. When used in high concentrations, allopurinol has shown to also scavenge hydroxyl radicals and prevent free radicals formation (Kelen and Robertson, 2010). In the neonatal rat, allopurinol administration 15 minutes after hypoxia-ischaemia has led to a reduction in cerebral oedema and long-term brain injury (Palmer et al., 1993).

Magnesium – magnesium sulfate is an NMDA receptor antagonist, therefore preventing excitotoxicity via inhibition of excessive calcium influx into the cell (Spandou et al., 2007). Magnesium has also been associated with direct action on mitochondrial activity, anticonvulsant properties and increase in cerebral blood flow (Enomoto et al., 2005). A randomized, controlled trial of magnesium sulfate, where the fetus of women at risk of preterm delivery was exposed to magnesium, has shown a reduction in the risk of cerebral palsy among survivors (Rouse et al., 2008). A second study by Doyle et al., has shown that antenatal magnesium sulfate administration to women at risk of preterm birth, has again showed neuroprotection and reduction in neuromotor disorders (Doyle et al., 2009).

Melatonin – melatonin, a neurohormone secreted from the pineal gland, has shown to significantly reduce infarct volume. This is considered to be a result of its ant-oxidant property, as melatonin has shown to reduce infarct volume and levels of oxidative stress without affecting blood-brain barrier integrity in adult rat models of middle cerebral artery occlusion (Sinha et al., 2001; Pei et al., 2003). In fetal rat models of bilateral utero-ovarian artery occlusion, melatonin has also shown a neuroprotective effect with reduction of oxidative mitochondrial dysfunction within the brain (Wakatsuki et al., 2001). Melatonin is also neuroprotective in the neonatal mouse (Husson et al., 2002), however, more recent studies have shown that melatonin protection may also be due to inhibition of inflammatory response following hypoxia-ischaemia (Pei and Cheung, 2004). Sheep hypoxia-ischaemia studies have shown the beneficial effect of melatonin administration both directly into fetus and into the mother (Miller et al., 2005; Welin AK et al., 2007). Furthermore, melatonin also reduced brain injury in a study using LPS-sensitization prior to hypoxia-ischaemia. This study showed that another free radical scavenging agent, **N-acetylcysteine (NAC)** provides a higher degree of neuroprotection in this synergistic mode of injury (Wang et al., 2006). However, NAC has been associated with adverse reactions in humans, thus limiting its use in humans (Heard, 2008), whereas melatonin shows minimal toxicity in humans (Lin and Lee, 2009).

Xenon – this is a noble gas occurring in Earth's atmosphere. Xenon is not only a safe anesthetic, but a study by Franks et al. has shown its role as a neuroprotective agent, via inhibition of the excitatory NMDA receptor channels (Franks et al., 1998). Additionally, recent studies have shown that xenon reduces isoflurane-associated cell death (Ma et al., 2007). A study where laboring rats were exposed to xenon four hours prior to intrauterine perinatal asphyxia revealed that the pups had a reduction in c-Fos expression in the dorsal horn following Morris water maze test at P50 (Yang et al., 2012).

Secondary neuroprotection – therapeutic window

Experimental animal studies have identified that hypoxia-ischaemia encephalopathy is a biphasic brain energy failure form of insult, where delayed neuronal cell death accounts for the larger part of cell death in this type of injury. Therefore, therapeutic strategies targeting this latent window period could possibly prevent or ameliorate secondary energy failure. Hypothermia has already been established as a clinical standard treatment used to prevent secondary energy failure. Other treatment candidates include the aforementioned melatonin, xenon and allopurinol, as well as erythropoietin.

Erythropoietin – studies using erythropoietin (EPO) after hypoxic-ischaemic insult have demonstrated its efficacy as a neuroprotective agent. This glycoprotein hormone is known to not only control erythropoiesis, but also, to modulate inflammatory response, as well as having vasogenic effects (Gonzales and Ferriero, 2007). Administration of erythropoietin following hypoxic-ischaemic insult leads to increase in oligodendrogenesis, thus attenuating white matter injury and promoting neurogenesis (Iwai et al., 2010). Erythropoietin's neuroprotective effect is thought to occur through a decrease in susceptibility to glutamate toxicity together with stimulation of anti-apoptotic factors and reduction in inflammation and NO-mediated injury (Palmer et al., 1999). Furthermore, a randomized controlled study using 167 infants revealed that low-dose erythropoietin administration (300 or 500 U/Kg) was deemed safe and resulted in improved neurological outcome in sufferers of moderate and severe neonatal encephalopathy at 18 months of age (Zhu et al., 2009).

Tertiary neuroprotection – post-injury recovery

Despite prevention of perinatal brain damage being the most desired for of treatment, promotion of plasticity and repair are also important aspects in neuroprotection, as quite often there is an absence of early markers of perinatal brain damage. Endogenous capacity in the neonatal brain is reduced following

hypoxic-ischaemic injury; however recent studies have suggested the use of stem cell transplantation to ameliorate brain damage (Velthove et al., 2009). Additionally, use of mesenchymal stem cells have also been suggested, as these cells are able to secrete several trophic factors, including colony stimulating factor-1 (CSF-1), vascular endothelial growth factor (VEGF) and brain derived neurotrophic factor (BDNF) (Rivera et al., 2006; Wei et al., 2009).

Combined therapies

The use of adjuvant therapies to hypothermia treatment has been extensively studied, with some of the most promising neuroprotective agents including melatonin and xenon. Melatonin has been shown to augment hypothermia protection in a piglet model of perinatal asphyxia. Results of this study showed that treated animals had improved cerebral metabolism and reduced cell death (Robertson et al, 2013). Another piglet model of hypoxia-ischaemia showed that, 50% xenon started 2 hours after insult improved brain neuroprotection when used in conjunction with hypothermia (Chandrasekaran et al., 2010). In a neonatal rat hypoxic-ischaemic study, using both hypothermia and xenon at 40% concentration or greater, has shown a synergistic neuroprotective effect, with improved neuromotor function at 30 days of age, thus suggesting a long-term protection role (Ma et al., 2005).

It is important to note that most of these treatments are only considering the hypoxic-ischaemic spectrum, and not the added damage caused by infection. It is widely accepted that there is a low prevalence of neonatal encephalopathy to a higher occurrence of maternal intrapartum pyrexia, which in turns makes a randomized controlled trial to determine a neuroprotective effect in maternal cooling difficult due to high recruiting numbers required. However, this matter does need to be addressed, as this is a relatively common obstetric complication (Giles and Donald 2005).

Concluding remarks

It is now evident that part of the multifactorial aetiology of neonatal encephalopathy is attributed to the modulating effect of infection to subsequent hypoxic-ischaemic insult. Furthermore, different synergistic animal models have been able to consistently reproduce the brain injury patterns associated with term neonatal encephalopathy. Unfortunately, despite advances in medical care and scientific research, the number of cerebral palsy sufferers has remained constant throughout the last 20 years. Therapeutic hypothermia is so far the only clinically accepted treatment; however, its success is only moderate. In terms of experimental therapies, a lot of different pharmacological and non-pharmacological agents are being tested and showing promising results. However, there has been a constant increase in shift in this research, where a number of research groups acknowledging that optimizing hypothermia with adjuvant agents hold the highest promising in preventing or ameliorating perinatal brain damage.

AIMS OF THE PROJECT

As outlined, perinatal brain damage is one of the main precursors to cerebral palsy and other neurological disorders. Several studies have demonstrated the involvement of infection/inflammation in the aetiology of perinatal brain damage. Furthermore, there has been growing evidence demonstrating the synergistic role of infection/inflammation and hypoxia-ischemia, where infection appears to sensitize the immature brain to subsequent hypoxic-ischemic insult, thus exacerbating neonatal brain damage and the risk of developing cerebral palsy.

Previous studies in this laboratory have demonstrated that the TNF family of pro-inflammatory cytokines consisting of TNF α , LT α and LT β play a role in the aetiology of perinatal brain injury. This original study demonstrated the up-regulation of these three cytokines following endotoxin-sensitization alone. Moreover, homozygous deletion of the whole gene cluster led to an abolishment in microglial activation and consequent inflammation in the murine model of infection/inflammation and hypoxic-ischemic insult (Kendall et al., 2011b).

Therefore, in this project we wanted to further characterize the role of the individual cytokines members of the TNF cluster and investigate which one or combination of, is mediating this LPS sensitization of the neonatal brain. For this purpose, the study was divided into three parts:

1. Test LPS-mediated sensitization in multiple background mouse strains (C57BL/6, 129SVJ, BALB/c, CD1 and FVB). As there would be a number of transgenic strains required for the study, and hypoxic-ischemic studies have demonstrated the variability of the model, we wanted to first test the efficacy of the experimental design, and see which strain

background/s would provide the optimal model of combined infection and hypoxic-ischemic insult.

2. In the second part, we wanted to identify which of the three cytokines TNF α , LT α or LT β is responsible for the LPS-mediated sensitization to subsequent hypoxia-ischemia seen previously. For that, we would use global single gene deletion for each of the three cytokines, as well as deletion of TNF α gene in myeloid granulocytes and macrophages – excluding microglial cells.
3. Originally, the third part of this study would have consisted of pharmacological inhibition of the identified-mediating cytokine. However, results on the individual cytokines deletion were dissimilar from what was observed in the TNF cluster deletion. Since the TNF cluster deletion also produces a very severe structural defect of the peripheral immune system, a new approach was made, where instead we would look at the acquired cellular immune system, and specifically, the T cell component. The aim is to see whether or not deleting a key component of the immune system would reproduce the same abolishment of LPS-mediated sensitization to subsequent hypoxic-ischemic insult seen in the TNF cluster deletion.

CHAPTER 2: GENERAL MATERIALS & METHODS

Animals

All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and approved by the Home Office. All animals used in this project were housed in the UCL Biological Services Unit following approved husbandry protocols. Experimental breeding pairs were mated in-house, and kept under standard conditions, i.e. with a 12 hour light-dark cycle, constant temperature (21-23°C) and humidity (60%±5), with access to pelleted food and water ad libitum. Several lines of wild type and genetically engineered mice were used in this study, and were sourced from a number of different laboratories and are summarised in Table 2.1.

Mouse strain	Background	Source
C57BL/6	C57BL/6	Charles River, UK
129SVJ	129SVJ	Charles River, UK
BALB/c	BALB/c	Charles River, UK
CD1	CD1	Charles River, UK
FVB	FVB	Charles River, UK
LT α global	C57BL6/129SVJ	The Jackson Laboratory, USA
LT β global	C57BL6/129SVJ	The Jackson Laboratory, USA
TNF α global	C57BL6/129SVJ	The Jackson Laboratory, USA
TNF α flox	C57BL/6	Dr Sergei Nedospasov, Bundesinstitut für Risikobewertung, Berlin, Germany
MLys CRE	C57BL/6	Dr Sergei Nedospasov, Bundesinstitut für Risikobewertung, Berlin, Germany
Nude	BALB/c	Charles River, UK

Table 2.1: Summary of wild type and transgenic mice.

3Rs: replacement, reduction and refinement

Due to the nature of this study, the use of animals defined as protected under the UK ASPA was essential. Every effort was made to comply with the 3Rs strategy of animal experimentation. Animals were mated after 6 weeks of age. Good husbandry is part of refinement of animal experimentation and therefore important to standardise: Cages were monitored on a regular basis by the BSU

staff and myself not only for signs of pregnancy, but also for signs of stress and sickness. Breeding pairs were kept to a maximum of 3 animals per cage (1 male, 2 females) to avoid crowding, as well as stamping on the litters. Once the pups were injected with either LPS or saline, the males were separated, and only returned once pups were collected for perfusion, in order to avoid cannibalism of the litters. Spare heterozygotes were kept at a maximum of 2/3 per cage, and a maximum of 1 cage with males and a second cage with females per strain. Additionally, to improve refinement and avoid boredom, all cages had housing with nesting material, one cardboard tube and one wood block. Enrichment diet was scattered throughout the bedding every Friday. Experimental groups were kept at around n=10 as this sample size was demonstrated in previous studies in our group to produce statistically significant results. Furthermore, the use of LPS endotoxin - a robust inducer of inflammation - reduced the high variability seen in the neonatal model of hypoxia-ischaemia. The only instance where more animals were used was in the NUDE study, as due to difficulty in breeding these animals together with the extra cost of maintaining such animals in IVC cages in quarantine rooms, we were unable to keep spare animals, and an extra litter was obtained from two females before the experiment was terminated, for safety measure. All experimental groups used the already established (in our lab) 48h post-insult time point, thus reducing the need for more data sets, as our research group has shown that this time point gives reliable changes in response to injury in both HI versus naïve, as well as HI+LPS versus HI+saline. For the histological assessments, 5 sections 10 sections apart were used per stain. As 50 consecutive sections were collected (see cryostat sectioning), this allowed the possible use of 10 different histological/immunohistological markers, thus avoiding the need to use extra animals. Furthermore, all markers used represented relevant questions to be addressed in this model of perinatal brain damage, once again ensuring avoidance of unnecessary using extra animals (Kendall et al., 2006; Kendall et al., 2011a; Kendall et al., 2011b).

Global gene deletion

TNF α – This mutation was developed by replacing part of the muTNF α gene, including the ATG translation initiation codon of the first exon, with a MC1neio ρ A cassette. The construct was electroporated into 129SVJ-derived embryonic stem cells (ESCs), and the correctly targeted ESCs were injected into C57BL/6 blastocysts. Resultant chimeras were consecutively crossed with C57BL/6 mice to achieve a homozygous colony (Pasparakis et al., 1996). These homozygous TNF α KO mice were obtained from Jackson Laboratory, USA. Animals were mated in-house with wild type C57BL/6 (Charles River, UK) to obtain a F1 heterozygote generation for breeding purposes. Heterozygous breeding produced F2 generation consisting of WT (controls), heterozygote and homozygote (KO) animals for the TNF α gene deletion. Heterozygous pups were not employed in this study.

LT α – Targeted mutation of the LT α gene was attained through partial replacement of LT α transcripts, introducing an earlier stop codon, with a neomycin resistant cassette. Similarly to the TNF α mutation, the targeting vector was electroporated into 129SVJ-derived ESCs, and successfully targeted ESCs were injected into C57BL/6 blastocysts. Continuous crossing with C57BL6 mice resulted in LT α homozygote generation (de Togni et al., 1994). Homozygous LT α KO mice were obtained from Jackson Laboratory, USA. As with the TNF α mutants, these animals were mated in-house with wild type C57BL/6 (Charles River, UK) to obtain the F1 heterozygote generation required for this study. Heterozygous breeding pairs produced F2 generation consisting of WT (controls), heterozygote and homozygote (KO) animals for the specific LT α gene deletion. Heterozygous pups were not included in this study.

LT β – In order to obtain LT β mutants, a 0.7 kb fragment of the gene, thus truncating the coding sequence at codon 153, was replaced with a neomycin resistant cassette. The construct was electroporated into 129SVJ-derived ESCs,

and the effectively targeted ESCs were injected into C57BL6 blastocysts similarly to the development of both TNF α and LT α mutants (Koni et al., 1997). Heterozygous LT β (+/-) mice were obtained from Jackson Laboratory, USA. These animals were mated in-house with C57BL/6 (Charles River, UK) to obtain F1 generation containing the heterozygote animals required for this study. Heterozygous breeding pairs produced F2 generation comprising of WT (controls), heterozygote and homozygote (KO) animals for the LT β gene deletion. Heterozygous littermates were not used in this study.

Athymic nude mouse – The recessive nude (nu) mutation consists of a single base pair (bp; G) deletion in the hepatocyte nuclear factor 3 (HNF-3)/forkhead homolog 11 gene (Segre et al., 1995), a member of the FOX gene family (Kaestner et al., 2000). This spontaneous mutation results in lack of thymus, the mice are unable to produce T cells, and that makes them immunodeficient. Therefore, all nude animals were kept in individually ventilated cages (IVCs) under quarantine for the duration of this study. Due to the nature of the mutation leading to the nude phenotype (single base pair deletion), pyrosequencing technology would be required to maintain a heterozygous breeding strategy. Considering that this is a recessive mutation and lacks phenotypic differences between WT and heterozygote animals, heterozygous (nu/+) females were mated with a homozygous recessive (nu/nu) male (Charles River, UK). Female homozygous nude mice are not effective breeders and fail to lactate. Experimental cohorts consisting of homozygous mutants (nu/nu) and littermate controls (nu/+) were generated with normal Mendelian frequency (50:50).

Cell-specific gene deletion

Cell-specific gene deletion animals were generated using Cre-Lox recombination. Transgenic mice carrying floxed allele for the TNF α gene were crossed with MLys::CRE +/- transgenic animals expressing cre recombinase under the myeloid-

expressing granulocyte/macrophage promoter (Clausen et al., 1999) twice in order to obtain littermates that were TNF α flox/flox MLys CRE -/- (controls) or TNF α flox/flox MLys CRE +/- (knockouts) cell-specific deletion. The loxP sites flanking the TNF α gene are recognised by MLys::CRE recombinase, which excises TNF α in MLys-expressing granulocyte/macrophages (excluding microglia) to generate homozygous cell-type specific deletion of TNF α gene.

Genotyping

Upon weaning, all transgenic mice used for breeding or colony maintenance were identified through ear-notch, and less than 0.5mm of their tail-tip biopsied for DNA extraction. Experimental transgenic pups had the tail-tips biopsied at perfusion. DNA was extracted by adding 20 μ l of 20 μ g/ml Proteinase K (Promega, UK) and 750 μ l of extraction buffer (5% 1M Tris, 20% 0.5M EDTA, 1% SDS, 3.3% 3M NaCl) to each tail and incubating at 55 $^{\circ}$ C overnight (O/N) in a rotating water-bath. Subsequently, samples were briefly vortexed and placed on ice, before being centrifuged at 16900g for 4min. 600 μ l isopropanol was added to the supernatant and the solution mixed by inversion. Samples were centrifuged for a further 1min (16900g) with resulting pellets washed with 70% ethanol (EtOH; v/v; VWR, UK) and centrifuged for further 1min (16900g). After air drying for 2h, the DNA samples were re-suspended in 50 μ l of Tris-EDTA buffer (TE; Promega, UK), incubated in a rotating water-bath for one hour at 65 $^{\circ}$ C and stored at 4 $^{\circ}$ C.

Polymerase chain reaction (PCR) DNA amplification was carried out using a 25 μ l Qiagen PCR master mix (2.5 μ l 10X buffer, 5 μ l Q solution, 0.25 μ l 25mM dNTP (Fermentas, UK), 0.125 μ l of each primer (1nM/ μ l; Invitrogen, UK; see table 2.2), 0.2 μ l Taq polymerase) and 1 μ l of DNA previously diluted to 5 μ g/ μ l in DEPC water according to DNA concentration using a FLUOstar Omega microplate reader (BMG Labtech, UK). The thermocycling conditions for each PCR were optimised on the T-Gradient PCR machine (Biometra, Germany).

PCR products were separated using 2% agarose-TAE gel electrophoresis. 1g agarose (Eurogentec) was added to 50ml 1x TAE buffer (0.4M Tris, 0.2M sodium acetate, 20mM EDTA, pH), boiled and ethidium bromide (EtBr; Invitrogen, UK) was added to a final concentration of 0.2µg/ml. PCR samples were mixed with loading dye (1x TAE, 50% glycerol, 0.025% bromophenol blue) immediately prior to well loading (10µl) and run against a 100bp DNA ladder (5µl; NEB, UK) for 40min at a constant voltage of 100V. Samples were then visualised with a UV transilluminator (BioDoc-It 220 Imaging system, UVP, UK).

Primer name	Annealing temp (°C)	Positive band	Sequence
LTα WT Fwd	60°C	229bp	5'- CCT TGT TGG TAA ACT TCT GCC -3'
LTα WT Rev	60°C		5'- AAG AGA GCA CAA GAC ATT GGG -3'
LTα MU Fwd	60°C	280bp	5'- CTT GGG TGG AGA GGC TAT TC -3'
LTα MU Rev	60°C		5'- AGG TGA GAT GAC AGG AGA TC -3'
LTβ Common	60°C		5'- CCT GTA GTC CAC CAT GTC G -3'
LTβ WT Rev	60°C	120bp	5'- GAG ACA GTC ACA CCT GTT G -3'
LTβ MU Rev	60°C	330bp	5'- CCT GTT CAA TGG CCG ATC C -3'
TNFα Common	60°C		5'- TAG CCA GGA GGG AGA ACA GA -3'
TNFα WT Rev	60°C	183bp	5'- AGT GCC TCT TCT GCC AGT TC -3'
TNFα MU Rev	60°C	318bp	5'- CGT TGG CTA CCC GTG ATA TT -3'
TNFαflox 41	60°C	Flx 400bp	5'- TGA GTC TGT CTT AAC TAA CC -3'
TNFαflox 42	60°C	WT 350bp	5'- CCC TTC ATT CTC AAG GCA CA -3'
MLys Common	60°C		5'- CTT GGG CTG CCA GAA TTT CTC -3'
MLys WT Rev	60°C	345bp	5'- TTA CAG TCG GCC AGG CTG AC -3'
MLys MU Rev	60°C	Near 1kb	5'- CCC AGA AAT GCC AGA TTA CG -3'

Table 2.2: PCR reaction primers, annealing temperature, band size and sequences. **Abbreviations:** Fwd – forward, MU – mutant, Rev – reverse and WT – wild type.

Because nude mice have a second defect, i.e., are born with functional but faulty hair growth follicles, resulting in a hairless appearance, their genotype could be inferred visually (see Figure 2.1).



Figure 2.1: Untyped Nude Mice are macroscopically differentiated at P9 prior to perfusion. Mice heterozygous (nu/+) for the nude mutation are physically larger and covered with white fur, whereas homozygous (nu/nu) mutants are smaller in size and also hairless.

Animal model

Levine first demonstrated the need to combine hypoxic and ischemic insults in order to cause significant lesion to the adult rat brain. Surviving rats showed considerable histological damage to the cerebral cortex, striatum and thalamus, with the hippocampus being the most severely affected region. However, the corpus callosum and corpus striatum white matter tracts remained largely unaffected (Levine, 1960). Rice et al (1981) then adapted this model and generated a neonatal HI model. Postnatal day 7 (P7) rats underwent unilateral carotid artery occlusion by surgical ligation (ischaemia) followed by 3.5h exposure to 8% oxygen (hypoxia). This combined HI insult resulted in grey matter damage in a manner similar to the adult rat model, as well as substantial WMI on the ipsilateral side, and occasional appearance of WM damage on the contralateral brain hemisphere.

HI surgery

Our laboratory has since been able to successfully adapt and reproduce this HI model using P7 mice, where pups are exposed for a total of either 30min (moderate) or 60min (severe) hypoxic insult (Kendall et al., 2011a). Animals were anaesthetised with isoflurane (5% induction, 1.5% maintenance, 1L/min; Abbott, UK). Following a midline incision to the neck the left common carotid artery was exposed and permanently occluded through ligation with 8/0 polypropylene suture (Ethicon, UK). The incision was closed using tissue glue. Pups were allowed to recover at 36°C before being returned to their respective dams to nurse for 1.5h. The animals were then placed in a sealed hypoxic chamber and exposed to a constant flow of humidified 8% oxygen/92% nitrogen (3L/min) at 36°C for 30min. Finally, after 10min recovery at 36°C, the mice were returned to their dams until point of sacrifice.

LPS injection

Our research group has effectively established a synergistic model of infection/inflammation and HI. Administration of LPS at 0.3µg/g 12h prior to HI (30min 8% oxygen exposure) insult resulted in a significant exacerbation of tissue loss compared to either HI or LPS alone (Kendall et al., 2011b). For the purposes of this study, a new batch of LPS was titrated. Intraperitoneal (ip) injections of 0.3µg/g, 0.6µg/g, 1µg/g and 10µg/g were administered followed 12h later by HI insult and 48h survival. Animals that received 0.3-0.6µg/g were associated with a survival rate of 6/6, 4/6 at 1µg/g and 0/6 at 10µg/g. This gave optimal sensitization dose of 0.6µg in 10µl/g BW administered intraperitoneally (titration was performed by Dr Mariya Hristova prior to the commencement of this project, see Figure 2.2) 12h prior to HI insult. All pups were randomly allocated to receive ip injections of either LPS (*Escherichia coli*, serotype 055:B5; Fluka, UK) or 10µl/g of 0.9% sterile normal saline (vehicle).

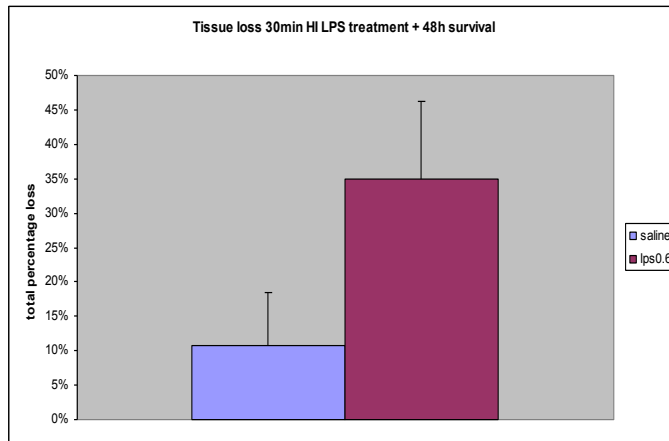


Figure 2.2: Comparison between saline- and LPS-treated (0.6µg/g) mice. Comparison between saline- and LPS-treated (0.6µg/g) mice. Saline controls (n=6) had limited infarct volume 48h post combined LPS+HI injury, whereas LPS pre-treated littermates (n=6) demonstrates a significant increase in infarct volume as demonstrated by Nissl stain used for volume measurement ($p < 0.05$). Graph reproduced with permission.

Perfusion fixation

48h post-HI animals were terminally anaesthetised with ip injection of 10µl/g BW pentobarbital sodium (Euthatal, Merial, UK). Paw withdrawal reflex was used to determine extent of anaesthesia and tail-tips collected for genotyping. A midline incision was made under the xiphoid process, exposing the peritoneal cavity. The diaphragm was incised at the nearest its junction with the anterior abdominal wall. The ribcage was cut laterally and lifted exposing the heart. A 26-gauge needle attached to a peristaltic pump (Gilson, UK) was inserted at the base of the apex of the heart and into the left ventricle. A solution of cold (4°C) 4% paraformaldehyde in 0.1M phosphate buffer (PFA/PB, pH 7.4) was pumped through the heart for 2.5min at a constant flow rate of 0.02L/min. Brains were extracted and post-fixed in 4% PFA/PB on a rotator (8 rpm) for 1h, followed by overnight rotation immersion in 30% sucrose (Fluka, UK) for cryoprotection. The forebrains were frozen on dry ice (BOC, UK) for further use (Möller et al., 1996).

Cryostat sectioning

Frozen forebrain sections were fixed onto a Leica CM 1900 cryostat chuck using optimal cutting temperature (OCT) compound (Tissue Tek, Netherlands). The chuck temperature was set at -15°C whereas the chamber temperature was set at -20°C. A small circular whole was punctured through the lower half of the contralateral hemisphere using a 26-gauge needle to allow distinction between

the ipsilateral and contralateral brain hemispheres. 50 sequential coronal sections of 40µm thickness, starting from the fusion point of the corpus callosum, were collected onto labelled 0.5% gelatinised glass slides (Thermo Scientific, UK) and stored at -80°C.

Immunohistochemistry

Brain sections were retrieved from -80°C, rehydrated in double distilled water (ddH₂O) and spread flat onto the slide under a dissection microscope using fine brushes. After fan drying for 40min at room temperature (RT), sections were circled with PAP pen (DAKO, Cambridgeshire), then incubated for 5min in 4% PFA and transferred into 0.1M PB. For antigen retrieval, sections were immersed for 2min each in 50% acetone, 100% acetone, 50% acetone, twice in 0.1M PB, and once in 0.1% bovine serum albumin/0.1M PB (PB/BSA) (Sigma, UK). The sections were then blocked in 5% goat serum (Sigma, UK) in PB for 30min at RT to match the source of the secondary antibody. The block was removed and the sections were incubated with primary antibody O/N at 4°C using dilutions obtained through previous titration.

On day 2, the primary antibody was washed from the slides by serial immersions in PB/BSA, PB, PB and PB/BSA. The appropriate biotinylated secondary antibody was pre-incubated with mouse serum (1:50; Serotec, UK) for 30min at 37°C before diluted to 1:100 in PB/BSA. The sections were then incubated with the secondary antibody for 1h at RT (see table 2.3 for list and source of antibodies used). Sections were washed by consecutive immersion in PB/BSA, PB/BSA, PB, PB and incubated for 1h at RT with Avidin-Biotinylated horse radish peroxidase Complex (ABC; 1:100; Vector, UK). The sections were washed in 4 changes of 10mM PB and the reaction was visualized with diaminobenzidine (DAB; Sigma, UK) and hydrogen peroxide (H₂O₂; Sigma, UK). This reaction mixture consisted of 0.5 g/l DAB, 1:3000 dilution of 30% H₂O₂ in PB, and allowed to react for 3-4min at RT. The intensity of the staining was monitored under a light microscope (Zeiss).

DAB reaction was stopped by immersing the slides once in 10mM PB, and twice in ddH₂O. After re-spreading and drying at RT, the sections were immersed 3x in xylene and mounted on a glass cover slip using Depex (BDH, UK).

Antigen	Antibody	Dilution	Manufacturer
Primary			
Alpha M (αMβ2 integrin)	Monoclonal Rat Anti Mouse CD11b	1:5000	Serotec, UK
Glial fibrillary acidic protein (GFAP)	Polyclonal Rabbit Anti GFAP	1:6000	Dako, UK
Secondary			
Rabbit Ig	Biot. anti-rabbit IgG, goat polyclonal	1:100	Vector, UK
Rat Ig	Biot. anti-rat IgG, goat polyclonal	1:100	Vector, UK

Table 2.3: List of antibodies used for immunohistochemistry.

Antibody specificity

The specificity raised against α M β 2 integrin has been previously confirmed in our laboratory using appropriate KO mice, where homozygous mutants demonstrated a lack of microglial α M immunoreactivity throughout the brain (Makawana et al., 2009). The GFAP antibody used in the current study is also well defined with long established functional characterization (Eng et al., 2000).

Terminal Transferase-Mediated d-UTP Nick End-Labeling (TUNEL)

Sections were rehydrated, spread and fixed as described in the above immunohistochemistry protocol. The slides were then incubated in 3% H₂O₂ in methanol (15min), and washed in 0.1M PB. The antigen retrieval followed the IHC protocol. Slides were incubated with terminal deoxytransferase (TdT) and deoxyuridine triphosphate (dUTP) solution (0.1% TdT, 0.15% dUTP, 1% cacodylate buffer; Roche, UK) for 2h at 37°C. The reaction was stopped in TUNEL stop solution (300mM NaCl, 300mM Sodium Citrate) for 10min. Slides were washed in 3x 0.1M PB and incubated for 1h at RT in ABC solution. Sections were then

washed 4 times in 10mM PB and the reaction was visualized using DAB prepared with cobalt nickel in the presence of H₂O₂ for 3-4min. Reaction was stopped and slides covered as described previously.

Nissl staining

Sections were rehydrated in ddH₂O, spread and allowed to dry completely. Slides were then fixed in 4% FA (BDH, UK) O/N followed by 70% EtOH O/N. Cresyl violet solution (1%) was prepared using 4g cresyl violet powder (BDH, UK) in 40ml 100% EtOH, shaken and inverted for 15min. This mixture was added to warm 360ml ddH₂O and mixed on a warmed magnetic stirrer plate for 20min. Once cooled, the solution was filtered O/N using Whatman size 4 filter paper. On day 3, slides were immersed in the 1% cresyl violet solution for 3min. Excess cresyl violet solution was removed by washing twice in running water. The slides were then dehydrated by consecutive immersion (2min each) in increasing concentrations of EtOH (70%, 90%, 96%, 96% with glacial acetic acid (10 drops, de-stain), 100%), isopropanol, and three washes in xylene. Slides were then covered using Depex as described previously.

Tissue Analysis

Infarct size

From each forebrain, 5 Nissl stained sections 400µm apart were photographed using x1 magnification and imported into Optimas 6.5 image analysis software (MediaCybernetics, Bothwell, WA). As cresyl violet labels both extra nuclear RNA granules and cell nuclei, the intact areas of the dorsoparietal cortex, pyriform cortex, hippocampus, striatum, thalamus and external capsule regions were delineated bilaterally using the free-hand tool. The area in pixel was obtained using the histogram tool (see Figure 2.3), and converted into mm² in Excel. The volume was calculated by multiplying the area in mm² by 400µm. These volumes were used to calculate the percentage of intact forebrain tissue as

ipsilateral/contralateral volume x 100, or infarct size using the contralateral/ipsilateral volume x 100 formula.

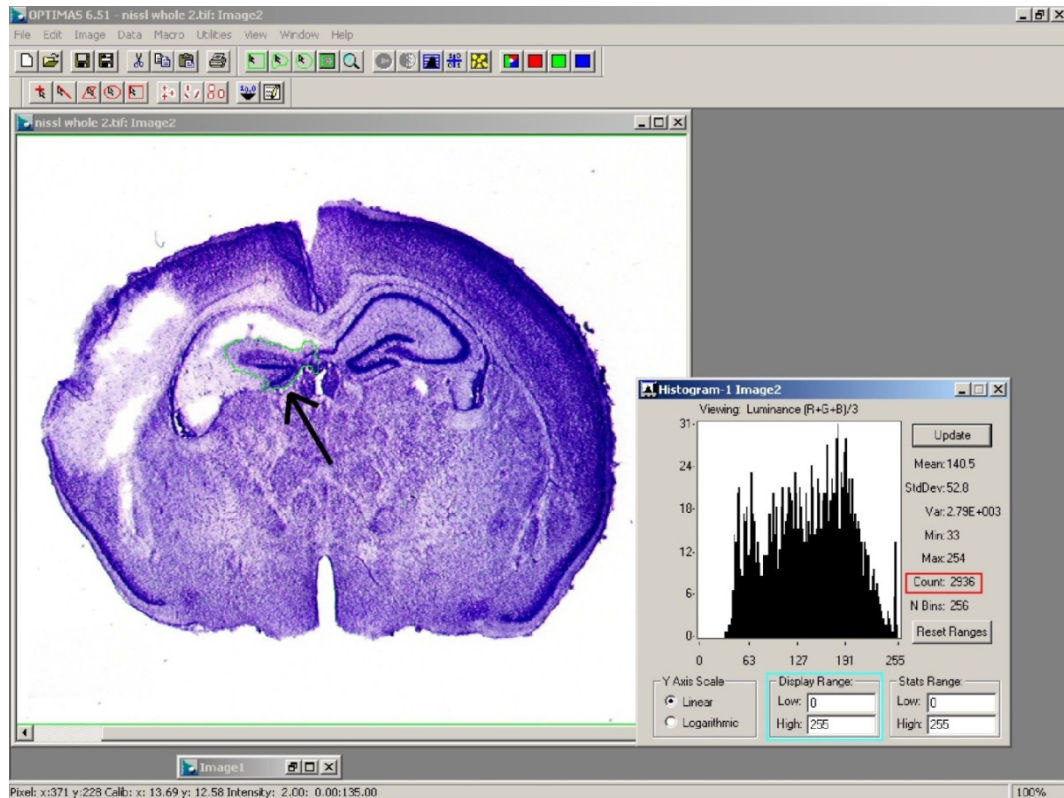


Figure 2.3: Nissl staining infarct volume measurement. The represented region of interest, ipsilateral hippocampus was delineated using the free hand tool (green delineation indicated by black arrow). Luminance histogram was reviewed to ensure a 0-255 range (blue box). Count values (red box) were transferred into an Excel spreadsheet for conversion and assessment. Contralateral hemisphere (right) appears intact following LPS-sensitization to subsequent hypoxic-ischemic insult (C57BL/6 wild type animal).

Injury score

Microglial activation (α M immunoreactivity) and neuronal cell loss (Nissl stain) were scored using a scoring system previously established in our laboratory (Kendall et al., 2006) on a light microscope at x10 magnification (see Figure 2.4). The semi-quantitative scoring system, as well as the infarct volume assessment was performed blindly in the dorsoparietal cortex, pyriform cortex, hippocampus, striatum and thalamus for both α M and Nissl stains. The external capsule was assessed only for the α M stain but not for Nissl due to lack of Nissl bodies outside neuronal soma. A scale of 0-4 was used for both α M and Nissl

individually, as described on table 2.4, and the two score were combined to give a total of 0-8 point injury score.

Score	alphaM immunoreactivity
0	No microglial activation
1	Focal activation
2	Mild diffuse activation, occasional amoeboid macrophages present
3	Widespread activation, predominant amoeboid macrophages present
4	Tissue loss
Score	Nissl (Cresyl Violet) Staining
0	No damage
1	Minimal evidence of damage with no discernible infarct
2	Small infarct (<50%) of the affected brain region
3	Large infarct (>50%) of the affected brain region
4	Total neuronal cell loss

Table 2.4: Brain Injury Score. There is a direct correlation between the level of microglial activation and neuronal cell loss (adapted from Kendall et al., 2006).

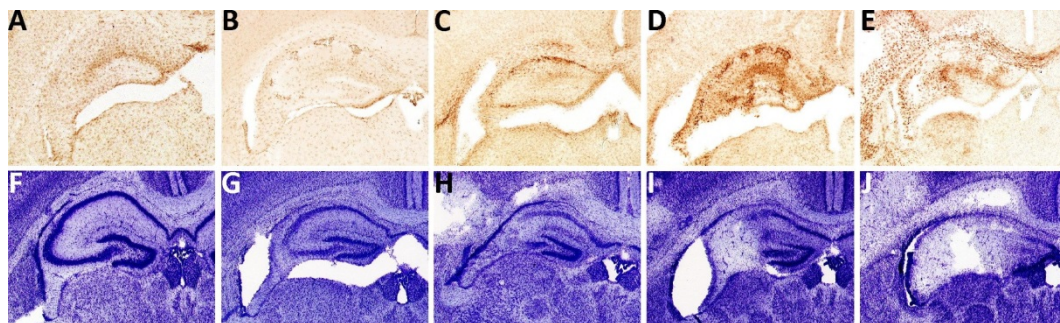


Figure 2.4: α M and Nissl immunoreactivity grades in the dorsoparietal cortex. Brain regions were scored for α M immunoreactivity based on different appearance and morphology as none (A), focal ramified (B), mild diffuse predominantly ramified (C), widespread predominantly amoeboid (D) and total activation, predominantly amoeboid and accompanied by tissue loss (E). Nissl scoring was distinguished as no damage (F), minimal damage (G), small infarct (H), large infarct (I) and total neuronal cell loss along with tissue loss (J).

Cell death

The presence of cell death involving DNA fragmentation was detected through quantification of the number of TUNEL positive nuclei in 3 separate fields (x20 magnification) for each of the six brain regions (dorsoparietal cortex, pyriform cortex, hippocampus, striatum, thalamus and external capsule), on both the ipsilateral and contralateral brain hemispheres. Subsequently, for each region, an

average for the 3 separate fields was taken, as well as for the 5 sections per brain, representing the number of TUNEL positive cells per region within a group.

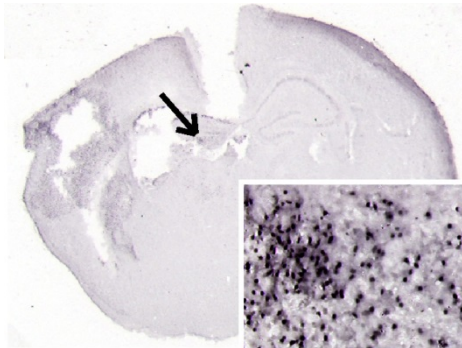


Figure 2.5: TUNEL staining of an LPS-treated mouse brain. LPS treatment (0.6 μ g/g) followed by neonatal hypoxia-ischemia leads a substantial increase in DNA fragmentation-associated cell death in the ipsilateral brain region (left). There are evident TUNEL+ cells in the ipsilateral dorsoparietal cortex, hippocampus (highlighted), striatum, thalamus and external capsule. Close-up of the hippocampal region shows a considerably high number of TUNEL+ cells.

Luminosity

A Sony 3CCD colour video camera (AVT-Horn; Aachen, Germany) was used to assess 3 fields at x20 magnification for each of the dorsoparietal cortex, pyriform cortex, hippocampus, striatum, thalamus and external capsule brain regions of brain sections stained for the presence of the GFAP marker of astrogliosis. Images of both ipsilateral and contralateral regions and for the glass were captured at x20 magnification, using Optimas 6.5 software. The individual regions of the captured images were delineated using the free-hand tool and mean and standard deviation values for optical luminosity were obtained using the histogram tool. The luminance histogram was consistently checked to ensure values were within the 0-255 range (see figure2.5).

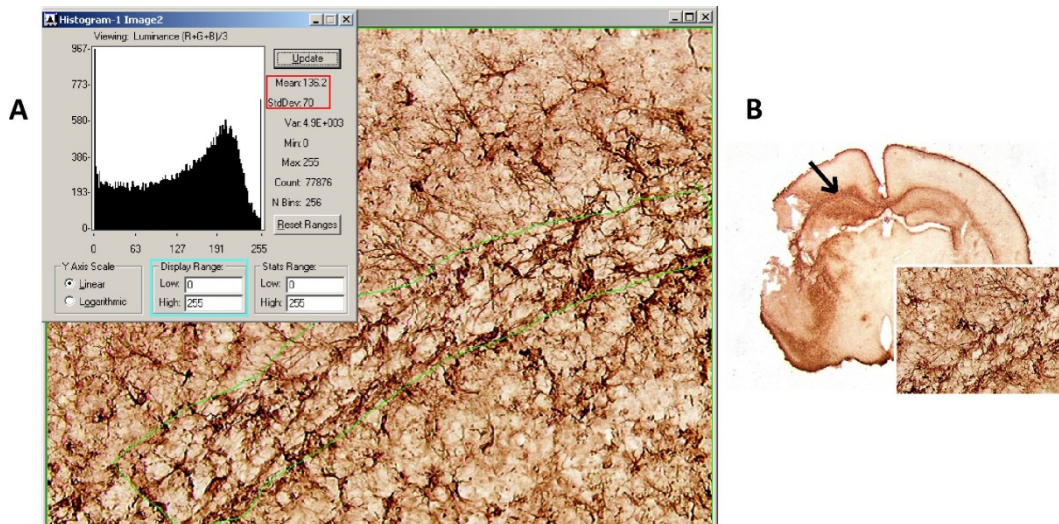


Figure 2.6: GFAP staining optical luminosity measurement. **A:** The represented region of interest, ipsilateral white matter was delineated using the free hand tool (green delineation indicated by black arrow), with luminance histogram reviewed at 0-255 range (blue box). Mean and standard deviation values (red box) were transferred into an Excel spread sheet for assessment. **B:** Full brain section of wild type C57BL/6 animal treated with LPS (0.6 μ g/g) prior to hypoxic-ischemic insult, with close up of region of interest. Denote that contralateral hemisphere (B, right) demonstrates minimal GFAP synthesis following LPS-sensitization to subsequent hypoxic-ischaemic insult.

The regional raw intensity of the stain was determined using the mean – standard deviation algorithm (Möller et al., 1996; Kloss et al., 2001) in both ipsilateral and contralateral brain hemispheres. The background staining intensity was also measured for each slide using the same algorithm. Final stain intensity for each brain region was calculated by the difference between raw staining and background staining intensities. Average values for each region were then compared between the different groups and hemispheres for statistical assessment.

Statistical analysis

The average \pm standard error of the mean (SEM) was recorded for all data. Statistical analysis for infarct size, alphaM immunoreactivity and neuronal loss, TUNEL+ counts and GFAP luminosity between all groups, and between the ipsilateral and contralateral brain hemispheres, was initially tested for normal distribution using PRISM GraphPad 5. As none of the data appeared to be

normally distributed, the Mann Whitney test was used. $P < 5\%$ was considered to represent a significant difference between groups.

CHAPTER 3: THE ROLE OF DIFFERENT STRAIN BACKGROUNDS IN BACTERIAL ENDOTOXIN-MEDIATED SENSITIZATION TO SUBSEQUENT HYPOXIC-ISCHAEMIC NEONATAL BRAIN DAMAGE

Abstract

The development of transgenic mouse strains led to their application in models that mimic human disease. Studies showed variability in the hypoxic-ischaemic mouse model as well as varying responses to the hypoxic-ischaemic insult in different mouse strains. To address this variability, we compared five strains commonly used (C57BL/6, 129SVJ, BALB/c, CD1 and FVB).

P7 pups were subjected to unilateral carotid occlusion followed by continuous 30min 8% O₂ exposure at 36°C. 12h prior, 2/3 of the pups received a single intraperitoneal LPS injection (0.6µg/g) (HI LPS) or saline (HI saline) as a control. 1/3 of the pups underwent hypoxia-ischaemia alone (controls) (HI alone).

Both C57BL/6 and 129SVJ strains were non-responsive to HI alone or HI saline, whereas BALB/c demonstrated a moderate response, and both CD1 and FVB revealed the highest brain damage. LPS pre-sensitization led to a substantial increase in overall brain infarction, immune response and cell death in all five strains. Interestingly, saline treatment also led to an increase in brain damage, particularly in the FVB background strain.

Individual Strain Results

C57BL/6

C57BL/6 is the most widely used inbred strain, not only as a general-purpose strain but also for the generation of transgenic mice carrying both spontaneous and induced mutations. For this study wild type C57BL/6 breeding pairs were

used and the experimental litters were split into three groups: HI alone (n=10), combined saline and HI (HI saline, n=11) and combined endotoxin and HI insult (HI LPS, n=11).

Infarct Volume

Extent of brain damage was assessed as percentage of ipsilateral/contralateral surviving tissue. The results obtained demonstrated minimal tissue damage in HI alone and HI saline animals, with no difference in total infarct size ($15\pm 7\%$; $8\pm 4\%$) between both groups. However, when comparing the HI saline control group with LPS pre-treated animals there was a significant increase in total tissue loss from $8\pm 4\%$ to $39\pm 8\%$ ($p < 1\%$) in the latter. Observation of the different brain regions demonstrated that LPS pre-treated animals had significantly higher tissue loss in all six ipsilateral brain regions when compared to their HI saline littermate controls: The dorsoparietal cortex showed an increase in infarct from $5\pm 5\%$ to $40\pm 8\%$ ($p < 1\%$), pyriform cortex from $7\pm 5\%$ to $31\pm 9\%$ ($p < 5\%$), hippocampus from $23\pm 6\%$ to $66\pm 11\%$ ($p < 1\%$), striatum from $10\pm 5\%$ to $34\pm 9\%$ ($p < 5\%$), thalamus from $2\pm 1\%$ to $26\pm 8\%$ ($p < 1\%$) and external capsule from 10 ± 7 to $53\pm 10\%$ ($p < 1\%$) (see figure 3.1.A).

Injury Score

Combined histological assessment of alphaM staining and Nissl neuronal cell loss showed minimal brain injury in both ipsilateral HI alone (1.5 ± 0.9) and HI saline (1.2 ± 0.4) animals. However, ipsilateral comparison between HI saline and HI LPS littermates revealed a significant increase in microglial activation and neuronal cell loss in the latter (4.9 ± 0.9 , $p < 1\%$). Furthermore, this surge in brain damage was evident in all assessed forebrain regions. The dorsoparietal cortex revealed an increase in injury score from 0.5 ± 0.4 to 5.1 ± 1.0 ($p < 0.1\%$), pyriform cortex from 0.3 ± 0.2 to 3.6 ± 0.9 ($p < 1\%$), hippocampus from 1.7 ± 0.8 to 6.3 ± 1.0 ($p < 1\%$), striatum from 0.9 ± 0.4 to 4.9 ± 0.9 ($p < 5\%$), thalamus from 0.3 ± 0.1 to 3.5 ± 1.0 ($p < 5\%$) and external capsule from 3.2 ± 0.8 to 6.2 ± 1.0 ($p < 5\%$) (see figure 3.1.B).

Observation of the contralateral brain hemisphere revealed a lack of brain injury in all three groups: HI alone (0.0 ± 0.0), HI saline (0.1 ± 0.0) and HI LPS (0.2 ± 0.1) (see figure 3.1.C).

TUNEL+ Cells

Quantification of DNA fragmentation-associated cell death has shown minimal TUNEL+ cells in the contralateral brain hemisphere of all three experimental groups: HI alone (0.0 ± 0.0), HI saline (0.0 ± 0.0) and HI LPS (0.3 ± 0.2) (see figure 3.1E). Minimal numbers of TUNEL+ cells were also observed on the ipsilateral brain hemisphere for HI alone (2.7 ± 1.2) and HI saline (6.9 ± 2.7) control groups. Conversely, LPS pre-sensitization has led to a significant increase in the number of cells undergoing fragmentation when compared to HI saline littermate controls (37.4 ± 17.5 , $p<5\%$). This decrease in cell survival was also evident in the ipsilateral dorsoparietal cortex, where the number of TUNEL+ cells increased from 3.7 ± 0.6 to 64.5 ± 26.3 ($p<1\%$), hippocampus from 22.4 ± 8.6 to 58.7 ± 17.1 ($p<5\%$) and external capsule from 2.1 ± 0.6 to 17.6 ± 6.0 ($p<5\%$) (see figure 3.1.D).

GFAP Immunoreactivity

Optical luminosity assessment showed that stress of saline injection had an effect on astrogliosis in the contralateral brain hemisphere, where there was an overall significant increase in astrocyte immunoreactivity from 13.5 ± 1.0 to 28.1 ± 3.2 ($p<0.1\%$). This saline-associated increase in GFAP synthesis was also significant in the dorsoparietal (from 12.4 ± 0.6 to 23.9 ± 3.1 , $p<0.1\%$) and pyriform cortex (from 11.4 ± 0.8 to 25.2 ± 3.3 , $p<1\%$), hippocampus (from 19.2 ± 1.8 to 42.0 ± 3.2 , $p<0.1\%$), striatum (from 10.6 ± 0.7 to 22.9 ± 3.7 , $p<0.1\%$) and external capsule (from 19.4 ± 1.3 to 44.2 ± 4.2 , $p<0.1\%$). LPS-treated animals did not appear overall more affected than saline littermate controls (33.4 ± 3.8), with the only exception being the thalamus, where there was an LPS-associated increase in astrocytic activation from 10.4 ± 1.5 to 18.8 ± 3.1 ($p<5\%$) (see figure 3.1.G). Observation of the ipsilateral hemisphere has also shown an overall saline-

mediated increase in GFAP immunoreactivity (from 22.3 ± 4.2 to 40.5 ± 4.8 , $p < 1\%$). This was seen in most regions, with the exception of thalamus: Dorsoparietal (from 19.9 ± 4.4 to 33.3 ± 4.3 , $p < 5\%$) and pyriform cortex (from 17.8 ± 2.2 to 36.6 ± 4.4 , $p < 1\%$), hippocampus (from 36.3 ± 6.7 to 64.6 ± 6.9 , $p < 5\%$), striatum (from 18.0 ± 4.4 to 33.0 ± 4.1 , $p < 1\%$) and external capsule (from 32.2 ± 6.4 to 61.9 ± 5.7 , $p < 1\%$) brain regions. Further assessment of the ipsilateral hemisphere showed an overall significant LPS-sensitization effect (62.4 ± 8.3 , $p < 5\%$) when compared to saline pre-treatment. This increase was also significant in most ipsilateral brain regions, where the dorsoparietal cortex demonstrated an increase to 59.1 ± 8.1 ($p < 5\%$), hippocampus to 89.4 ± 13.4 ($p < 5\%$), striatum to 57.2 ± 6.8 ($p < 5\%$) and thalamus to 41.3 ± 7.8 ($p < 1\%$) (see figure 3.1.F).

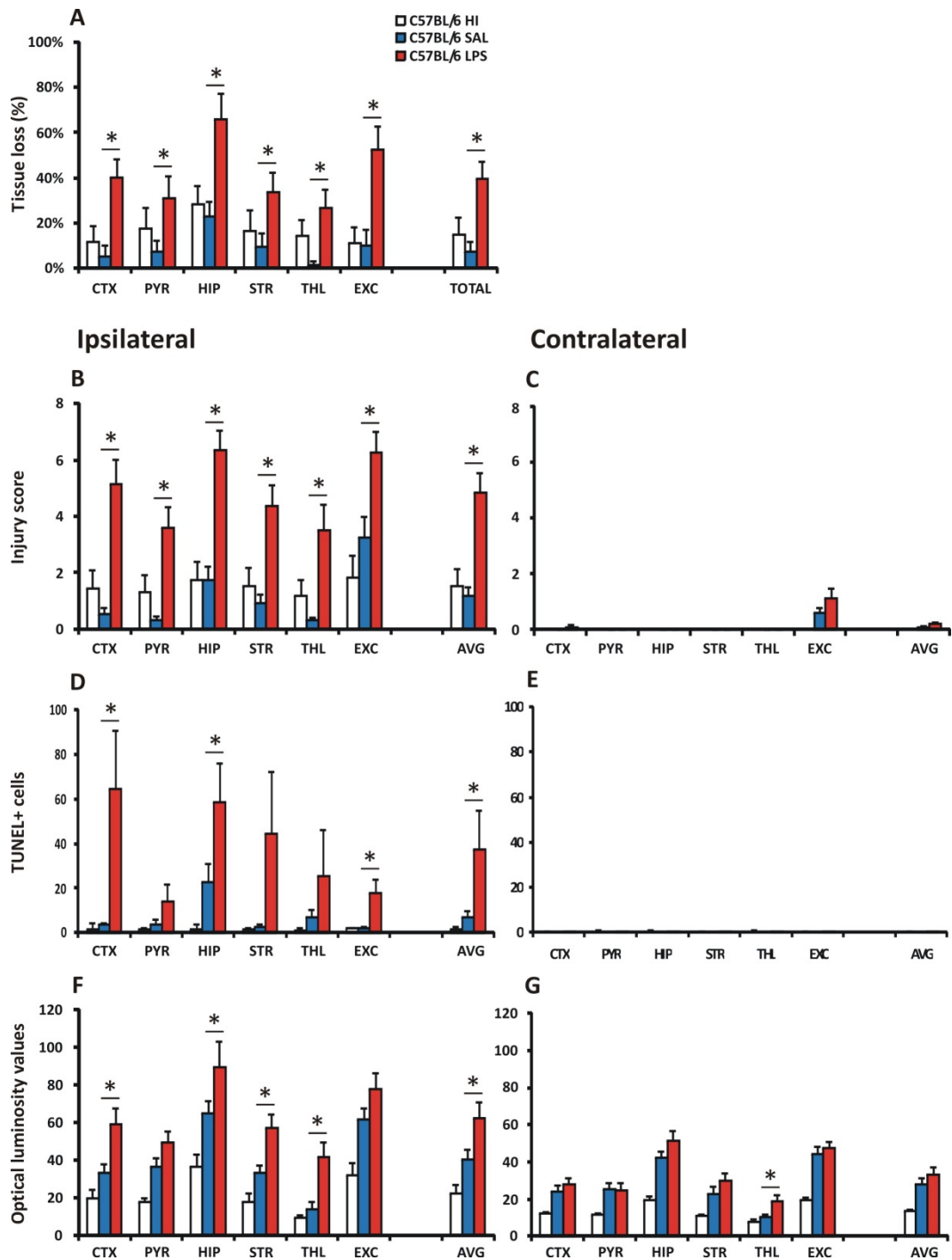


Figure 3.1: Effects of LPS endotoxin treatment (0.6 μ g/g) in wild type C57BL/6 mice followed by neonatal hypoxic-ischemic brain. Animals injected with saline served as controls. **A:** Infarct volume assessment of individual forebrain regions and total ipsilateral hemisphere infarction, as percent of contralateral hemisphere, showed that compared to saline-treated littermates, LPS sensitization (LPS) led to a total significant increase in tissue infarction, which was significant in all assessed ipsilateral brain regions. Minimal tissue loss was observed in both HI alone (HI) and HI saline (SAL) groups. **B:** Ipsilateral brain injury score assessment revealed minimal microglial inflammatory response and neuronal cell death in both HI alone and HI saline groups. LPS-treated animals had demonstrably higher overall brain injury, which was also evident in all assessed ipsilateral brain regions. **C:** Contralateral brain injury assessment demonstrated no microglial inflammation or tissue loss in any of the three groups. **D:** DNA fragmentation cell death was only

associated with LPS-treatment. Overall TUNEL+ cell quantification was significantly higher than in HI saline littermate controls; this was also significant in 50% of assessed brain regions **E**: However, none of the three treatments elicited DNA fragmentation-associated cell death in the contralateral brain hemisphere. **F**: Astroglial immunoreactivity assessment using optical luminosity values showed a saline-associated increase in GFAP synthesis in the ipsilateral brain hemisphere. This reached significance in most brain regions (significance not shown). LPS-treated further exacerbated astrogliosis. HI LPS group showed a significant overall increase in astroglial activity, which was also significant in most ipsilateral brain regions. **G**: Contralateral astroglial immunoreactivity assessment also demonstrated a significant overall saline-associated stress, which was significant for most contralateral brain regions. LPS-treated animals did not appear more affected, with only thalamus showing a significant increase in astrogliosis when compared to HI saline controls. * $p < 5\%$ in Mann Whitney test for the LPS-treated wild type vs. knockout groups, $n=10$ for HI alone, $n=11$ for HI saline and $n=11$ for HI LPS. **Abbreviations**: CTX- dorsoparietal and PYR – pyriform cortex, HIP – hippocampus, STR – striatum, THL – thalamus, EXC – external capsule.

129SVJ

The 129SVJ strain is widely used in the production of targeted mutation, as multiple embryonic stem cell lines are derived from 129SVJ mice. In this study a wild type 129SVJ breeding strategy was used, and the offspring were separated into three groups: HI alone ($n=10$), HI saline ($n=10$) and HI LPS ($n=10$).

Infarct Volume

Results looking at infarction revealed minimal tissue loss in both HI alone and HI saline groups ($3 \pm 2\%$ and $4 \pm 1\%$ respectively). However, comparison between HI saline and HI LPS animals demonstrated that the latter were highly sensitized to LPS treatment prior to HI injury with a total increase in tissue infarction from $4 \pm 1\%$ to $29 \pm 9\%$ ($p < 1\%$). Assessment of the individual brain regions showed that this increase was also significant in the dorsoparietal ($3 \pm 1\%$ to $32 \pm 10\%$, $p < 5\%$) and pyriform cortex ($3 \pm 2\%$ to $32 \pm 10\%$, $p < 1\%$), striatum ($1 \pm 5\%$ to $31 \pm 11\%$, $p < 5\%$) and external capsule ($3 \pm 2\%$ to $27 \pm 10\%$, $p < 5\%$) (see figure 3.2.A).

Injury Score

Brain injury score assessment showed no microglial activation or neuronal cell loss in the ipsilateral brain hemisphere of HI alone and HI saline animals (0.1 ± 0.1 and 0.7 ± 0.7 respectively). Conversely, HI LPS animals not only showed a

significant overall increase in alphaM and neuronal cell death (2.7 ± 1.1 , $p < 5\%$), but also, this increase in brain injury reached statistical significance in the dorsoparietal (from 0.8 ± 0.5 to 3.7 ± 1.1 , $p < 5\%$) (see figure 3.2.B). None of the three experimental groups – HI alone, HI saline and HI LPS – demonstrated brain injury in the contralateral brain hemisphere (0.0 ± 0.0 , 0.0 ± 0.0 and 0.1 ± 0.1 respectively) (see figure 3.2.C).

TUNEL+ Cells

Average quantification of TUNEL+ cells showed minimal counts for HI alone (0.0 ± 0.0), HI saline (0.2 ± 0.2) and HI LPS (2.1 ± 1.2) in the contralateral brain hemisphere (see figure 3.2.E). Assessment of the ipsilateral hemisphere revealed that HI (0.0 ± 0.0) and HI saline (1.5 ± 1.5) insults also elicit minimal TUNEL+ cell death. Ipsilateral observation of the HI LPS group demonstrated that LPS pre-treatment led to a visible increase in DNA fragmentation-associated cell death when compared with HI saline controls (from 1.3 ± 1.3 to 15.0 ± 7.6). However, this never reached significance (see figure 3.2.D).

GFAP Immunoreactivity

Astrogliosis assessment revealed minimal astrocytic immunoreactivity in the contralateral brain hemisphere of the three experimental groups: HI alone (18.9 ± 1.5), HI saline (27.3 ± 4.4) and HI LPS (29.9 ± 4.4). Despite both HI saline and HI LPS showing a small trend towards increased astrogliosis, this was never significant (see figure 3.2.G). Furthermore, ipsilateral comparison between HI alone and HI saline animals once again demonstrated minimal astrocytic activation and no difference between both groups (21.9 ± 1.9 and 30.1 ± 6.2 respectively). Ipsilateral assessment of HI LPS animals demonstrated an overall substantial increase in GFAP immunoreactivity when compared to HI saline littermate controls (from 3.01 ± 6.2 to 47.3 ± 8.8 , $p < 5\%$). This was further evident in the dorsoparietal (from 21.6 ± 4.6 to 45.3 ± 9.1 , $p < 5\%$) and pyriform cortex (from 17.9 ± 4.5 to 31.3 ± 5.5 , $p < 5\%$) ipsilateral brain regions (see figure 3.2.F).

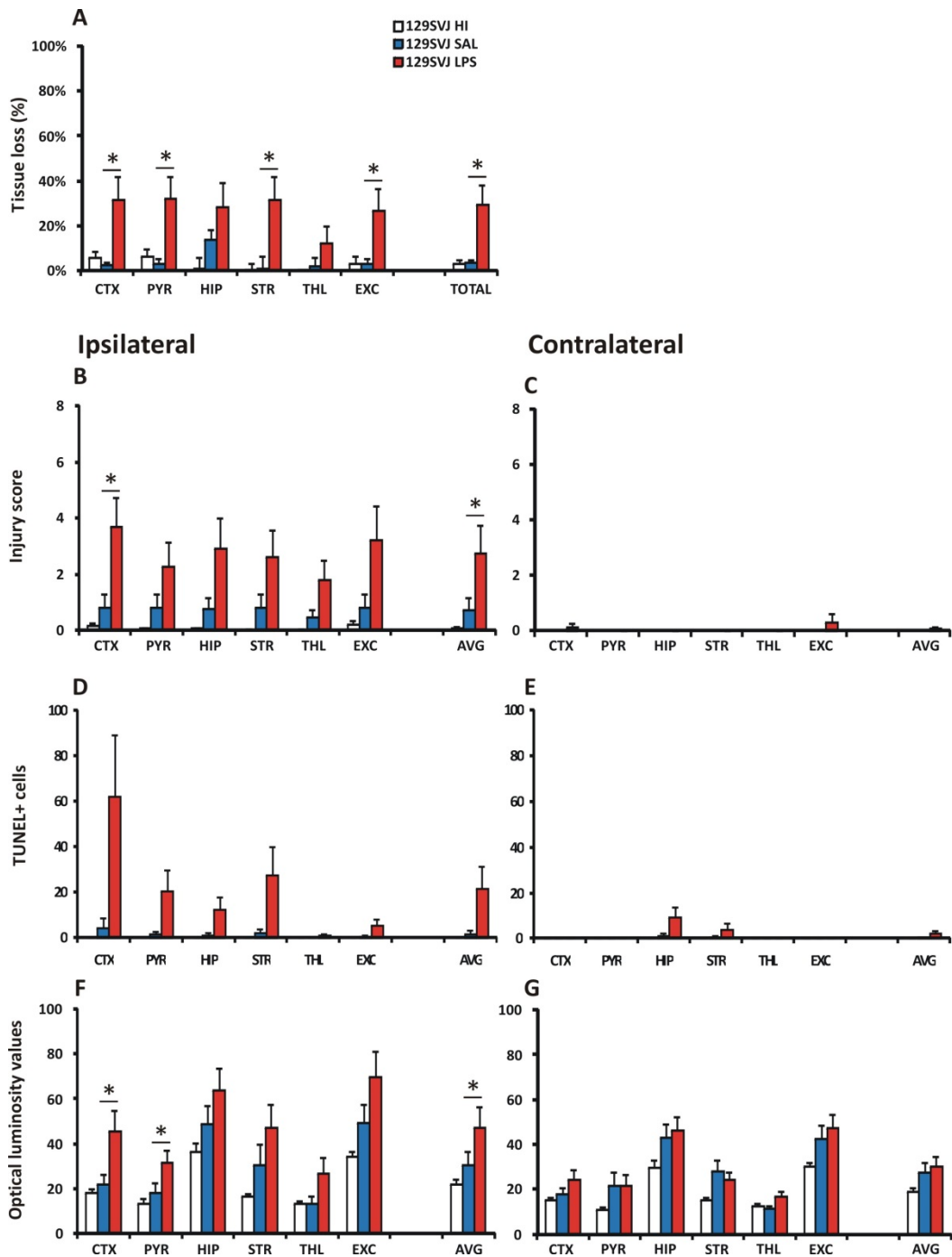


Figure 3.2: Effects of LPS endotoxin treatment (0.6µg/g) in wild type 129SVJ mice followed by neonatal hypoxic-ischemic brain. Animals injected with saline served as controls. **A:** Infarct volume assessment of individual forebrain regions and total ipsilateral hemisphere infarction, as percent of contralateral hemisphere, showed that compared to saline-treated littermates, LPS sensitization (LPS) led to a total significant increase in tissue infarction, which was significant in most assessed ipsilateral brain regions, with only exception being the thalamus, which only reached a trend towards increased infarction. Minimal tissue loss was observed in both HI alone (HI) and HI saline (SAL) groups. **B:** Ipsilateral brain injury score assessment revealed minimal microglial inflammatory response and neuronal cell death in both HI alone and HI saline groups. LPS-treated animals had demonstrably higher overall brain injury that was also significant in both

dorsoparietal and pyriform cortex brain regions. **C:** Contralateral brain injury assessment demonstrated no microglial inflammation or tissue loss in any of the three groups. **D:** DNA fragmentation cell death was only associated with LPS-treatment. TUNEL+ cell quantification was visibly higher than in HI saline littermate controls; this increase in cell death was significant in the dorsoparietal cortex and hippocampus ipsilateral brain regions **E:** None of the three injuries elicited DNA fragmentation-associated cell death in the contralateral brain hemisphere. **F:** Ipsilateral astroglial immunoreactivity assessment using optical luminosity values showed minimal increase in GFAP synthesis in both HI alone and HI saline groups. Conversely, LPS administration led to a significant overall increase in astrogliosis. This increase in GFAP synthesis was significant in both dorsoparietal and pyriform cortex. **G:** Contralateral astroglial immunoreactivity assessment showed minimal astrogliosis in the HI alone group with only a small increase for both HI saline and HI LPS groups. * $p < 5\%$ in Mann Whitney test for the LPS-treated wild type vs. knockout groups, $n=10$ per group. **Abbreviations:** CTX- dorsoparietal and PYR – pyriform cortex, HIP – hippocampus, STR – striatum, THL – thalamus, EXC – external capsule.

BALB/c

BALB/c, another commonly used inbred strain was also studied in this project. Once again, littermate offspring of wild type BALB/c breeding pairs were divided into three experimental groups: HI alone ($n=11$), HI saline ($n=9$) and HI LPS ($n=10$).

Infarct Volume

Infarct volume assessment showed moderate tissue loss with no difference between total HI alone ($21 \pm 8\%$) and total HI saline ($36 \pm 11\%$) animals. Interestingly, assessment of the HI LPS group revealed only a trend in increased tissue necrosis when compared to HI saline controls ($47 \pm 9\%$ and $36 \pm 11\%$ respectively) (see figure 3.3.A).

Injury Score

Ipsilateral combined brain injury score demonstrated overall minimal microglial activation and neuronal cell loss in the HI alone group (2.3 ± 0.9). Despite a small increase in overall injury score in the HI saline group, this was still minimal (3.8 ± 1.2). Pre-treatment with LPS revealed only a trend towards increased microglial activation and neuronal cell loss when compared to HI saline controls (from 3.8 ± 1.2 to 5.4 ± 1.0) (see figure 3.3.B). Furthermore, none of the three insults (HI alone, HI saline and HI LPS) were enough to elicit brain injury in the

contralateral brain hemisphere (0.0 ± 0.0 , 0.1 ± 0.0 and 0.2 ± 0.0 respectively) (see figure 3.3.C).

TUNEL+ Cells

Results for HI alone (0.0 ± 0.0), HI saline (0.0 ± 0.0) and HI LPS (0.2 ± 0.1) animals have demonstrated that none of these insults were enough to cause DNA fragmentation-associated cell death in the contralateral brain hemisphere (see figure 3.3.E). Ipsilateral quantification of TUNEL+ cells showed minimal counts for both HI alone (21.0 ± 11.6) and HI saline (35.3 ± 15.0) animals. Comparison between HI saline control group and HI LPS animals revealed an average significant increase in TUNEL+ cells on the latter from 35.3 ± 15.0 to 96.8 ± 31.3 ($p < 5\%$). Closer observation of the assessed ipsilateral brain regions showed that significance was also reached in the striatum (from 31.3 ± 20.7 to 132.2 ± 52.2 , $p < 5\%$) and external capsule white matter (from 7.0 ± 2.6 to 26.2 ± 8.3 , $p < 5\%$) (see figure 3.3.D).

GFAP Immunoreactivity

In terms of GFAP immunoreactivity, optical luminosity values revealed no difference between HI alone (35.7 ± 5.1), HI saline (25.7 ± 2.8) and HI LPS (28.0 ± 3.6) in the contralateral brain hemisphere (see figure 3.3.G). HI LPS pre-treatment (57.1 ± 8.0) also failed to elicit an increase in astrogliosis in the ipsilateral brain hemisphere when compared to HI saline (49.7 ± 7.2) controls (see figure 3.3.F).

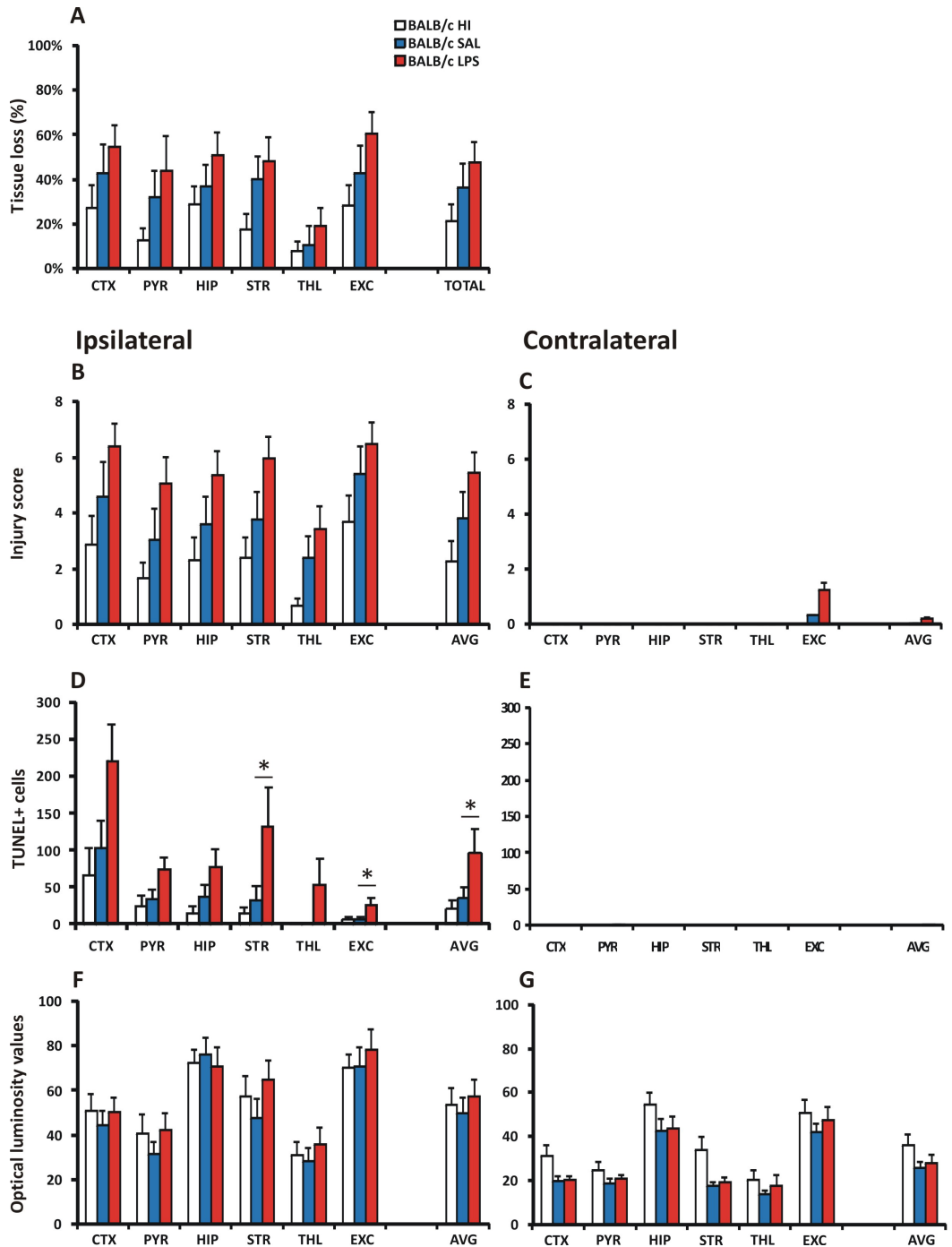


Figure 3.3: Effects of LPS endotoxin treatment (0.6 μ g/g) in wild type BALB/c mice followed by neonatal hypoxic-ischemic brain. Animals injected with saline served as controls. **A:** Infarct volume assessment of individual forebrain regions and total ipsilateral hemisphere infarction, as percent of contralateral hemisphere, showed that both HI alone (HI) and HI saline (SAL) groups had moderate tissue loss, with no significant difference between both groups. LPS sensitization (LPS) led to a further increase in tissue infarction; however, this never reached significance. **B:** Ipsilateral brain injury score assessment revealed minimal microglial inflammatory response and neuronal cell death in both HI alone and HI saline groups. LPS-treated animals showed only a small increase in overall brain injury. **C:** Contralateral brain injury assessment demonstrated no microglial inflammation or tissue loss in any of the three groups. **D:** DNA fragmentation cell death was only associated with LPS-treatment. Overall TUNEL+ cell quantification was significantly

higher than in HI saline littermate controls; this was also significant in the striatum and external capsule ipsilateral brain regions. **E:** None of the three groups – HI alone, HI saline and HI LPS – had demonstrable DNA fragmentation-associated cell death in the contralateral brain hemisphere. **F:** Ipsilateral astroglial immunoreactivity assessment demonstrated equally moderate astrogliosis in all the groups (HI alone, HI saline and HI LPS). **G:** Contralateral astroglial immunoreactivity assessment demonstrated equally reduced GFAP synthesis. * $p < 5\%$ in Mann Whitney test for the LPS-treated wild type vs. knockout groups, $n=11$ for HI alone, $n=19$ for HI saline and $n=10$ for HI LPS. **Abbreviations:** CTX- dorsoparietal and PYR – pyriform cortex, HIP – hippocampus, STR – striatum, THL – thalamus, EXC – external capsule.

CD1

The CD1 mouse is a strain that, unlike C57Bl/6, 129SvJ, BALB/c or FVB, is outbred. It is commonly used in research, including hypoxic-ischaemic insult studies, where importance is laid on having a background that may generate a more diverse response. Offspring of wild type CD1 breeding pairs were divided into HI alone ($n=10$), HI saline ($n=12$) and HI LPS ($n=10$).

Infarct Volume

Infarct measurements showed moderate tissue loss of $46 \pm 12\%$ in HI alone and of $39 \pm 9\%$ in HI saline animals. This tissue necrosis was further increased in animals pre-treated with LPS. LPS sensitization led to a total increase in tissue infarction to $86 \pm 5\%$ when compared to HI saline controls ($p < 1\%$). This was also evident in all assessed brain regions: Dorsoparietal (from $38 \pm 9\%$ to $83 \pm 4\%$, $p < 1\%$) and pyriform cortex (from $30 \pm 13\%$ to $89 \pm 11\%$, $p < 1\%$), hippocampus (from $55 \pm 9\%$ to $90 \pm 6\%$, $p < 5\%$), striatum (from $46 \pm 14\%$ to $93 \pm 5\%$, $p < 5\%$), thalamus (from $30 \pm 11\%$ to $76 \pm 15\%$, $p < 5\%$) and external capsule (from $32 \pm 6\%$ to $81 \pm 5\%$, $p < 0.1\%$) (see figure 3.4.A).

Injury Score

Average ipsilateral injury score of HI alone and HI saline littermates demonstrated similarly high microglial activation and neuronal cell loss in both groups (4.7 ± 1.0 and 4.9 ± 0.9 respectively). There was a significant overall increase in ipsilateral brain injury in HI LPS animals (from 4.9 ± 0.9 to 6.8 ± 0.8 ,

$p < 5\%$), this was also significant most ipsilateral brain regions: Piriform cortex (from 3.6 ± 0.8 to 6.6 ± 0.6 , $p < 5\%$), hippocampus (from 4.9 ± 0.7 to 6.7 ± 0.5 , $p < 5\%$), striatum (from 5.0 ± 0.9 to 6.8 ± 0.5 , $p < 5\%$) and thalamus (from 3.0 ± 0.7 to 6.5 ± 0.6 , $p < 5\%$) when compared to HI saline controls (see figure 3.4.B). Observation of the contralateral brain hemisphere showed no microglial activation or neuronal cellular loss in any of the three experimental groups (HI alone, HI saline and HI LPS) (see figure 3.4.C).

TUNEL+ Cells

Observation of the contralateral brain hemisphere revealed a reduced number of TUNEL+ cells for all three experimental groups. HI alone had an average of 0.0 ± 0.0 , HI saline 0.0 ± 0.0 and HI LPS 1.0 ± 0.5 TUNEL+ cells (see figure 3.4.E). Assessment of the ipsilateral brain hemisphere showed a small similar increase in DNA fragmentation-associated cell death staining for both HI alone (27.6 ± 15.1) and HI saline (19.3 ± 9.1) animals. Comparison between ipsilateral HI saline and HI LPS animals revealed an LPS-mediated sensitization effect, where there was an average increase to 84.6 ± 27.7 ($p < 5\%$) in TUNEL+ cells in the latter. Furthermore, this increase was also evident in hippocampus (from 17.6 ± 11.3 to 101.8 ± 29.2 , $p < 5\%$), striatum (from 28.0 ± 15.1 to 126.7 ± 52.6 , $p < 5\%$) and thalamus (from 0.0 ± 0.0 to 126.9 ± 33.2 , $p < 0.1\%$) ipsilateral brain regions (see figure 3.4.D).

GFAP Immunoreactivity

Direct quantification of GFAP immunoreactivity revealed no difference in optical luminosity values between contralateral HI alone (28.9 ± 3.0) and HI saline (29.7 ± 3.9) animals. HI LPS group showed an overall significant increase in astrogliosis (48.3 ± 5.2 , $p < 5\%$), which was also significant in the dorsoparietal cortex (from 20.7 ± 1.9 to 36.1 ± 5.2 , $p < 1\%$), striatum (from 20.1 ± 2.3 to 36.5 ± 4.2 , $p < 5\%$), thalamus (from 15.7 ± 2.2 to 41.6 ± 5.2 , $p < 1\%$) and external capsule (from 15.7 ± 2.2 to 41.6 ± 5.2 , $p < 5\%$) contralateral regions when compared to littermate saline controls (see figure 3.4.G). Assessment of the occluded brain hemisphere

has shown no difference in overall astrocyte immunoreactivity between HI alone (57.2 ± 10.6) and HI saline (56.8 ± 7.9) animals. Comparison between the latter and littermate HI LPS animals revealed a significant increase in GFAP synthesis to 86.1 ± 11.6 ($p < 1\%$) in the LPs treated animals. This was also significant in the dorsoparietal cortex (from 45.0 ± 6.5 to 89.6 ± 16.9 , $p < 1\%$), striatum (from 56.2 ± 7.3 to 82.9 ± 15.4 , $p < 5\%$) and thalamus (from 31.0 ± 6.0 to 75.0 ± 9.4 , $p < 1\%$) brain regions (see figure 3.4.F).

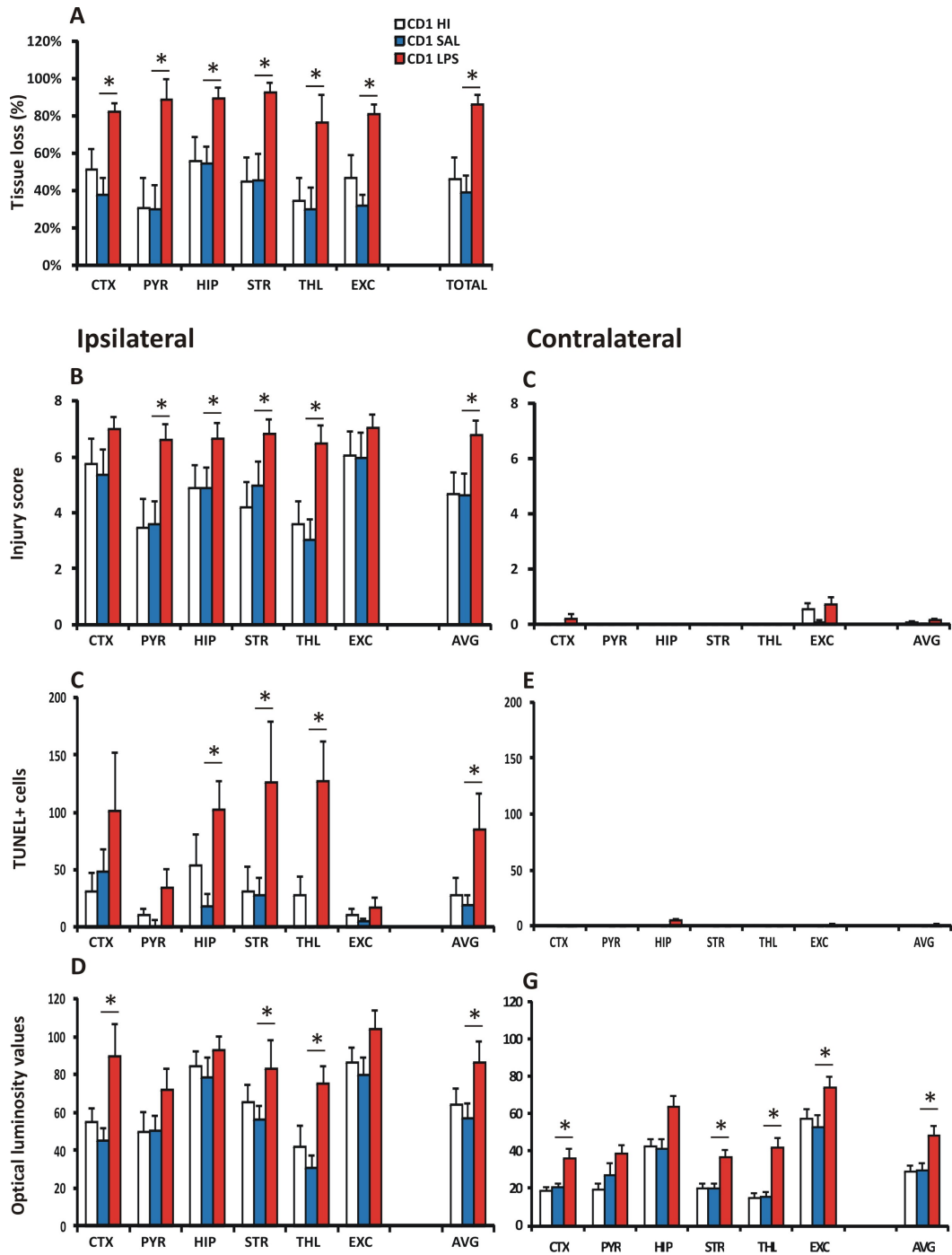


Figure 3.4: Effects of LPS endotoxin treatment (0.6 μ g/g) in wild type CD1 mice followed by neonatal hypoxic-ischemic brain. Animals injected with saline served as controls. **A:** Infarct volume assessment of individual forebrain regions and total ipsilateral hemisphere infarction, as percent of contralateral hemisphere, showed that compared to saline-treated littermates, LPS sensitization (LPS) led to a total significant increase in tissue infarction, which was significant in all assessed ipsilateral brain regions. Moderate tissue loss was observed in both HI alone (HI) and HI saline (SAL) groups. **B:** Ipsilateral brain injury score assessment revealed equally high microglial inflammatory response and neuronal cell death in both HI alone and HI saline groups. LPS-treated animals demonstrated a further increase in brain injury. This LPS-associated increase reached significance in the ipsilateral thalamus brain region. **C:** Contralateral brain injury assessment demonstrated no microglial inflammation or tissue loss in any of the three groups. **D:** There was

minimal DNA fragmentation cell death in both ipsilateral HI alone and HI saline groups. DNA fragmentation cell death was significantly increased with LPS-treatment, this was also significant in 50% of assessed brain regions **E**: However, none of the three insults (HI alone, HI saline and HI LPS) elicited DNA fragmentation-associated cell death in the contralateral brain hemisphere. **F**: Ipsilateral astroglial immunoreactivity assessment using optical luminosity values showed no difference in GFAP synthesis between HI alone and HI saline groups. LPS treatment however, led to an overall increase in astrogliosis, which reached significance in the ipsilateral dorsoparietal cortex and thalamus brain regions. **G**: Contralateral astroglial immunoreactivity assessment showed minimal astroglial activation on both HI alone and HI saline groups. LPS-treated animals showed a trend towards increased GFAP synthesis which reached significance in the dorsoparietal cortex and thalamus brain regions. * $p < 5\%$ in Mann Whitney test for the LPS-treated wild type vs. knockout groups, $n=10$ for HI alone, $n=12$ for HI saline and $n=10$ for HI LPS. **Abbreviations**: CTX- dorsoparietal and PYR – pyriform cortex, HIP – hippocampus, STR – striatum, THL – thalamus, EXC – external capsule.

FVB

FVB mice are another suitable background strain used in transgenic research. Similarly to the previous background strains studied, offspring of wild type FVB animals were divided into three experimental groups: HI alone ($n=9$), HI saline ($n=11$) and HI LPS ($n=11$).

Infarct Volume

Ipsilateral brain hemisphere of HI alone and HI saline animals had considerable tissue infarction of around 50% over the contralateral hemisphere ($41 \pm 10\%$ and $50 \pm 9\%$ respectively). LPS sensitization led to a further substantial increase in tissue infarction to $87 \pm 3\%$ ($p < 1\%$). Further comparison between HI LPS animals and HI saline littermate controls demonstrated that this exacerbation in tissue necrosis was evident in all six assessed brain regions: Dorsoparietal (from $52 \pm 7\%$ to $83 \pm 4\%$, $p < 1\%$) and pyriform cortex (from $55 \pm 14\%$ to $99 \pm 1\%$, $p < 5\%$), hippocampus (from $59 \pm 10\%$ to $90 \pm 5\%$, $p < 5\%$), striatum (from $57 \pm 11\%$ to $95 \pm 2\%$, $p < 1\%$), thalamus (from $27 \pm 13\%$ to $74 \pm 10\%$, $p < 1\%$) and external capsule (from $36 \pm 9\%$ to $82 \pm 4\%$, $p < 0.1\%$) (see figure 3.5.A).

Injury Score

Results showed no alphaM staining or Nissl neuronal cell loss in the contralateral brain hemispheres of mice in any of the three experimental groups: HI alone, HI

saline and HI LPS (see figure 3.5.C). However, comparison between ipsilateral HI alone and HI saline, revealed a saline injection-associated stress for the latter group, with a significant increase in average brain injury score from 2.5 ± 0.6 to 5.7 ± 1.0 ($p < 5\%$). The pyriform cortex (from 2.0 ± 0.5 to 5.7 ± 0.9 , $p < 5\%$), hippocampus (from 1.8 ± 0.5 to 5.8 ± 1.0 , $p < 5\%$), striatum (from 2.1 ± 0.5 to 5.9 ± 0.9 , $p < 5\%$) and external capsule (from 2.8 ± 0.6 to 6.5 ± 1.0 , $p < 5\%$) regions were also significantly more affected in the HI saline group when compared to HI alone controls. Ipsilateral comparison of saline and LPS treatment revealed a further significant increase in overall brain injury score to 8.0 ± 0.0 ($p < 1\%$) in the latter. The most significantly affected ipsilateral brain regions to LPS administration were the dorsoparietal (from 6.3 ± 1.0 to 8.0 ± 0.0 , $p < 5\%$) and pyriform cortex (from 5.7 ± 0.9 to 8.0 ± 0.0 , $p < 5\%$), hippocampus (from 5.8 ± 1.0 to 8.0 ± 0.0 , $p < 5\%$), striatum (from 5.9 ± 0.9 to 8.0 ± 0.0 , $p < 5\%$) and thalamus (from 4.1 ± 1.0 to 7.8 ± 0.2 , $p < 5\%$) (see figure 3.5.B).

TUNEL+ Cells

None of the three experimental insults led to DNA-fragmentation-associated cell death in the contralateral brain hemisphere. HI alone revealed minimal average TUNEL+ counts of 1.6 ± 1.6 , HI saline 2.8 ± 2.8 and HI LPS 3.7 ± 2.3 (see figure 3.5.E). However, observation of the occluded brain hemisphere revealed an increase in TUNEL+ cells for both HI alone (70.5 ± 35.8) and HI saline (82.1 ± 22.7), with no difference between the two groups. Further assessment of the ipsilateral brain hemisphere revealed a substantial overall increase in TUNEL+ cells to 194.1 ± 31.2 in the LPS group when compared to HI saline littermate controls ($p < 5\%$). This increase in DNA fragmentation was significant in most brain regions, with the dorsoparietal cortex showing an increase in TUNEL+ cells from 163.0 ± 38.1 to 376.5 ± 55.6 ($p < 1\%$), pyriform cortex from 54.3 ± 12.7 to 132.2 ± 21.1 ($p < 1\%$), hippocampus from 101.4 ± 23.1 to 248.3 ± 22.3 ($p < 0.1\%$), thalamus from 61.4 ± 32.2 to 158.1 ± 33.3 ($p < 5\%$) and external capsule from 16.8 ± 6.3 to 51.1 ± 12.1 ($p < 5\%$) (see figure 3.5.D).

GFAP Immunoreactivity

Contralateral optical luminosity assessments showed similar moderate optical luminosity values between HI LPS and both HI alone (58.5 ± 3.6) and HI saline (58.5 ± 2.6) animals (see figure 3.5.G). LPS pre-treatment (59.2 ± 3.3) did not lead to an increase in astrocytic activation in the contralateral brain hemisphere. However, results in the ipsilateral hemisphere showed that both HI alone (90.2 ± 8.1) and HI saline (82.7 ± 5.7) had a similar and strong astrocytic immunoreactivity. Furthermore, comparison between HI saline controls and HI LPS animals demonstrated a significant overall increase in GFAP synthesis in the latter (99.3 ± 6.8 , $p < 5\%$), this was also evident in the dorsoparietal cortex, where there was a significant increase in astrogliosis from 77.7 ± 5.5 to 98.4 ± 8.3 ($p < 5\%$), hippocampus from 89.8 ± 4.3 to 111.2 ± 4.7 ($p < 1\%$), thalamus from 62.3 ± 6.6 to 94.3 ± 6.6 ($p < 1\%$) and external capsule from 100.3 ± 5.0 to 118.2 ± 7.2 ($p < 5\%$) brain regions (see figure 3.5.F).

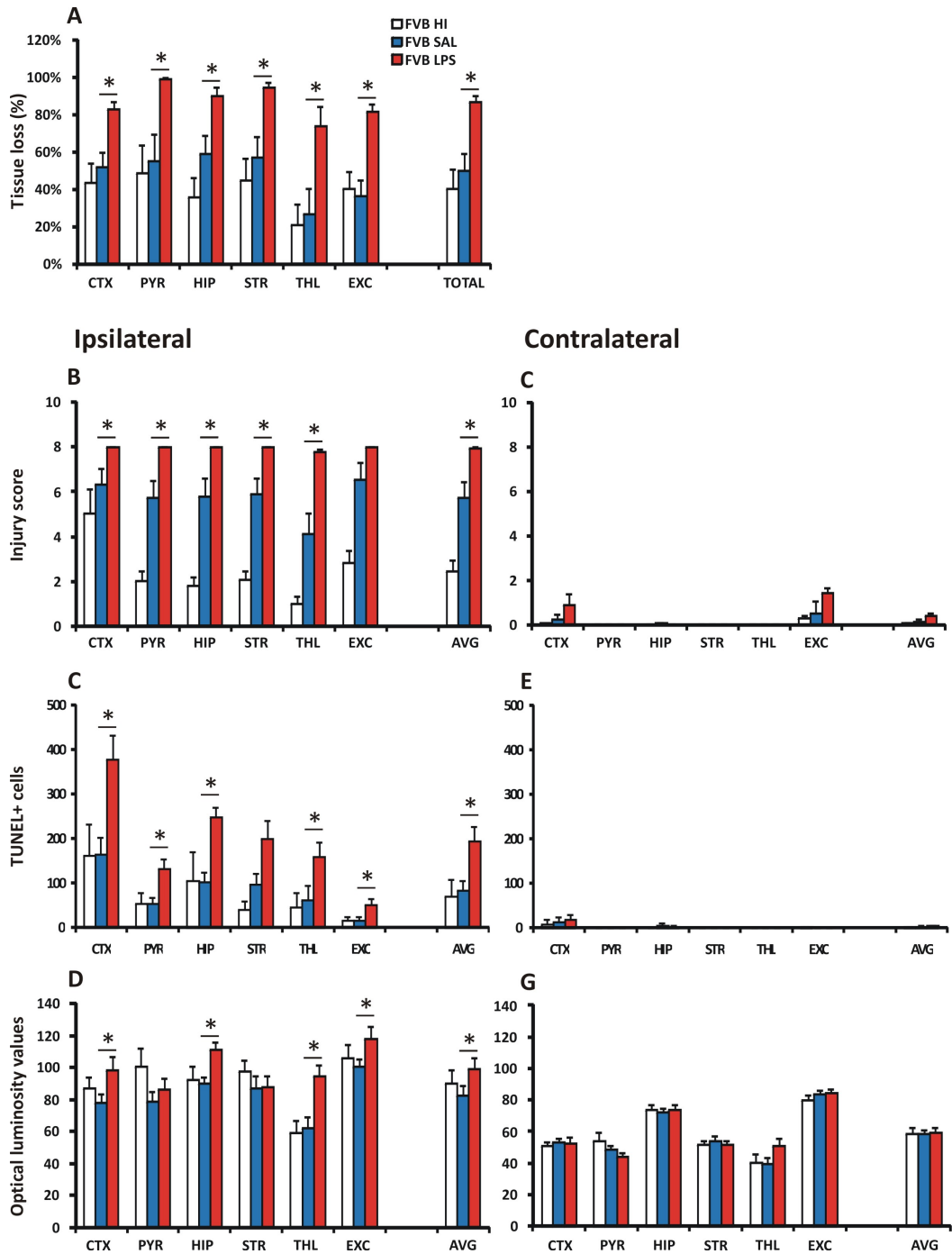


Figure 3.5: Effects of LPS endotoxin treatment (0.6 μ g/g) in wild type FVB mice followed by neonatal hypoxic-ischemic brain. Animals injected with saline served as controls. **A:** Infarct volume assessment of individual forebrain regions and total ipsilateral hemisphere infarction, as percent of contralateral hemisphere, showed that both HI alone (HI) and HI saline (HI SAL) groups had substantial tissue loss. Compared to saline-treated littermates, LPS sensitization (LPS) led to a further total significant increase in tissue infarction, which was significant in all assessed ipsilateral brain regions. **B:** Ipsilateral brain injury score assessment revealed minimal microglial inflammatory response and neuronal cell death in the HI alone. However, there was a significantly visible saline stress-associated increase in brain injury. This was significant in most ipsilateral brain regions, with only exception being the dorsoparietal cortex, where only a trend was visible. LPS-treated animals had an even higher overall brain injury which was also significant in all assessed ipsilateral brain regions. **C:** Contralateral brain injury assessment demonstrated no

microglial inflammation or tissue loss in any of the three groups. **D:** There was minimal DNA fragmentation cell death in both ipsilateral HI alone and HI saline groups. However, LPS treatment led to an overall significant increase in TUNEL+ cell quantification, which was significant in most ipsilateral brain regions, with pyriform cortex as the only exception, where only a trend towards increased TUNEL+ cell death was visible. **E:** However, none of the three treatments elicit DNA fragmentation-associated cell death in the contralateral brain hemisphere. **F:** Ipsilateral astroglial immunoreactivity assessment using optical luminosity values showed similar and visible GFAP synthesis in both ipsilateral HI alone and HI saline groups. LPS-treated further exacerbated astrogliosis. HI LPS group showed a significant overall increase in astroglial activity, which was also significant in 50% of the assessed ipsilateral brain regions. **G:** Contralateral astroglial immunoreactivity assessment demonstrated no difference in GFAP synthesis between HI alone, HI saline and HI LPS groups. * $p < 5\%$ in Mann Whitney test for the LPS-treated wild type vs. knockout groups, $n=9$ for HI alone, $n=11$ for HI saline and $n=11$ for HI LPS. **Abbreviations:** CTX- dorsoparietal and PYR – pyriform cortex, HIP – hippocampus, STR – striatum, THL – thalamus, EXC – external capsule.

Differences between C57BL/6, 129SVJ, BALB/c, CD1 and FVB in combined inflammation and hypoxia-ischemia injury

Infarct Volume

All five strains showed a robust LPS-mediated sensitization to subsequent HI injury in the ipsilateral brain hemisphere, with higher tissue necrosis in LPS-treated animals when compared to saline littermate controls. C57BL/6, CD1 and FVB strains showed the highest level of LPS-mediated sensitization, with not only an overall significant increase in tissue infarction, but also, this increase in tissue necrosis was still evident in all assessed brain regions. 129SVJ strain also had a total significant increase in tissue loss in LPS-treated animals when compared to HI saline littermates; this significance was evident in almost all regions with the exception of thalamus. Interestingly, BALB/c LPS-treated animals, despite showing a visible trend towards increased tissue infarction, this increase failed to reach significance when compared to HI saline littermates. Overall comparison of saline treated groups over the five different strains has shown that saline administration prior to HI injury demonstrated leads to minimal or no increase in tissue infarction when compared to HI alone littermate controls.

Injury Score

In terms of brain injury assessment, again LPS pre-treatment led to a significant increase in microglial activation and neuronal cell loss in all five strains. C57BL/6 showed the highest overall increase as well as significant increase for all ipsilateral brain regions when compared to saline-treated controls. 129SVJ and BALB/c demonstrated only moderate significant LPS-sensitization. In the 129SVJ study only the dorsoparietal cortex was particularly affected. However, this was significant enough to lead to an overall significant increase in combined injury score for this strain. BALB/c background strain revealed only a trend towards increased brain injury associated with LPS pre-sensitization. Similarly to C57BL/6 animals, the CD1 and FVB strains were particularly affected by LPS administration prior to HI insult. Not only there was a significant overall increase in ipsilateral brain injury score in HI LPS animals, this was also significant in most assessed brain regions, with only exception being the dorsoparietal cortex in the CD1 LPS animals and external capsule white matter in FVB LPS animals, where only a trend towards increased brain injury was reached in the LPS treated animals when compared to saline littermate controls. Interestingly, saline-associated increase in brain damage was seen in FVB saline-treated animals, where there was a significant overall increase in alphaM staining and Nissl neuronal cell loss, when compared with HI alone controls. This saline damaging effect was also seen in most ipsilateral brain regions: pyriform cortex, striatum, thalamus and external capsule. All five background strains demonstrated minimal brain injury in the contralateral brain regions irrespective of type of insult (HI alone, HI saline and HI LPS).

TUNEL+ Cells

Once again, LPS administration prior to HI insult demonstrated a significant damaging effect. Quantification of DNA fragmentation-associated cell death using TUNEL+ cell assessment has shown that LPS pre-treatment led to a substantial increase in TUNEL+ cells in the ipsilateral brain hemisphere of all five

different background strains. Despite not reaching significance, 129SVJ LPS animals showed a visible trend in increased DNA fragmentation-associated cell death. At least three of the seven assessments (overall, dorsoparietal and pyriform cortex, hippocampus, striatum, thalamus and external capsule) were significantly more affected in BALB/c background strain, with four regions in both C57BL/6 and CD1 studies being particularly affected. The FVB strain demonstrated the most significant increase in TUNEL+ cells, where six of the seven different comparisons had significant increase in HI LPS-associated TUNEL+ cell death when compared to HI saline controls. Saline stress-associated effect is not seen in the TUNEL+ cell assessment. Contralateral brain hemisphere observation demonstrated no HI, HI saline or HI LPS-induced DNA fragmentation-associated cell death.

GFAP Immunoreactivity

Optical luminosity values also revealed LPS-mediated sensitization as causal to an increase in astrocytic activation. Both C57BL/6 and FVB background strains were overall particularly affected, with five out of the six individual ipsilateral brain regions also being significantly affected. This LPS-induced astrogliosis was also overall strongly evident in the CD1 and 129SVJ with three and four of the assessed brain regions being significantly affected respectively. Conversely, BALB/c animals revealed no LPS-mediated sensitization effect in astrocytic activation in the ipsilateral brain hemisphere when compared to saline controls. Assessment of the contralateral brain hemisphere revealed an LPS-mediated increase in astrogliosis only on the thalamus of C57BL/6 animals, and on four out of seven assessments in the contralateral brain regions of CD1 HI LPS animals when compared to saline littermate controls.

Ipsilateral saline-associated increase in astrogliosis only occurred in C57BL/6 animals. This was significant in most brain regions as well as overall. Observation of the contralateral brain hemisphere in all five strains showed no significant

difference between HI alone and HI saline littermates most strains, with only exception being C57BL/6 animals. In a similar manner to what was observed in the ipsilateral hemisphere, the same brain regions appeared to be detrimentally affected by saline administration: dorsoparietal and pyriform cortex, hippocampus, striatum and external capsule, as well as overall.

Discussion

The P7 mouse model of hypoxic-ischaemic insult is widely used as a model of neuropathology that mimics, to a certain degree of fidelity the pathology of hypoxic-ischaemic injury occurring in human term neonates. With the addition of transgenic mice, this model has become particularly relevant in attempting to establish the pattern of injury evolution and assessing efficacy of particular treatments. As advance in science and medicine have shown the aetiology of perinatal brain damage to be multifactorial and not only a result of hypoxic-ischaemic insult, the neonatal mouse model of hypoxic-ischaemic encephalopathy has been modified to include an inflammatory sensitizing element, known to occur in some human cases of perinatal brain damage.

Previous research have shown that genetic strain influences the degree of brain damage in neonatal mouse models of hypoxic (Li et al., 2008; Li et al., 2009; Li et al, 2013), ischaemic (Comi et al., 2013) and hypoxic-ischaemic (Sheldon et al., 1998) insults. Additionally, studies looking at inflammatory response in different mouse strains, once again demonstrated that genetic background plays a role in cytokine synthesis (Lambertsen et al., 2002; Hoover-Plow et al., 2008; Gibb et al., 2011; Browne et al., 2012) as well as inflammatory cell recruitment (White et al., 2002; Hoover-Plow et al., 2008). However, no study to date has investigated whether this variation in response is also seen in the synergistic model of inflammation and neonatal hypoxic-ischaemic insult. The current study has not

only been able to replicate previous research in regards to the differences in hypoxic-ischaemic response by different isogenic strains (Sheldon et al., 1998), but also to demonstrate that despite the consistent occurrence of LPS-mediated sensitization across the multiple strains used, the level of sensitization-mediated exacerbation in brain damage also differs between different genetic strains. Interestingly, some of the strains appeared to be affected by intraperitoneal administration of sterile 0.9% saline as a control. This novel finding can be of relevance, as saline treatment may not always represent a true control.

Hypoxia-ischaemia differs significantly between different mouse strains

Several ischaemia studies have demonstrated that cerebrovascular architecture differs not only between different mouse background strains (Barone et al., 1993; Fujii et al., 1997; Maeda et al., 1998; Maeda et al., 1999; Beckmann, 2000), but also within the same strain (Beckmann, 2000). Particularly, a study by Nicolau Beckmann (2000) using non-invasive magnetic resonance angiography (MRA) to detect differences in cerebrovascular anatomy in adult mice, has demonstrated not only a difference in arterial architecture between C57BL/6, CD1, CBA and 129SVJ mouse strains, but also within the CD1 strain (Beckmann, 2000). Both Fujii et al., 1997 and Maeda et al., 1998 have used intravascular perfusion of carbon black ink to describe cerebrovascular anatomical differences between C57BL/6 and 129SVJ inbred strains, where the former also described an incompletely formed circle of Willis in C57BL/6 mice (Fujii et al., 1997; Maeda et al., 1998). These studies suggested that this difference in cerebrovascular anatomy could explain, in part, the larger brain infarction seen in C57BL/6 compared to 129SVJ mice following both transient and permanent middle cerebral artery occlusion, as this artery supplies a significantly larger territory in C57BL/6 animals (Connolly et al., 1996). Another study comparing BDF, CFW and BALB/c strains' response to focal cerebral ischaemia, showed a difference in response between each of these strains. Furthermore, BALB/c mice were significantly more affected, not only in terms of cerebral infarction, but also in

increased mortality when compared to the other two (Barone et al., 1993). However, all aforementioned studies were performed in adult mice and used a different ischaemic insult methodology. Comi et al., 2009, used a more similar approach to the study performed in this project, where unilateral carotid ligation was performed in P12 CD1 and C57BL/6 mice. This group had previously shown that CD1 mice exhibited moderate to severe brain injury in the cortex, hippocampus, striatum and thalamus brain regions as well as seizure-related behaviour (Comi et al., 2004). In the more recent comparison study, it was observed that the C57BL/6 were much less vulnerable than CD1 mice to both brain injury and seizures (Comi et al., 2009). Similarly, a study looking at the different responses of adult C57BL/6 and CD1 mice in acute hypoxia has shown a strain-related difference in response to insult. Zwemer et al., 2006, described a higher tolerance and survival to acute hypoxic insult in the C57BL/6 strain when compared to CD1 mice. Indirect calorimetry as well as respiratory exchange ratio and fluorometric assay of plasma ketones showed that C57BL/6 and CD1 mice respond differently to hypoxic insult. Whereas CD1 animals lowered their mass-specific oxygen consumption and carbon dioxide production, C57BL/6 mice maintained both oxygen consumption and carbon dioxide production, but switched rapidly fuel to ketone metabolism. Despite lowering metabolic demand being considered generally more efficient, it appears that maximizing oxygen usage may be more advantageous in an episode of acute severe hypoxia (Zwemer et al., 2006).

The only other study to date to have looked at the effects of hypoxia-ischaemia in multiple mouse strains was conducted by Sheldon and colleagues, where histological assessment conducted five days after P7 hypoxic-ischaemic insult revealed that 129SVJ mice had minimal brain injury, in both 30min and 60min set-ups. The same study showed that C57BL/6 animals had moderate brain damage following 30min hypoxia, which was increased when animals underwent 60min hypoxia (Sheldon et al., 1998). This C57BL/6 increased susceptibility to a

more severe hypoxia was also seen in a study by Kendall et al. 2006 where 48h post-HI insult, animals that underwent 60min hypoxia following unilateral carotid occlusion, had substantially more brain damage than the 30min hypoxia group (Kendall et al., 2006). Sheldon et al. 1998 also showed that out of the three assessed strains, C57BL/6, 129SVJ and CD1, the latter was the most sensitive strain to 30min hypoxic-ischaemic insult. Interestingly, CD1 mice showed no increase in brain damage after 60min hypoxia; however this strain displayed an increase in death post hypoxic-ischaemic insult (Sheldon et al., 1998).

Similarly to what was seen in both neonatal and adult ischaemic insults, adult hypoxic and neonatal hypoxic-ischaemic studies, this study has shown that there is a strain-dependent variation response to hypoxic-ischaemic insult. This project has shown that 48h post-HI, the most sensitive strain appears to be the FVB strain. Furthermore, the same trend is seen between this and previous studies, where CD1 animals appeared to be the second most affected strain, with higher degree of infarction, followed by BALB/c, C57BL/6 and lastly 129SVJ. In this particular study, histological assessment of brain damage looking at tissue infarction, combined injury score, TUNEL-associated cell death and astrogliosis has shown that both inbred strains C57BL/6 and 129SVJ have the least brain damage in response to hypoxic-ischaemic insult alone. In respect to individual assessments, C57BL/6 animals had an average of 15% tissue infarction, 1.5 combined injury score, 2.7 TUNEL+ cell counts and a minimal optical luminosity value (OLV; GFAP immunoreactivity) of 22.3. 129SVJ animals were even further resistant to hypoxia-ischaemia alone, with 3%, 0.1, 0.0 and 21.9 respectively. However, this response was moderately increased in BALB/c animals, where there was an overall increase in infarction to 21%, combined alphaM and Nissl injury score to 2.3, 21 TUNEL+ cells and 53.6 OLV. There was a further increase in brain damage seen in the CD1 and FVB outbred strains. CD1 mice demonstrated an infarct volume of 46%, injury score of 4.7; TUNEL+ counts of 27.3 and OLV of 63.9. Similarly, FVB animals demonstrated an elevated increase in infarction

(41%), combined injury (2.5), TUNEL+ cells (70.5) and GFAP immunoreactivity (90.2). Interestingly, contrary to what was observed previously (Sheldon et al., 1998) and despite this varying degree of brain damage, none of the animals used in this study succumbed to the experimental design, irrespective of strain background.

Saline-stressor effect in the different strain backgrounds

Interestingly, different assessments performed in this study have shown a saline injection-associated susceptibility in different strains as seen in the C57BL/6 GFAP immunoreactivity assessment, where this effect was also observed in the contralateral hemisphere. Combined microglial activation and neuronal cell loss injury score assessment in FVB mice also showed a saline-associated increase in damage. As the saline used in the experimental design was obtained from the BSU veterinary specifically as an intravenous infusion, therefore removing the risk of endotoxin contamination, it was concluded that other stress factors were causing this sensitivity to sterile saline administration.

Stressful events are known to cause short and/or long-term adverse effects both in terms of behavioural but also in neurochemical outcomes, including influencing circulating and brain cytokine activity (Gibb et al., 2008; Audet et al., 2010). Furthermore, individual animals are known to demonstrate different sensitivity to stress, and this difference in response is also noticeable between different animal strains, where BALB/c animals are known to be highly stress reactive in comparison to the C57BL/6 strain (Anisman et al., 2001; Anisman et al., 2008). In a study where exposure to a dominant mouse was used as a social stress alone or in combination with different acute immune challenges to investigate immune response, it was shown that social stressor combined with saline administration led to an increase in the production of plasma corticosterone in both C57BL/6 and BALB/c animals when compared to respective no stress groups. Assessment of hippocampal expression of IL-1, IL-6

and TNF α cytokines showed a trend in increased IL-6 expression in C57BL/6 animals that again underwent stress challenge combined with saline administration (vehicle). Furthermore, both no stress and stress C57BL/6 groups had a higher level of IL-6 cytokine production when compared to respective BALB/c groups. As expected, the increase in corticosterone and cytokines was visibly-significantly higher in animals, both C57BL/6 and BALB/c, that were also subjected to LPS administration instead of saline (Gibb et al., 2011). Another study used forced swim-stress combined with peripheral administration of either LPS or saline as a control. C57BL/6 and BALB/c mice were forced to swim 15min instead of the usual 6min, thus leading to an effective physical and psychological stressor in rodents (Connor et al., 1997). It was demonstrated that the forced-swim stressor alone (plus saline) significantly raised corticosterone levels in both C57BL/6 and BALB/c mice. There was also a small increase in hippocampal IL-1 β mRNA expression in BALB/c swim-stress animals. This study also demonstrated LPS-mediated upregulation of IL-1 β and TNF α in both strains, but to a greater extent in the BALB/c animals, which interestingly was attenuated in the presence of swim-stressor, thus indicating an altered immune activation in BALB/c animals when compared to the more normo-sensitive C57BL/6 strain (Browne et al., 2012). Therefore, it is possible that in the current study, the stress associated with night-time separation from the dam together with intraperitoneal administration of saline could have an effect in the immune response, which is seen in the assessment of immune-associated markers for astrogliosis and microglial activation of C57BL/6 and FVB strains.

Interestingly, the psychological and/or physical stress studies aforementioned have demonstrated that great care is required when creating an experimental design involving an immune response (Gibb et al., 2011; Brown et al., 2012). These studies have demonstrated that multiple factors, including environmental changes can lead to an upregulation of the immune system, which can then sensitize the organism to subsequent insults such as LPS and hypoxia-ischaemia.

It was also shown that a same stress-factor that could exacerbate the response to a subsequent immune challenge could instead dampen it depending on the background strain used (Browne et al., 2012). Subsequently, the present study has also shown how relevant it is to understand the importance of using a proper control, and also to understand, that the use of saline, or possibly any other vehicle does not always equate to a controlled unadulterated response.

Strain impact on combined inflammation and hypoxic-ischaemic insults

As the principal aim of this study was to understand the role of inflammation and the individual members of the TNF cluster of cytokines using multiple single gene knockout strains, it was decided to first address if the variability seen in hypoxic, ischaemic and hypoxic-ischaemic models would also occur in the combined inflammation and hypoxia-ischaemia model, and if so, could this have an effect in the interpretation of results.

This study, as well as previous research (Hagberg et al., 2002; Mallard et al., 2003; Kendall et al., 2011b; van de Looij et al., 2012), has shown that bacterial endotoxin successfully sensitizes the neonatal brain to subsequent hypoxic-ischaemic injury, as all five strains, C57BL/6, 129SVJ, BALB/c, CD1 and FVB demonstrated strong LPS-mediated sensitization to subsequent hypoxic-ischaemic insult. Additionally, it was shown that the mouse strain background used has an effect in the degree of brain damage. LPS pre-treatment significantly increased tissue infarction, microglial activation, cell death and astrogliosis in the C57BL/6 strain. 129SVJ also demonstrated an overall significant increase in tissue infarction, microglial activation and neuronal cell death injury score, and astrogliosis; with also a visible trend towards increased TUNEL+ cell quantification after LPS treatment. BALB/c mice also showed an overall trend towards increased brain damage in the LPS group; however this was only significant in the TUNEL assessment. CD1 mice also showed substantial increase in brain damage associated with LPS administration; this was particularly evident

in terms of tissue infarction and TUNEL+ cell quantification. Finally, FVB LPS-treated mice showed maximal brain damage, with significant increase in tissue loss, microglial activation and neuronal cell loss brain injury and DNA fragmentation-associated cell death. Despite LPS-associated increase in astrocyte activation only demonstrating an overall trend, astrogliosis was still significantly increased in 50% of the assessed brain regions for the FVB strain.

After initial observation of strain-mediated variation in response to insult and correlating these observed differences, at least partially, with anatomical differences, particularly in cerebrovascular architecture, further studies looked also at inflammatory effect and variation between different genetic mouse strains. Cytokines are an integral part of the inflammatory response and are upregulated following both neonatal and adult insult. In an adult mouse study looking at the difference between C57BL/6 and BALB/c in the synthesis of the pleiotropic cytokine TNF α within the focal and border zone of cerebral infarction has demonstrated that degree of TNF α synthesized by microglia-macrophages is strain dependent. However, this variation in cellular TNF α production is not present in terms of time-related response and cellular source. Interestingly, this group has shown that BALB/c mice had larger infarct area with significantly fewer TNF α -producing microglia (Lambertsen et al., 2002). TNF α is a known activator of the vascular endothelium, T cells, B cells (Ettinger et al., 1998), as well as stimulator of adhesion molecules and chemokines culminating in infiltration of blood-borne leukocytes into the CNS (Sedwig et al., 2000). Additionally, this cytokine is an endogenous pyrogen, inducer of cell death (Gaur and Aggarwal, 2003) and further activates microglia and macrophages stimulating not only its own production, but also that of other pro-inflammatory cytokines such as IL-1 β and IL-6, as well as nitric oxide (Kendall et al., 2011b), all known mediators of cerebral ischaemia. This finding could potentially explain the results seen in the current project where, despite an obvious increase in brain damage in the BALB/c strain, the immune profile, as seen through the combined injury score

and astrogliosis assessments, never reached significance in the animals pre-treated with LPS when compared to saline-treated controls, as opposed to what was seen in the C57BL/6, CD1 and FVB strains. Genetic differences can cause a variation in the sensitivity to insult and inflammation and alteration in the balance between pro- and anti-inflammatory cytokines, and consequently affect the microglial and astroglial immune response, activation and recruitment of inflammatory cells.

However, both this current (48h) and the Lambertsen et al., (12h and 24h) studies have only looked at assessments post-insult. A study where TNF α was inhibited prior to 1h middle cerebral artery occlusion in CD1 mice, showed that 23h later the infarct area of mice pre-treated with monoclonal neutralizing anti-murine TNF α antibody was significantly smaller than that of vehicle-treated mice. Additionally, expression of intracellular adhesion molecule-1 (ICAM-1) was also significantly reduced in the inhibited group (Yang et al., 1998). The same reduction in infarction was observed in BALB/c mice pre-treated with TNF receptor linked to polyethylene glycol - which binds to and inhibits TNF α - prior to permanent middle cerebral artery occlusion (Nawashiro et al., 1997). Conversely, hybrid C57BL/6x129 mice, which were deficient in TNF receptors, demonstrated higher focal cerebral infarction, and epileptic seizures following focal cerebral ischaemia (Bruce et al., 1996; Gary et al., 1998). These different studies show a TNF α modulatory effect on ischaemic-induced brain damage irrespective of strain background. Therefore, despite a reduced number of TNF α synthesizing microglia seen in BALB/c animals after ischaemic insult (Lambertsen et al., 2002) correlating to a less significant increase inflammatory response in the current combined inflammation and hypoxic-ischaemic study, it has been shown that this does not equate to a correlation in reduced infarct size, as albeit not significant, there was a substantial infarction in the BALB/c LPS group. The same substantial increase in tissue necrosis was also observed in the other assessed strains, which could be an indicative that if the different strains have a

genetic varying effect on TNF α synthesis, this occurs only hours later and not immediately after insult.

Of all five strains assessed (C57BL/6, 129SVJ, BALB/c, CD1 and FVB), the one that appears to be the least affected is the 129SVJ. In the current study, both HI alone and HI+SAL groups have demonstrated minimal response, and despite a substantial to significant increase in damage-associated markers, this increase was generally smaller than what was observed in the other four strains. This reduced response has been explained in part due to the cerebrovascular anatomical differences observed in 129SVJ mice (Fujii et al., 1997; Maeda et al., 1998; Beckmann, 2000). However, more recent studies looking at the immune profile of different mouse strains have shown that 129SVJ strain has a defect in inflammatory cell recruitment (White et al., 2002; Hoover-Plow et al., 2008). White et al. 2002, administered thioglycollate medium into the peritoneum of both male and female C57BL/6, 129SVJ, BALB/c and CD1 mice to test inflammatory cell recruitment 4 days after injection. All four strains revealed different total cell recruitment, where the C57BL/6 demonstrated to be the most responsive, followed by BALB/c and CD1, and 129SVJ mice appeared to be the least affected. Further assessment on both C57BL/6 and 129SVJ mice using multiple time points (4h, 1d, 2d, 3d, 4d and 5d) revealed that both strains had similar neutrophil recruitment at 4h post-inject, with significant macrophage recruitment appearing only 24h after injection. Interestingly, 129SVJ had highest cell count 4h post-injection with numbers falling progressively at later time points. The opposite effect was seen in the C57BL/6 strain, where the total number of cell counts rose steadily up to 48h following injection, where it reached a plateau up to 4d post-injection with gradual reduction started at day 5 (White et al., 2002). In a study by Hoover-Plow et al. 2008, thioglycollate was also used as an inflammatory stimulus in A/J, C57BL/6 and 129SVJ animals. In this study, A/J animals had the lowest number of recruited leucocytes and macrophages, followed by 129SVJ animals, when compared to C57BL/6 mice

(Hoover-Plow et al., 2008). A study by Medina and North, further confirms this reduced 129SVJ ability to recruit macrophages in a model of *Mycobacterium tuberculosis*, where 129SVJ mice were significantly more susceptible than C57BL/6 animals (Medina and North 1998). Therefore, this observed reduced macrophage recruitment and resistance to *M. tuberculosis* further demonstrates defective macrophage recruitment and/or function, which could further explain its diminished response not only to HI alone and HI+SAL, but also in the LPS ability to induce as robust an immune response when compared the other four strains.

C57BL/6 as the optimal strain in combined inflammation and hypoxic-ischaemic insult

C57BL/6 and 129SVJ are the most commonly used parent strains in the creation of transgenic mice. As explained above, both C57BL/6 and 129SVJ had minimal response to either HI alone or combine HI and saline treatments. However, C57BL/6 showed a higher degree of LPS-mediated brain injury. This is in concordance with previous findings. A study by Maeda et al. has shown anatomical differences in adult C57BL/6 and 129SVJ inbred strains. Location of anastomoses was determined using carbon black stained latex showed that C57BL/6 animals had a larger vascularised area supplied by the middle cerebral artery, which could potentially explain the larger infarct volume in these animals when compared to 129SVJ after middle cerebral artery occlusion (Maeda et al., 1998). Furthermore, inflammation cell recruitment studies have demonstrated a reduced 129SVJ strain ability to recruit immune cells (White et al., 2002; Hoover-Plow et al., 2008) and produce TNF α (Lambertsen et al., 2002), a pro-inflammatory cytokine that is well established as playing a role in this particular type of insult. This makes the use of the 129SVJ strain less than desirable in this particular study, where the objective is to further understand the role of the individual members of the TNF cluster of cytokines in sensitizing the immature brain to subsequent hypoxic-ischaemic insult.

BALB/c animals, although responsive to LPS sensitization, only had a moderate increase in the studied damage-associated markers when compared to HI alone and HI+SAL. Additionally, its baseline response to both controls was relatively high, thus again making this strain less than ideal for the current project as the purpose is to require LPS administration in order to elicit a response. This same principle excludes both CD1 and FVB animals, as their baseline response (HI alone and HI+SAL) were too high. Furthermore, C57BL/6 strain appears to be the most well characterised isogenic strain, which allows for better understanding of its response to different types of insult. Therefore, for the purpose of this research, it was decided to maintain animals whenever possible in a C57BL/6 background, as this particular background demonstrated a sufficiently high response to LPS sensitization, not only across all four histological assessments, but also within the six different forebrain regions being assessed. This choice thus would allow clear comparison between LPS and saline (control) groups, with the added benefit that the chosen strain - C57BL/6 - is not sensitive enough to have a baseline response to the moderate 30min hypoxic-ischaemic insult. Furthermore, keeping the strains in this background would avoid unnecessary rebreeding and crossing of animals to a new background, thus improving our use of the 3Rs as, with the exception of NUDE animals, all other transgenic strains were obtained in the C57BL/6 background.

Conclusion

This study not only has been able to reproduce similar results to previous work looking at the effects of using different mice strains in different insults, it has also further consolidated that genetic background influences response to both a hypoxic-ischaemic as well as an immune-sensitized hypoxic-ischaemic injury. The observed differences in response to such insults could assist in future studies attempting to explain the high variation in the neonatal mouse model of hypoxia-ischaemia as well as foetal/neonatal response to inflammation and

reduced oxygen-blood supply, as it has been established that careful selection of the experimental strain is required. Cerebral blood vascularity and immune sensitivity are known factors involved in response to insult, which appear to also play a role in this synergistic model. However, other factors such as activation of early transcription factors such not be excluded as possible denominators in perinatal brain damage resulting from both hypoxia-ischaemia and combined inflammation and hypoxia-ischaemia injury, and therefore should be investigated in future studies. Furthermore, great care is required when considering the control groups, as this study has shown that saline, a commonly used vehicle, may also elicit an immune response, possibly due to stress-associated activation of the immune system.

CHAPTER 4: THE ROLE OF TNF α , LT α AND LT β CYTOKINES IN ENDOTOXIN-MEDIATED NEONATAL HYPOXIC-ISCHAEMIC BRAIN DAMAGE

Abstract

Infection and hypoxic-ischaemic (HI) insult share inflammatory mechanisms. However, because live infection is frequently a too rapid and catastrophic event, the pathophysiological effects of infection are normally modelled using some standard pathogen such as endotoxins, particularly the *E. coli* lipopolysaccharide (LPS). Systemic injection of LPS up-regulates inflammation-associated molecules, including the TNF cluster of pro-inflammatory cytokines. Our group has explored the role of this cluster in terms of the LPS signalling pathway, and shown that its deletion abolishes LPS sensitization to HI.

In this study, we wanted to investigate individual effects of the TNF α , LT α and LT β cluster members, using either global gene deletion, or macrophage-specific deletion of TNF α with MLys::Cre. P7 littermate wild type and homozygous knockout offspring of heterozygous mice underwent HI insult, consisting of carotid occlusion followed by 30min 8% oxygen exposure. 12h prior, 0.6 μ g/g LPS or saline was administered intraperitoneally.

LPS pre-treatment resulted in significant neuronal injury and infarct, inflammation, cell death and astrogliosis in wild type littermates of all four experimental groups. LT α homozygous mutants revealed a clear reduction in LPS-mediated sensitization. Conversely, global LT β deletion had a detrimental effect, with significantly increased brain damage. TNF α ^{-/-} showed a trend towards greater damage, but deletion just in MLys⁺ macrophages was strongly protective, pointing to a dual role for the TNF α gene depending on which cell-type it is expressed.

Lymphotoxin α deletion results in a marked reduction in brain damage following combined endotoxin and hypoxic-ischaemic insult

Generation of mutants lacking $LT\alpha$ gene

Animals homozygous for the deletion of the $LT\alpha$ gene were bred against animals that were WT with a C57BL/6 background. F1 generation obtained from this breeding allowed setting up of heterozygous breeding pairs. F2 offspring mice received a single intraperitoneal injection of either 0.6 μ g/g LPS, or saline as a control, 12h prior HI insult. 48h post HI insult, mice were transcardially perfused, and after PCR genotyping (see figure 4.1), only WT and homozygotes for $LT\alpha$ gene deletion were used for further investigation.

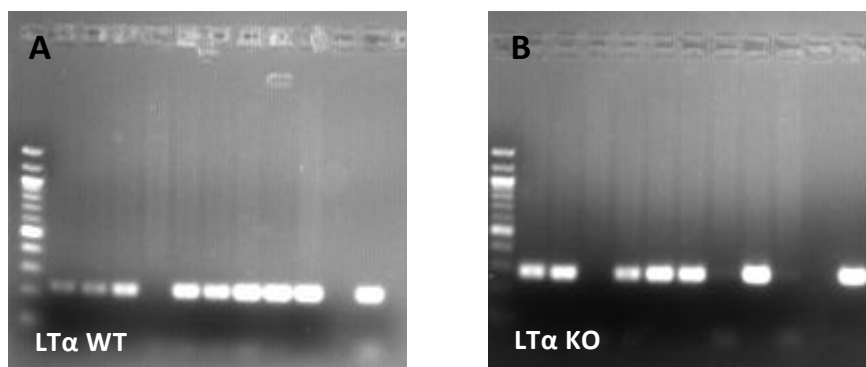


Figure 4.1: Generation of $LT\alpha^{+/+}$, $LT\alpha^{+/-}$ and $LT\alpha^{-/-}$ -mice. Global deletion of the $LT\alpha$ gene was achieved using a heterozygous breeding strategy. Agarose gel electrophoresis following PCR reaction allowed identification of the different genotypes through visualization of the fragment band sizes: $LT\alpha$ wild type allele at 229bp (A) and $LT\alpha$ mutant allele at 280bp (B). If an animal had both wild type (+/+) and knock out alleles (-/-) PCR product present, then it was a heterozygote (+/-) for the mutation.

$LT\alpha$ global gene deletion decreases tissue loss

As demonstrated by other studies (Kendall et al., 2011b) as well as in the previous study (chapter 3), LPS sensitization prior to HI insult leads to a significant overall increase in brain tissue damage in WT animals, including the C57BL/6 strain. Comparison between WT saline (n=10) and WT LPS (n=10) treated animals using total and regional measurements of infarct size as percentage of forebrain as ipsilateral/contralateral volume has shown a total

increase in tissue loss from $5\pm 2\%$ to $23\pm 5\%$ ($p < 1\%$). This was significant in the dorsoparietal cortex (from $5\pm 1\%$ to $25\pm 6\%$, $p < 5\%$), hippocampus (from $12\pm 4\%$ to $43\pm 7\%$, $p < 1\%$), striatum (from $7\pm 3\%$ to $22\pm 5\%$, $p < 5\%$), thalamus (from $-2\pm 2\%$ to $7\pm 3\%$, $p < 5\%$) and external capsule white matter (from $1\pm 2\%$ to $33\pm 10\%$, $p < 1\%$) brain regions. Furthermore, WT animals pre-sensitized with LPS endotoxin demonstrated the highest infarct volume of all four experimental groups: WT saline, WT LPS, $LT\alpha$ KO saline and $LT\alpha$ KO LPS, with a total tissue loss of $23\pm 5\%$. There was minimal tissue damage in $LT\alpha$ KO mice injected with saline ($4\pm 2\%$, $n=10$), with trend towards increase in KO animals pre-treated with LPS ($15\pm 5\%$, $n=10$). However, this difference did not reach significance. Comparison between the two LPS groups – $LT\alpha$ WT and $LT\alpha$ KO – revealed that the mutants had less necrotic tissue, with significant decrease in infarct size from $25\pm 6\%$ to $17\pm 7\%$ ($p < 5\%$) in the dorsoparietal cortex brain region (see figure 4.2.A).

$LT\alpha$ -/- animals have reduced injury response to combined LPS and HI insults

Once again, histological analysis confirmed the results previously observed in the WT C57BL/6 mice where pre-sensitization with bacterial endotoxin resulted in a marked increase in injury response on the ipsilateral brain hemisphere. WT animals pre-treated with LPS displayed a significant increase in brain injury and cell loss in comparison to WT saline-treated littermates (2.1 ± 0.5 vs. 0.4 ± 0.1 , $p < 1\%$). This was also significant in most brain regions: dorsoparietal (3.5 ± 0.9 vs. 0.2 ± 0.1 , $p < 1\%$) and pyriform cortex (0.6 ± 0.2 vs. 0.1 ± 0.1 , $p < 5\%$), hippocampus (3.8 ± 0.5 vs. 0.9 ± 0.2 , $p < 0.1\%$), striatum (1.3 ± 0.4 vs. 0.4 ± 0.2 , $p < 5\%$) and external capsule (2.4 ± 0.5 vs. 0.4 ± 0.3 , $p < 1\%$). Likewise, when LPS was administered to mice lacking both copies of the $LT\alpha$ gene, these showed a clear up-regulation in inflammation when compared to saline-treated KO animals (1.4 ± 0.4 vs. 0.4 ± 0.2 , $p < 5\%$). Additionally, $LT\alpha$ mutants showed a clear decrease in LPS sensitization-mediated response to HI in comparison to $LT\alpha$ WT animals. Microglial inflammation and cell death was markedly reduced in the pyriform cortex (from

0.6±0.2 to 0.1±0.1, p<5%) and in the hippocampus (from 3.8±0.5 to 2.5±0.6, p<5%) (see figure 4.2.B).

Ltα deletion reduces TUNEL+ cells

Neural cell death associated with nuclear DNA fragmentation was also studied in this project. The dorsoparietal and pyriform cortex, hippocampus, striatum, thalamus and external capsule white matter regions on both ipsilateral and contralateral brain regions were quantified for TUNEL positive cells. As previously demonstrated (see chapter 3) WT littermate animals pre-sensitized with LPS demonstrated higher TUNEL counts in the ipsilateral brain hemisphere when compared to WT saline-treated littermates. This increase was particularly significant in the dorsoparietal and pyriform cortex brain regions, with the number of TUNEL positive cells increasing from 12.8±2.8 to 152.4 (p<1%) and from 5.3±0.8 to 24.6±13.1 (p<5%) respectively. Comparison between the mutant groups revealed that LPS LTα KO animals were also more affected than KO littermates pre-treated with saline. This was principally evident in the hippocampus, where the number of TUNEL positive cells increased from 30.2±7.4 to 185.9±56.5 (p<1%). With the exception of the hippocampus and thalamus, there were generally less TUNEL+ cells in the LPS LTα mutants in comparison to WT LPS littermate controls. However, significance reduction was only reached in the pyriform cortex, where the number of TUNEL-associated cell death decreased from 24.6±13.1 to 5.0±1.7 (p<5%) (see figure 4.2.C).

Astrogliosis response to LPS pre-sensitization is not altered in LTα mutants

The final assessment performed in this study was observation of the levels of astrogliosis using optical luminosity measurement, 48h after combined inflammation and HI injury. In a manner similar to what was shown in the strain comparison study (chapter 3), bacterial endotoxin pre-sensitization of the WT littermates led to a clear increase in astrocyte immunoreactivity when compared to WT saline controls, with an average luminosity intensity of 26.0±3.1 in the WT

saline versus 39.8 ± 5.4 in the WT LPS group ($p < 1\%$). Separate analysis of the brain regions revealed that this increase was also significant in the dorsoparietal (from 19.8 ± 1.3 to 39.8 ± 5.9 , $p < 1\%$) and pyriform cortex (from 19.8 ± 2.1 to 26.6 ± 5.8 , $p < 5\%$), striatum (from 18.2 ± 2.3 to 41.1 ± 12.0 , $p < 5\%$) and external capsule (from 40.2 ± 4.3 to 54.3 ± 4.2 , $p < 5\%$). Similarly, observation for the $LT\alpha$ mutant groups revealed a significant increase in astrogilosis in the external capsule (from 44.4 ± 3.8 to 57.0 ± 4.9 , $P < 5\%$) as well as overall (from 27.8 ± 2.6 to 39.5 ± 6.0 , $p < 5\%$) on the $LT\alpha$ LPS KO animals. However, global deletion of the $LT\alpha$ gene did not alter astrocytic activation in the presence of LPS sensitization when compared to their WT LPS littermates (see figure 4.2.D).

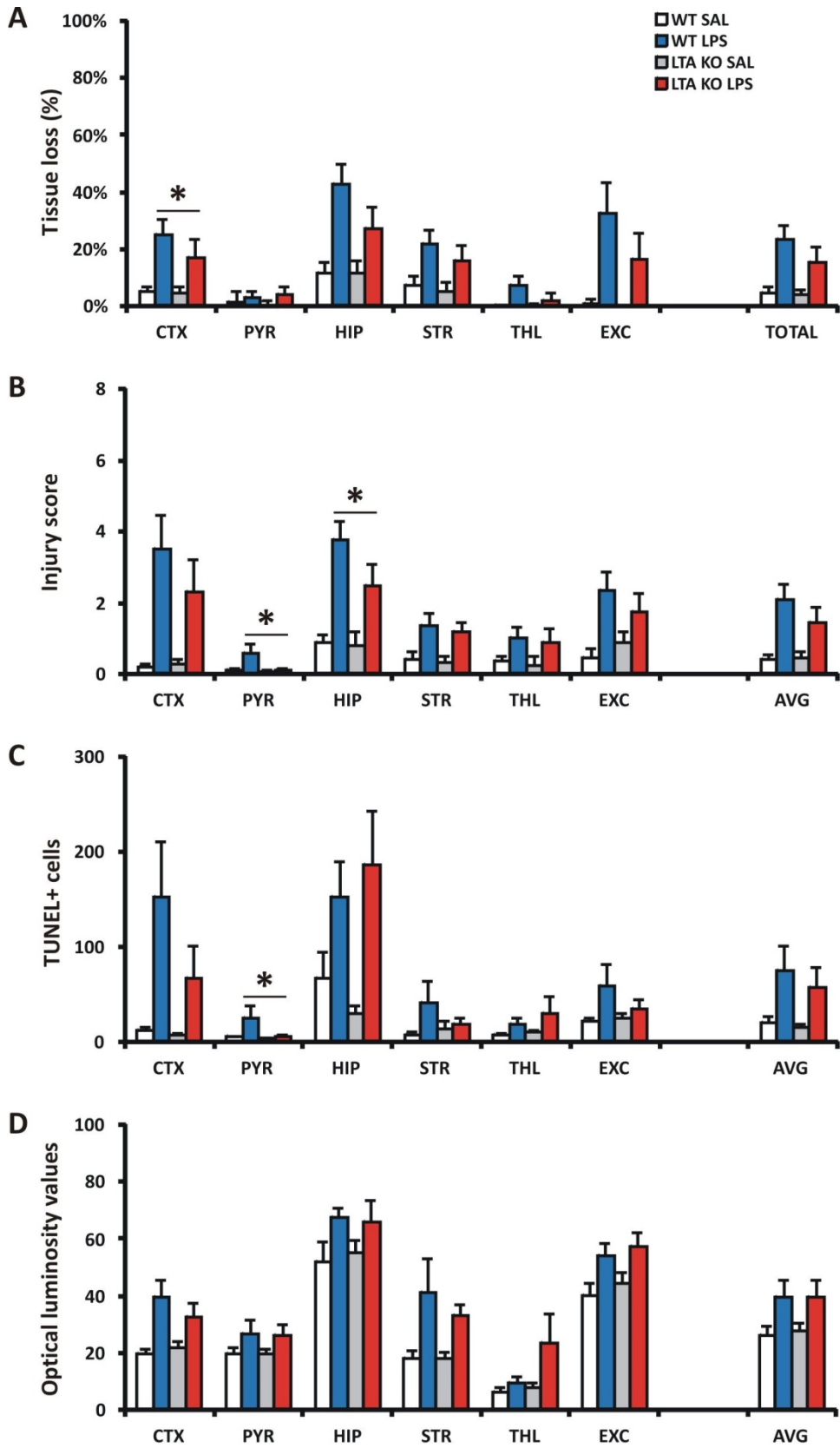


Figure 4.2: Effects of LT α global gene deletion on neonatal hypoxic-ischemic brain injury after pre-exposure to 0.6 μ g/g LPS endotoxin (LPS). Animals injected with saline (SAL) served as controls. **A:** Infarct volume assessment of individual forebrain regions and total ipsilateral

hemisphere infarction as percent of contralateral hemisphere, showed that compared to wild type LPS-treated littermates, global deletion of LT α gene led to a 50% reduction in tissue infarction, which reached significance in the dorsoparietal cortex. **B:** Brain injury score assessment of LT α gene deletion revealed an overall 50% reduction in microglial inflammatory response and neuronal cell death in the LPS knockouts compared to wild type LPS littermates. This reduction in brain injury score reached significance in the pyriform cortex and hippocampus ipsilateral brain regions. **C:** DNA fragmentation cell death assessment using TUNEL+ cell quantification demonstrated an overall decrease in LPS-mediated cell death in the LT α gene deficient mice with significant reduction reached in the pyriform cortex. **D:** Astroglial immunoreactivity assessment using optical luminosity values showed a minor decrease in GFAP synthesis in the LPS LT α knockouts when compared to LPS wild type littermates. Contralateral hemisphere not shown. * $p < 5\%$ in Mann Whitney test for the LPS-treated wild type vs. knockout groups, $n=10$ per group. **Abbreviations:** WT – wild type, KO – knockout, CTX- dorsoparietal and PYR – pyriform cortex, HIP – hippocampus, STR – striatum, THL – thalamus, EXC – external capsule.

Lymphotoxin β gene deletion has a detrimental role in the neonatal synergy model of endotoxin and hypoxia-ischaemia brain injury

It was originally believed that the animals received from Jackson laboratory for this study were homozygous for the global deletion of the LT β gene, as well as on the C57Bl/6 background. The P6 offspring of the homozygous breeder pairs were randomly allocated to receive LPS endotoxin and saline followed by HI insult 12h later, and allowed 48h survival. Their response to combined LPS and HI insult was compared to offspring of commercially provided C57BL/6 non-littermate mice that underwent the same type of injury. Previous studies established that C57BL/6 LPS-treated mice ($n=13$) showed endotoxin-sensitization and up-regulation of brain injury, whereas C57BL/6 saline animals ($n=13$) demonstrated minimal brain injury (3.1 ± 0.4 vs. 1.0 ± 0.2 , $p < 5\%$) (see figure 4.3.A).

However, in depth assessment of the LT β strain through PCR genotyping revealed that some of the animals used as breeding pairs were in fact heterozygous for the LT β gene. Therefore all three genotypes – WT, HET and KO – were inadvertently present in the F1 generation used in this first study. Comparison of all three different LT β genotypes showed that LPS-sensitization was lost in all three LPS groups (see figure 4.3.B). This preliminary study onto the LT β cytokine role in combined LPS+HI injury was performed by Drs Mariya Hristova, Giles Kendall and Sigrun Lange.

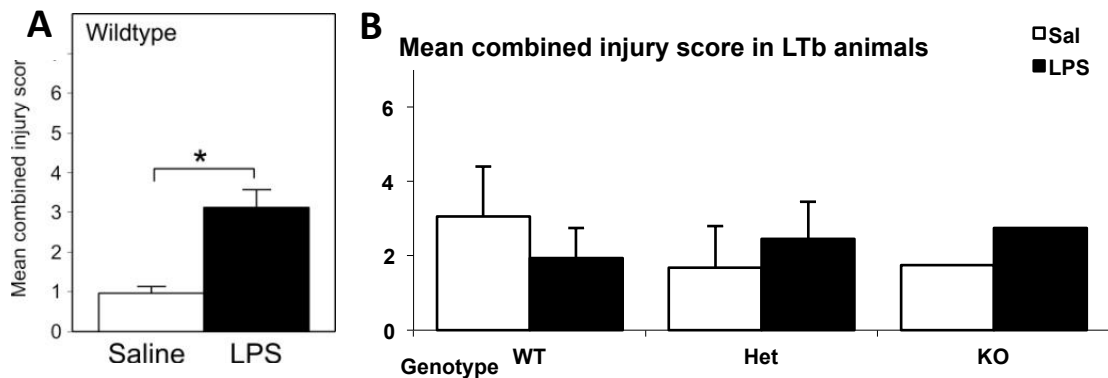


Figure 4.3: Overall and mean combined injury score of $LT\beta$ homozygous and heterozygous breeding. Overall brain injury scores (A) and combined injury score across the different forebrain regions (B). LPS administration in WT mice (n=13) led to sensitization of the neonatal brain to HI insult with significant increase in brain injury, whereas WT saline animals (n=13) had minimal brain injury (A). $LT\beta$ null animals did not show significant difference in the mean combined injury score in comparison to littermate saline-treated animals (B). *p<5% in one way ANOVA followed by post-hoc Tukey. **Abbreviations:** WT – wild-type, HET – heterozygous, KO – knockout. Graph reproduced with permission.

Heterozygous $LT\beta$ (+/-) breeding strategy

After observing that WT mice obtained in $LT\beta$ heterozygous breeding were non-responsive to endotoxin sensitization for HI injury, we turned to the working hypothesis that some strains can be more resistant to this combined insult, as seen on chapter 3. We first repeated the experiment by re-crossing only the $LT\beta$ heterozygote animals and used the F1 offspring containing both WT (controls) and KO littermates.

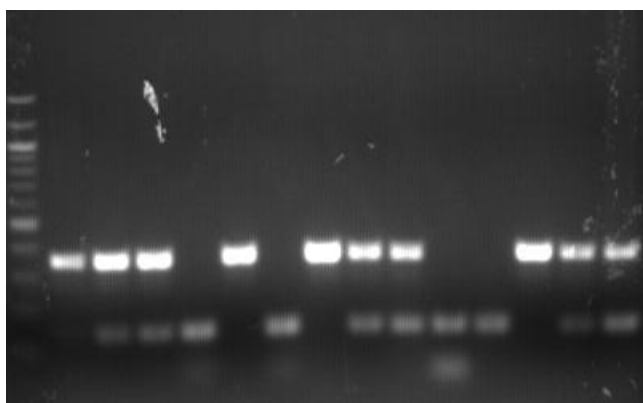


Figure 4.4: Generation of $LT\beta^{+/+}$, $LT\beta^{+/-}$ and $LT\beta^{-/-}$ mice. $LT\beta$ mice were bred in a heterozygous fashion in order to obtain $LT\beta^{+/+}$ and $LT\beta^{-/-}$ littermates. PCR reaction products were run on an agarose gel electrophoresis apparatus to detect the different band sizes. $LT\beta$ wild type allele has 120bp, whereas the $LT\beta$ mutant allele has a product size of 330bp. $LT\beta^{+/-}$ genotype had both wild type and mutant alleles present.

For this experiment, a new batch of LPS was required, with an optimal sensitization dose of 0.6µg/g (see chapter 2). Combined assessment of microglia activation and neuronal cell loss in animals that had undergone HI insult at P7 with pre-treatment of LPS and saline 12h prior revealed again a resistance in LPS sensitization on the WT group. The KO animals showed a trend towards increase in injury in some of the ipsilateral brain regions upon application of LPS, but this never reached statistical significance (see figure 4.5).

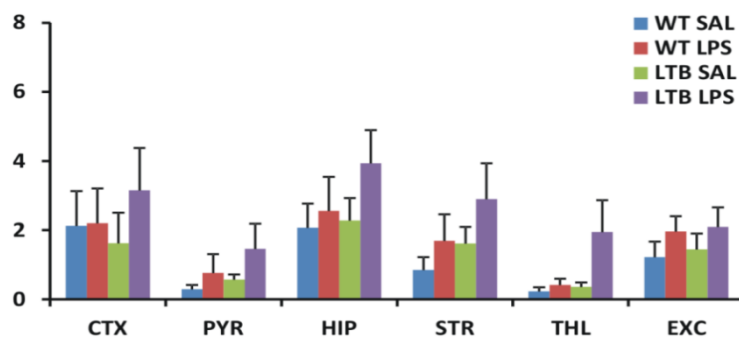


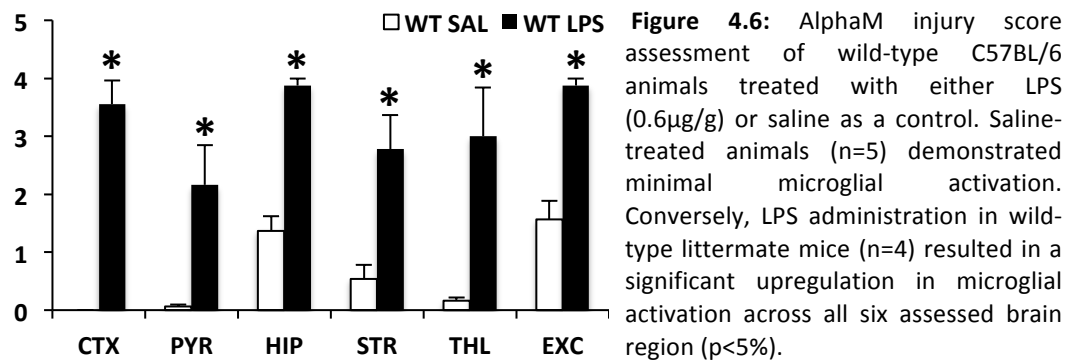
Figure 4.5: Combined injury score in WT and LTβ KO littermates pre-sensitized with saline and LPS (0.6µg/g) 12h before neonatal HI injury. LPS administration in WT mice (n=10) did not sensitize the neonatal brain to HI in comparison to WT saline-treated animals (n=10). LTβ

KO animals (n=10) showed a trend towards increase in the mean combined injury score in comparison to their WT LPS littermates. Saline KO animals (n=10) demonstrated minimal brain injury. The Y-axis shows brain injury score based on extent of infarction and microglial macrophage response. One way ANOVA test followed by post-hoc Tukey. *Abbreviations:* WT – wild-type, CTX - dorsoparietal and PYR - pyriform (PYR) cortex, HIP - hippocampus, STR - striatum, THL - thalamus (THL), EXC - external capsule.

Crossing of heterozygous LTβ-KO with commercially-provided C57BL/6

To address the issue with the non-responsiveness to LPS administration in the wild type littermates produced through heterozygous LTβ-breeding, the current LPS batch was tested to check whether or not it had lost its sensitizing property. AlphaM assessment demonstrated that this was not the case as there was significant microglial activation in all assessed brain regions (see figure 4.6). This finding raised two main concerns. Was this reduction caused by difference in strain background or due to gene deletion? If non-responsiveness was a result of strain background differences, this change in response is probably a dominant trait, as further crossing of the animals did not remove this lack of endotoxin sensitization. In an attempt to address this new hypothesis, F2 heterozygous mice were re-crossed with commercially provided C57BL/6 mice, shown to be

strong responders to endotoxin sensitization (see chapter 3 as well as figure 4.6). This procedure introduced a 50% C57BL/6 background into the strain. F1 offspring heterozygotes were crossed and the F2 generation mice that were either WT or homozygous for $LT\beta$ gene deletion were used in the LPS (0.6 μ g/g) + HI synergy murine model of perinatal brain damage.



Global deletion of the $LT\beta$ gene significantly increases cerebral tissue loss

As shown in figure 4.7.A, there was an overall increase in infarct volume in WT LPS animals (n=10) in comparison to the WT saline control group (n=10). However, this was only significant in the pyriform cortex (from 7 \pm 4% to 21 \pm 7%, p<5%) and hippocampus (from 18 \pm 5% to 45 \pm 9%, p<5%) ipsilateral brain regions. Observation of the mutant groups revealed a statistically significant increase in total infarct volume from 5 \pm 3% to 51 \pm 10% (p<0.1%) in $LT\beta$ KO mice treated with LPS (n=10) when compared to saline controls (n=10). This was apparent in all brain regions: Dorsoparietal from 3 \pm 5% to 52 \pm 10% (p<0.1%) and pyriform cortex from 1 \pm 6% to 43 \pm 12% (p<1%), hippocampus from 17 \pm 6% to 60 \pm 12% (p<1%), striatum from 11 \pm 5% to 60 \pm 11% (p<1%), thalamus from -41 \pm 3% to 31 \pm 10% (p<1%) and external capsule from 4 \pm 4% to 53 \pm 13% (p<1%). Additionally, LPS treated $LT\beta$ KO animals demonstrated a significant overall increase in tissue loss relating to WT LPS mice (from 23 \pm 7% to 53 \pm 13%, p<5%). When the ipsilateral forebrain regions of both WT and KO LPS groups were assessed separately, this significance was also evident in the dorsoparietal cortex (25 \pm 7% vs. 52 \pm 10%, p<5%), striatum (23 \pm 9% vs. 60 \pm 11%, p<1%), thalamus (110 \pm 6% vs. 31 \pm 10%, p<1%) and external capsule (23 \pm 7% vs. 53 \pm 13%, p<1%) (see figure 4.7.A).

LTβ mutants have higher neonatal brain injury when treated with LPS

Analysis of WT LTβ animals showed reduced brain injury when treated with saline. Conversely, WT littermate animals revealed significant sensitization and increase in inflammation when treated with LPS. This was particularly visible in the dorsoparietal cortex (1.4 ± 0.8 vs. 3.4 ± 1.1 , $p < 1\%$) and external capsule (2.5 ± 0.8 vs. 4.5 ± 1.1 , $p < 5\%$). Comparison between the two LTβ KO groups demonstrated a significant increase from 0.9 ± 0.6 in the saline group to 5.6 ± 1.2 in the endotoxin group ($p < 1\%$). This overall increase in inflammation and cellular loss was also noticeable in all six brain regions when assessed individually. In the dorsoparietal cortex there was an increase from 0.7 ± 0.7 to 5.6 ($p < 1\%$), pyriform cortex from 0.7 ± 0.7 to 5.4 ± 1.2 ($p < 1\%$), hippocampus from 1.2 ± 0.8 to 5.9 ± 1.1 ($p < 1\%$), striatum from 0.5 ± 0.4 to 5.5 ± 1.2 ($p < 0.1\%$), thalamus from 0.4 ± 0.4 to 5.5 ± 1.1 ($p < 0.1\%$) and external capsule white matter from 1.6 ± 0.8 to 6.0 ± 1.1 ($p < 1\%$). Evaluation of WT and LTβ KO animals pre-treated with LPS established that homozygous deletion of LTβ gene led to an overall increase in sensitization and inflammation in the ipsilateral brain hemisphere (3.1 ± 1.0 vs. 5.6 ± 1.2 , $p < 5\%$). This was particularly significant on the pyriform cortex (2.2 ± 0.9 vs. 5.4 ± 1.2 , $p < 5\%$), striatum (2.5 ± 0.8 vs. 5.5 ± 1.2 , $p < 0.1\%$) and thalamus (2.2 ± 0.9 vs. 5.5 ± 1.1 , $p < 5\%$) (see figure 4.7.B).

LTβ^{-/-} mice have a marked increase in TUNEL positive cells following LPS+HI insult

Quantification of TUNEL+ cells revealed only a small trend in increased cell death in the WT LPS group when compared to WT saline littermate controls. LTβ^{-/-} LPS treated animals revealed the highest number of cells undergoing DNA fragmentation. Comparison with the LTβ^{-/-} saline controls, which had an overall count of 0.8 ± 0.5 , the number of TUNEL+ cells was significantly increased to 43.5 ± 15.5 ($p < 0.1\%$). The same level of significance was also found in all ipsilateral brain regions assessed. The dorsoparietal cortex showed an increase from 1.1 ± 0.5 to 70.8 ± 21.7 ($p < 0.11\%$), pyriform cortex from 0.0 ± 0.0 to 29.8 ± 12.3

($p < 1\%$), hippocampus from 2.3 ± 1.5 to 42.1 ± 14.3 ($p < 1\%$), striatum from 0.5 ± 0.5 to 52.9 ± 19.7 ($p < 1\%$), thalamus from 0.0 ± 0.0 to 24.4 ± 12.3 ($p < 5\%$) and external capsule from 0.8 ± 0.7 to 41.0 ± 12.6 ($p < 0.1\%$). Furthermore, analysis between $LT\beta$ WT and $LT\beta$ KO endotoxin groups also showed a significant overall increase in TUNEL+ counts in the later, as well as in most assessed brain regions, with the exception of the hippocampus, which only showed a trend towards increased DNA fragmentation-associated cell death. The dorsoparietal cortex showed an average increase in TUNEL+ cells from 10.0 ± 5.8 to 70.8 ± 21.7 ($p < 1\%$), pyriform cortex from 2.7 ± 2.6 to 29.8 ± 12.3 ($p < 1\%$), striatum from 5.7 ± 3.0 to 52.9 ± 19.7 ($p < 1\%$), thalamus from 1.5 ± 1.0 to 24.4 ± 12.3 ($p < 5\%$) and external capsule from 11.0 ± 5.2 to 41.0 ± 12.6 ($p < 1\%$) (see figure 4.7.C).

Astrocyte immunoreactivity is unaffected by global deletion of the $LT\beta$ gene

GFAP immunoreactivity assessment showed an overall increase in astrogliosis in WT animals treated with LPS versus their WT saline littermate controls. The $LT\beta$ KO LPS group revealed the highest levels of immunoreactivity, and when compared with $LT\beta$ KO saline animals, this difference reached statistical significance in the dorsoparietal cortex (34.3 ± 4.7 vs. 58.0 ± 7.0 , $p < 1\%$), hippocampus (53.6 ± 6.4 vs. 75.4 ± 6.9 , $p < 5\%$), external capsule (60.0 ± 5.2 vs. 83.1 ± 6.9 , $p < 15\%$), as well as overall (45.3 ± 7.9 vs. 65.5 ± 8.0 , $p < 5\%$). However, observation of the two LPS groups – WT and $LT\beta$ KO – demonstrated only a small trend towards higher astrocytic activation on the mutant group (see figure 4.7.D).

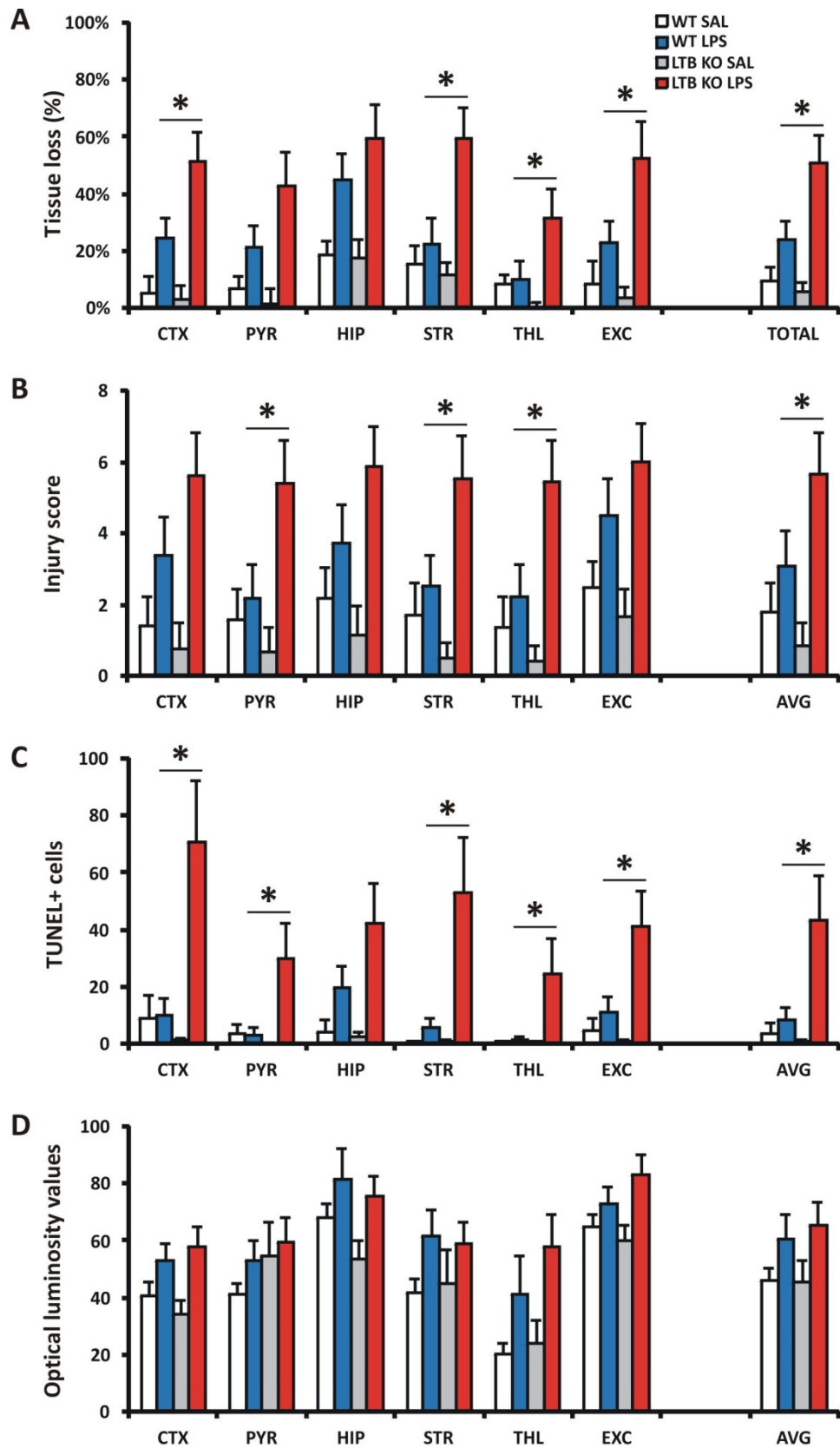


Figure 4.7: Effects of LTβ global gene deletion on neonatal hypoxic-ischemic brain injury after pre-exposure to 0.6μg/g LPS endotoxin (LPS). Animals injected with saline (SAL) served as controls. **A:** Infarct volume assessment of individual forebrain regions and total ipsilateral

hemisphere infarction as percent of contralateral hemisphere revealed substantial increase in tissue necrosis on the LPS $LT\beta$ mutants, which reached significance in the dorsoparietal cortex, striatum, thalamus and external capsule ipsilateral brain regions, as well as in total tissue infarction. **B:** $LT\beta$ brain injury score also demonstrated an increased detrimental effect in the LPS $LT\beta$ knockouts, with an overall significant increase in microglial activation and neuronal cell loss, which was also significant in the ipsilateral pyriform cortex, striatum and thalamus brain regions. **C:** DNA fragmentation cell death assessment using TUNEL+ cell quantification demonstrated a significant overall increase in cell death in the LPS-treated $LT\beta$ gene deficient mice. This detrimental effect was also significant in most ipsilateral brain regions, with only exception being the hippocampus, where only a trend towards increased TUNEL+ associated cell death was visible. **D:** Astroglial immunoreactivity assessment using optical luminosity values showed an overall trend towards increased astrocyte activation in the $LT\beta$ deficient mice treated with LPS when compared to LPS wild type littermates. Contralateral hemisphere not shown. * $p < 5\%$ in Mann Whitney test for the LPS-treated wild type vs. knockout groups, $n = 10$ per group. **Abbreviations:** WT – wild type, KO – knockout, CTX- dorsoparietal and PYR – pyriform cortex, HIP – hippocampus, STR – striatum, THL – thalamus, EXC – external capsule.

Tumour necrosis factor α : dual role in the sensitization of the neonatal brain to hypoxic-ischemic insult?

Effects of $TNF\alpha$ gene on endotoxin-mediated sensitization to HI insult and subsequent perinatal brain damage were assessed by global gene deletion, and by analysing cell-type specific deletion of this cytokine using the Cre-Lox recombination technique. The latter used the $MLys::CRE$ promoter, where $TNF\alpha$ was deleted in granulocytes/macrophages expressing $MLys$ promoter, therefore excluding brain microglia.

Homozygous breeding of $TNF\alpha$ mutants ($TNF\alpha^{-/-}$)

Similarly to the initial $LT\beta^{-/-}$ study, $TNF\alpha^{-/-}$ mice were compared with WT C57BL/6 non-littermate animals. This initial $TNF\alpha$ study, like the preliminary $LT\beta$ work was performed by Drs Mariya Hristova, Giles Kendall and Sigrun Lange. The same WT C57BL/6 animals were again used as non-littermate controls. Both P6 WT and P6 $TNF\alpha$ (KO) mice were pre-treated with either $0.3\mu g/g$ LPS or saline. 12h after endotoxin/vehicle administration, all experimental animals were exposed to HI insult and allowed 48h survival.

The results indicated that WT animals injected with saline showed negligible brain injury. Conversely, when treated with LPS, there was a significant increase

in mean combined injury score in the cortex ($p < 1\%$), hippocampus ($p < 0.1\%$), striatum ($p < 5\%$), thalamus ($p < 5\%$) and ipsilateral white matter ($p < 5\%$) brain regions. This significance was also present in the overall injury score ($p < 5\%$). Subjective comparison between the WT and TNF α KO saline groups revealed a significant increase in combined injury score in the latter. Statistical significance was reached in the dorsoparietal cortex ($p < 5\%$), hippocampus ($p < 1\%$), striatum ($p < 5\%$), thalamus ($p < 5\%$) and ipsilateral white matter ($p < 5\%$) regions, and also in the overall injury score ($p < 5\%$). In contrast, LPS treated TNF α KO animals demonstrated little further increase in damage in comparison to their saline littermate controls (see figure 4.8).

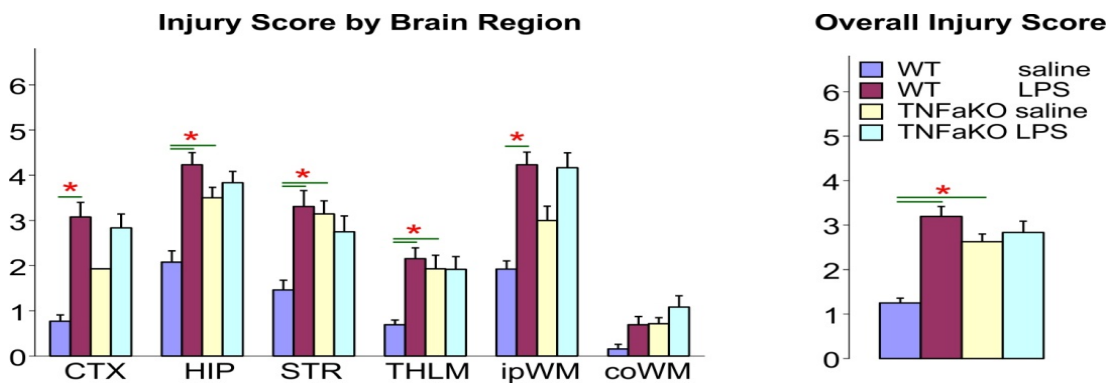


Figure 4.8: Combined injury score in WT C57BL/6 and homozygous TNF α null animals pre-treated with saline and LPS (0.3 μ g/g) 12h before neonatal HI injury.

Brain injury score across the different forebrain regions (left) and overall brain injury score (right). LPS administration in WT mice ($n=13$) led to sensitization of the neonatal brain to HI insult with significant increase in brain injury, whereas WT saline animals ($n=13$) had minimal brain injury. TNF α null animals ($n=14$) before HI insult did not show significant difference in the mean combined injury score in comparison to their saline treated littermate animals ($n=13$). * $p < 5\%$ in one way ANOVA followed by post-hoc Tukey. **Abbreviations:** CTX – cortex, HIP – hippocampus, STR – striatum, THLM – thalamus, IpWWM – ipsilateral and CoWM – contralateral white matter. Graph reproduced with permission.

Crossing of homozygous TNF α KO with WT C57BL/6 mice

Homozygous breeding of TNF α null animals thus provided two separate sets of results. Compared to WT C57BL/6 non-littermate mice, TNF α KO exhibited more severe injury at baseline, as well as a considerable reduction in LPS sensitization prior to HI. This latter finding raised two main questions. Was this reduction caused by difference in strain background or due to global deletion of the TNF α

gene? This issue led to a second experimental approach where homozygous TNF α KO mice were crossed with commercially provided C57BL/6 animals. The F1 heterozygous offspring were set up as the new breeding pairs with F2 progeny used in the LPS + HI experiment. In this second approach a new batch of LPS was required with a new optimal sensitization dose of 0.6 μ g/g (see chapter 2). All animals were injected randomly with LPS or saline, followed 12h later by HI insult, and once again allowed to survive for 48h. PCR genotyping allowed use of WT littermates as control (see figure 4.9.A). The new results showed that the LPS sensitivity previously seen on the WT C57BL/6 (see chapter 3) had now disappeared on the new experimental LPS WT littermate animals. In addition, there was a trend towards increased inflammation and cell loss on the LPS TNF α KO animals, but this did not reach statistical significance (see figure 4.9.B).

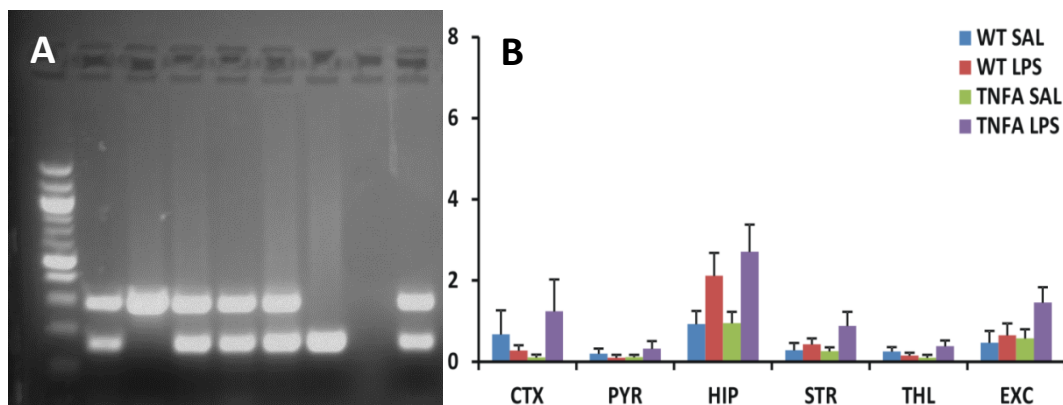


Figure 4. 9: TNF α gene deletion study using WT littermate controls. **A:** Generation of TNF α +/+, TNF α +/- and TNF α -/- mice. TNF α mice were bred in a homozygous knock out versus WT C57BL/6 manner in order to obtain TNF α +/+ and TNF α -/- littermates. PCR reaction products were run on an agarose gel electrophoresis apparatus to detect the different genotypes. TNF α wildtype allele has 180bp, whereas the TNF α mutant allele has a product size of 318bp. TNF α +/- genotype was identified through the presence of both +/+ and -/- alleles. **B:** Combined microglial activation and cell death injury score in WT and TNF α KO littermates pre-sensitized with saline and LPS (0.6 μ g/g) 12h before neonatal HI injury. LPS administration in WT mice (n=10) did not sensitize the neonatal brain to HI in comparison to WT saline-treated animals (n=10). TNF α KO animals (n=10) showed a trend towards increase in the mean combined injury score in comparison to their WT LPS littermates. Saline KO animals (n=10) demonstrated minimal brain injury. The Y-axis shows brain injury score based on extent of infarction and microglial macrophage response. *p<0.05 in one way ANOVA followed by post-hoc Tukey. *Abbreviations:* CTX - dorsoparietal and PYR - pyriform cortex, HIP - hippocampus, STR - striatum, THL - thalamus, EXC - external capsule.

Crossing of heterozygous TNF α with WT C57BL/6 mice

Once more, TNF α KO mice displayed no sensitization to HI when treated with LPS. Moreover, introduction of C57BL/6 background (by 50%) appeared to not be enough in terms of prompting endotoxin sensitization, as wild type LPS-treated littermates now demonstrated the same non-responsiveness as the homozygous mutants. Since it was shown that the current LPS batch had not lost its function (see figure 4.6), it was now assumed, just as with the LT β study, that if non-responsiveness was a result of strain background differences, this change in response is probably a dominant trait. In an attempt to address this new hypothesis, F2 heterozygous mice were re-crossed with C57BL/6. F3 heterozygous offspring were now used as the new tentative breeding pairs. The experimental design remained the same as the one described directly above, where F4 mice were injected with 0.6 μ g/g LPS or saline 12h before HI followed by 48h survival. Again, only WT and TNF α KO littermates were used for histological assessment.

TNF α gene ablation reveals tendency towards increased tissue infarction in LPS treated animals

Re-crossing homozygous TNF α null mice with WT C57BL/6 for two generations successfully re-established the LPS-mediated sensitization to subsequent HI, initially observed in the C57BL/6 animals. This effectively allowed further histological assessment of the molecular components involved in this combined model of neonatal brain injury.

Infarct volume assessment of the WT groups showed minimal necrosis in the saline group (n=9) and a trend towards increased tissue loss in animals that had been administered LPS (n=9) prior to HI insult. This was visible in all brain regions with significance reached in the hippocampus (26 \pm 8% vs. 45 \pm 5%, p<5%). TNF α KO saline treated animals (n=10) demonstrated minimal tissue loss, and when compared with TNF α KO LPS administered littermates (n=10), the latter

demonstrated a clear increase in brain injury. However, once again, this increase in LPS-sensitization did not reach significance. Furthermore, the results obtained did not reveal statistical significant difference between the WT and KO LPS groups, only a trend towards increase of tissue necrosis on the mutants (see figure 4.10.A).

Global deletion of TNF α gene leads to increased immune response and cell loss

This second change in experimental strategy also revealed a clear up-regulation in LPS-mediated sensitivity in WT LPS-administered vs. WT saline animals. Statistical significance was reached on the dorsoparietal cortex (1.0 ± 0.9 vs. 4.1 ± 1.2 , $p < 5\%$) and hippocampus (1.6 ± 0.9 vs. 4.5 ± 1.2 , $p < 5\%$). Additionally, this increase in endotoxin-mediated sensitivity was also observed on the TNF α KO LPS, when compared to TNF α KO saline controls. This was again apparent in brain injury on the dorsoparietal cortex (0.7 ± 0.6 vs. 5.2 ± 1.1 , $p < 1\%$) and hippocampus (1.5 ± 0.8 vs. 6.1 ± 0.9 , $p < 1\%$), as well as in the striatum (0.4 ± 0.2 vs. 3.6 ± 0.9 , $p < 1\%$) and external capsule (1.6 ± 0.5 vs. 6.4 ± 0.9 , $p < 5\%$). Comparison between the two LPS groups – WT vs. TNF α KO – demonstrated a trend towards increased brain injury in the mutant animals. However, this did not reach significance (see figure 4.10.B).

TNF α -/- animals have higher DNA fragmentation-associated cell death

LPS sensitization prior to HI caused a marked increase in DNA fragmentation positive cells in the WT group, with a significant increase in TUNEL+ cells in the hippocampus (65.7 ± 20.6 vs. 25.5 ± 12.5 , $p < 1\%$) when compared with WT saline littermate controls. Assessment of both mutant groups again demonstrated a markedly higher number of TUNEL+ cells in the TNF α KO LPS group, with statistical significance reached in the dorsoparietal cortex (4.1 ± 3.5 vs. 73.8 ± 18.3 , $p < 0.1\%$) and hippocampus (29.8 ± 8.3 vs. 84.0 ± 19.1 , $p < 0.1\%$) ipsilateral brain regions. Global TNF α gene deletion led to a higher number of TUNEL+ cells in the LPS group when compared to WT LPS littermates. Furthermore, this was

statistically significant in the dorsoparietal cortex, where the number of TUNEL+ cells rose from 33.4 ± 16.4 (WT LPS) to 73.8 ± 18.3 (TNF α -/- LPS, $p < 1\%$) (see figure 4.10.C).

TNF α global gene deletion has a moderate effect on astrocytic activation

Similarly to what has been shown in the previous TNF α global gene deletion assessments, LPS administration prior to HI insult led to an increase in reactive astrogliosis in the WT group when compared to its control (WT saline). Furthermore, this astrocytic activation was significantly higher in the striatum (from 23.5 ± 6.0 to 37.9 ± 4.3 , $p < 5\%$) and external capsule (from 42.5 ± 4.3 to 56.3 ± 2.8 , $p < 5\%$). Observation of the TNF α -/- saline and TNF α -/- LPS groups revealed the same rise in GFAP synthesis in the later, and significantly more astrocytic activation in the dorsoparietal cortex (from 19.1 ± 6.4 to 39.2 ± 12.4 , $p < 1\%$), striatum (from 23.4 ± 7.8 to 36.2 ± 11.5 , $p < 5\%$) and external capsule (from 47.4 ± 15.8 to 59.2 ± 18.7 , $p < 5\%$). Albeit the LPS mutants demonstrated a slightly higher astrocytic reactivity, this was not significant when compared with the WT LPS group (see figure 4.10.D).

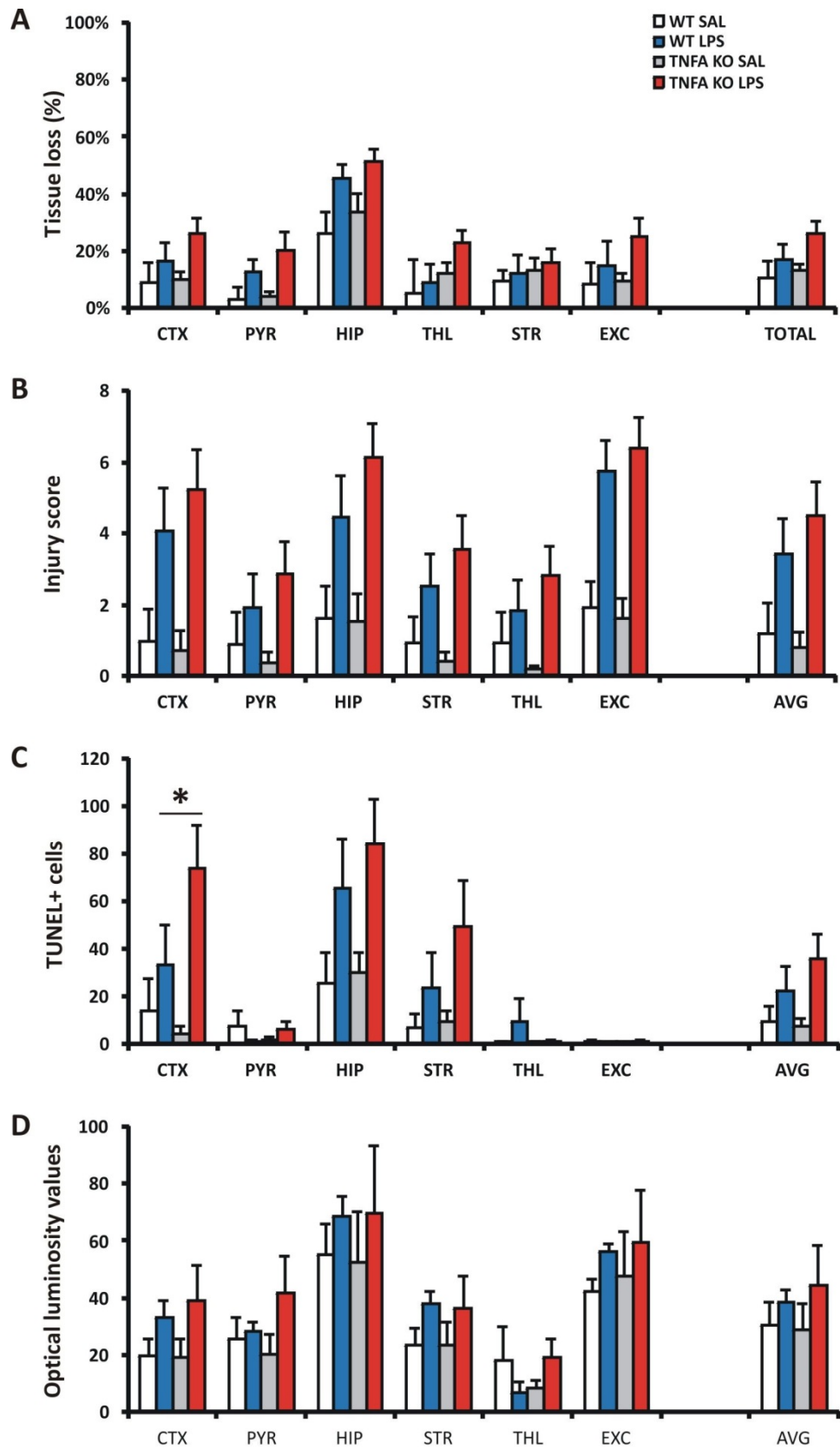


Figure 4.10: Effects of TNF α global gene deletion on neonatal hypoxic-ischemic brain injury after pre-exposure to 0.6 μ g/g LPS endotoxin (LPS). Animals injected with saline (SAL) served as controls. **A:** Infarct volume assessment of individual forebrain regions and total ipsilateral

hemisphere infarction as percent of contralateral hemisphere, showed that global deletion of TNF α gene leads to an overall trend towards increased tissue infarction following LPS administration. **B:** Brain injury score assessment of TNF α gene deletion revealed an overall trend towards increased inflammatory microglial activation and neuronal cell loss in all ipsilateral brain regions of LPS-treated animals. **C:** DNA fragmentation cell death assessment using TUNEL+ cell quantification demonstrated an overall increase in cell death in global TNF α gene deficient mice following LPS administration. This detrimental effect reached significance in the ipsilateral dorsoparietal cortex brain region. **D:** Astroglial immunoreactivity assessment using optical luminosity values showed an overall trend towards increase in GFAP synthesis in LPS-treated global TNF α mutants when compared to littermate LPS-treated wild type animals. Contralateral hemisphere not shown. *p<5% in Mann Whitney test for the LPS-treated wild type vs. knockout groups, n=9 for WT SAL, n=9 for WT LPS, n=10 for TNF α KO SAL and n=10 for TNF α KO LPS. **Abbreviations:** WT – wild type, KO – knockout, CTX- dorsoparietal and PYR – pyriform cortex, HIP – hippocampus, STR – striatum, THL – thalamus, EXC – external capsule.

Cell-type specific deletion of TNF α gene: MLys-expressing macrophages have a detrimental effect in perinatal brain after LPS-mediated HI

In order to further characterise the role of TNF α as a moderator of combined endotoxin and HI insult, a more precise approach was required, as it is well established that multiple cell-types produce TNF α . Knowing that microglia/macrophage play a primary and significant role in brain inflammation, the next aim was to target these cell-types specifically. For this purpose, we were able to obtain animals that were MLys::CRE. This promoter is expressed only in mature hematopoietic granulomyelomonocytic cells, i.e. granulocytes and macrophages, and not in microglia, as the latter originate from the yolk sac and not from the bone marrow (Mawhinney et al., 2012; Prinz and Priller, 2014).

First, animals that had two copies of loxP-tagged TNF α gene (TNF α f/f) and one copy of Cre recombinase under the control of the MLys promoter (+/-) were crossed with WT C57BL/6 animals. From the F1 generation, only the animals that had one copy of TNF α flox (flox/+) and were heterozygous for MLys::Cre were set up as the next breeding pairs. F2 offspring that had both MLys+/- and TNF α f/f were again crossed, and the F3 progeny, consisting solely of WT f/f (controls) and MLys+/- TNF α f/f (mutants) was used in the LPS + HI study (see figure 4.11). 12H prior to HI, P6 mice received ip LPS (0.6 μ g/g) injection or saline. After 48h

survival, animals were sacrificed and brain tissue collected for histological analysis.

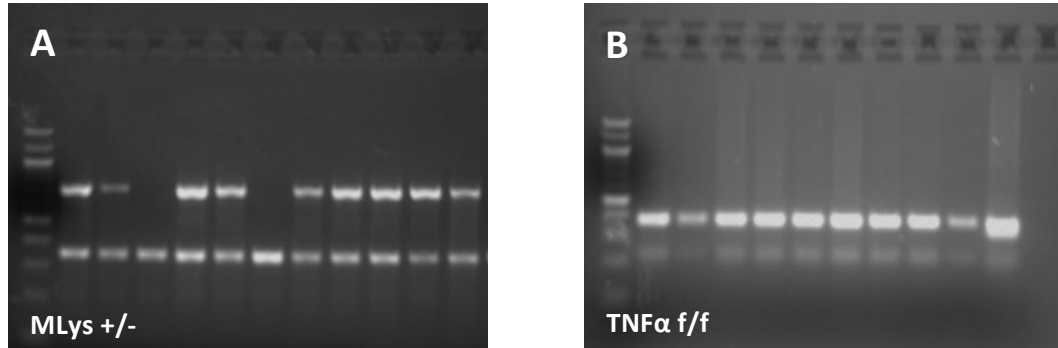


Figure 4.11: Generation of WT TNF α /f and MLys \pm /- TNF α /f mutants.

WT TNF α /f mice were bred with MLys \pm /- TNF α /f in order to obtain same genotype offspring. Agarose gel electrophoresis following PCR reaction allowed distinguishing the different genotypes through visualization of the different band sizes: MLys WT allele at 345bp and MLys KO allele near 1kbp (A); TNF α flox allele at 400bp and TNF α WT allele at 350bp (B).

Deletion of TNF α in MLys macrophages reduces tissue infarction

Assessment of neuronal cell loss by looking at infarct volume demonstrated successful LPS-sensitization in the WT group (n=10) with minimal tissue loss in the WT saline controls (n=10) ($39\pm 7\%$ and $12\pm 5\%$ respectively, $p < 1\%$). This increase in infarction was significant in most brain regions. The dorsoparietal cortex showed a significant increase from $10\pm 5\%$ to $44\pm 8\%$ ($p < 0.1\%$), pyriform cortex from $12\pm 9\%$ to $36\pm 8\%$ ($p < 5\%$), striatum from $16\pm 6\%$ to $35\pm 6\%$ ($p < 5\%$) and external capsule from $5\pm 8\%$ to $48\pm 11\%$ ($p < 1\%$). Saline administered MLys \pm /- TNF α /f animals had minimal tissue infarction ($9\pm 4\%$). Conversely, KO animals treated with LPS had an increase in tissue loss when compared to the KO saline, with significance in the external capsule (from $1\pm 5\%$ to $23\pm 6\%$, $p < 1\%$). Assessment between WT LPS and MLys \pm /- TNF α /f LPS groups clearly indicated that deletion of TNF α in MLys-expressing macrophages reduces tissue loss. MLys \pm /- TNF α /f LPS animals had significantly less necrotic tissue in the dorsoparietal ($44\pm 8\%$ vs. $25\pm 8\%$, $p < 5\%$) and pyriform cortex ($36\pm 8\%$ vs. $9\pm 6\%$, $p < 5\%$), and striatum ($35\pm 6\%$ vs. $20\pm 6\%$, $p < 5\%$) ipsilateral brain regions, with

significance also being reached in the overall ($39\pm 7\%$ vs. $23\pm 6\%$, $p<5\%$) tissue loss assessment between both LPS groups (see figure 4.12.A).

MLys^{+/-} TNF α /f animals have decrease brain injury following LPS+HI injury

Combined α M (activated macrophage/microglia) and Nissl (neuronal cell loss) injury scoring (see chapter 2) revealed successful LPS-mediated sensitization in the WT TNF α /f group. The ipsilateral dorsoparietal (5.8 ± 1.1 vs. 2.4 ± 0.9 , $p<5\%$) and pyriform cortex (5.2 ± 1.0 vs. 1.6 ± 0.8 , $p<5\%$), striatum (5.1 ± 1.1 vs. 1.1 ± 0.7 , $p<1\%$) and thalamus (4.5 ± 1.1 vs. 0.7 ± 0.5 , $p<0.1\%$) brain regions had significantly more brain injury in the WT LPS treated group when compared to WT saline injected animals. The same increase in α M activation and neuronal loss was seen in the MLys^{+/-} TNF α /f LPS animals when compared to the MLys^{+/-} TNF α /f saline controls. In this assessment, LPS KO animals had significantly higher brain injury in the dorsoparietal cortex (5.1 ± 1.1 vs. 0.9 ± 0.6 , $p<1\%$), hippocampus (6.3 ± 1.0 vs. 1.9 ± 1.0 , $p<1\%$) and external capsule (5.7 ± 1.0 vs. 2.2 ± 0.8 , $p<5\%$). Comparing both WT and MLys mutants after endotoxin administration highlighted a trend towards reduction of LPS-mediated sensitization to subsequent HI in MLys-expressing macrophages where the TNF α gene had been deleted. This decrease in injury reached statistical significance in the pyriform cortex (from 5.2 ± 1.0 to 2.7 ± 0.9 , $p<5\%$) and thalamus (from 4.5 ± 1.1 to 1.9 ± 0.7 , $p<5\%$) brain regions (see figure 4.12.B).

TNF α ^{-/-} in MLys macrophages ameliorates DNA fragmentation and cell death

TUNEL+ cell quantification comparison between the WT littermate groups showed minimal number of DNA fragment-associated cell death in the WT saline controls (42.6 ± 20.7). The average number of TUNEL+ cells was significantly increased to 182.3 ± 46.2 in the WT LPS administered group ($p<0.1\%$). Observation of the individually assessed brain regions showed that this significance was also present in the dorsoparietal (from 49.7 ± 30.4 to 363.9 ± 82.1 , $p<0.1\%$) and pyriform cortex (from 24.8 ± 20.0 to 105.1 ± 40.1 , $p<5\%$), striatum

(from 36.6 ± 24.1 to 253.9 ± 65.1 , $p < 1\%$), thalamus (from 18.0 ± 9.4 to 60.9 ± 20.0 , $p < 5\%$) and external capsule (from 22.9 ± 11.0 to 129.3 ± 30.4 , $p < 0.1\%$) ipsilateral brain regions. Subjective comparison between the KO groups –MLys^{+/-} TNF α /f saline and MLys^{+/-} TNF α /f LPS – also revealed a trend towards higher average number of TUNEL+ cells in the latter, however this increase never reached significance. Assessment of the LPS groups (WT and KO) demonstrated a significant reduction not only in the dorsoparietal (from 363.9 ± 82.1 to 158.3 ± 44.6 , $p < 0.1\%$) and pyriform cortex (from 105.1 ± 40.1 to 21.4 ± 9.7 , $p < 5\%$) and external capsule (from 129.3 ± 30.4 to 46.2 ± 16.6 , $p < 1\%$), but also overall (from 182.3 ± 46.2 to 87.3 ± 25.8 , $p < 5\%$) (see figure 4.12.C).

GFAP immunoreactivity is mildly reduced in MLys^{+/-} TNF α /f LPS animals

Finally, comparison between WT saline and WT LPS animals in terms of GFAP synthesis demonstrated an overall higher increase in astrocytic activation in the WT LPS group from 40.7 ± 5.2 to 53.7 ± 6.2 ($p < 5\%$). Assessment of the individual brain regions showed that this significance was also present in the pyriform cortex from 34.8 ± 5.4 to 55.2 ± 6.8 ($p < 5\%$) and striatum from 33.1 ± 4.6 to 51.4 ± 6.8 ($p < 5\%$). Comparison between the KO saline and KO LPS groups also showed the same overall increase in astrocyte reactivity from 38.1 ± 5.4 to 49.8 ± 5.8 ($p < 5\%$), as well as in the dorsoparietal cortex from 30.7 ± 5.8 to 50.9 ± 6.5 ($p < 5\%$). However, comparison between WT LPS and MLys^{+/-} TNF α /f LPS injected animals showed that astrocyte activation was only moderately affected by deletion of TNF α gene in MLys expressing macrophages, with the latter having slightly less GFAP synthesis (see figure 4.12.D).

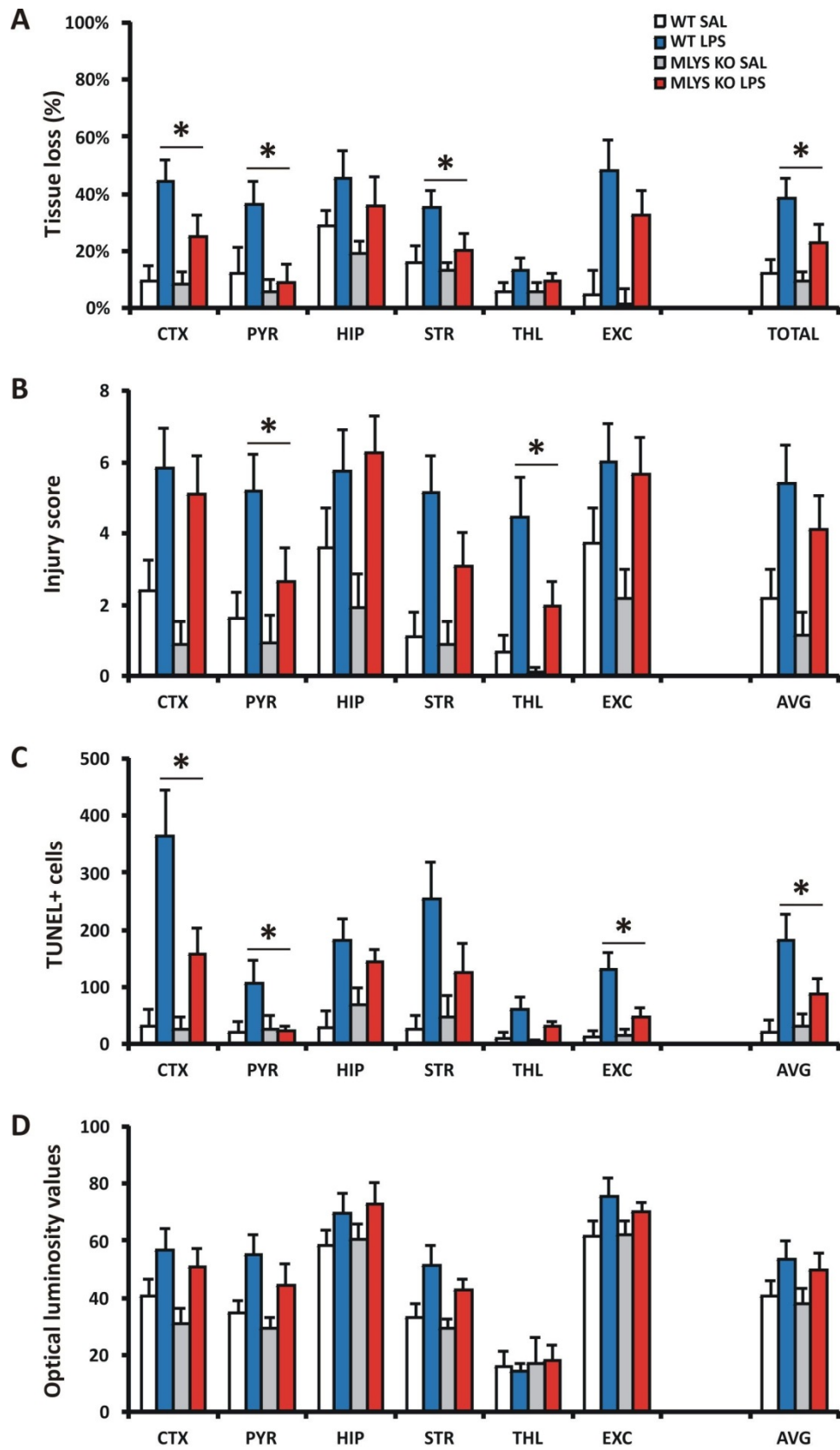


Figure 4.12: Effects of TNF α gene deletion in MLYs+ macrophages on neonatal hypoxic-ischemic brain injury after pre-exposure to 0.6 μ g/g LPS endotoxin (LPS). Animals injected with saline (SAL) served as controls. **A:** Infarct volume assessment of individual forebrain regions and total

ipsilateral hemisphere infarction as percent of contralateral hemisphere, showed that this cell specific deletion of the TNF α gene was not only significantly neuroprotective overall, but this significant decrease in tissue necrosis was also observed in the dorsoparietal and pyriform cortex and striatum ipsilateral brain regions of LPS-treated knockout animals. **B:** Brain injury assessment of TNF α ^{-/-} MLys^{+/-} animals revealed an overall trend towards reduced microglial inflammatory response and neuronal cell loss following LPS administration. This becomes significant in the pyriform cortex and thalamus ipsilateral brain regions. **C:** DNA fragmentation cell death assessment using TUNEL⁺ cell quantification demonstrated a significant overall decrease in cell death in the LPS-treated knockout group, with significant reduction also reached in the dorsoparietal and pyriform cortex, and external capsule ipsilateral brain regions. **D:** Astroglial immunoreactivity assessment using optical luminosity values showed a trend towards reduced GFAP synthesis in the LPS TNF α ^{-/-} MLys^{+/-} animals when compared to wild type LPS-treated littermates. Contralateral hemisphere not shown. *p<5% in Mann Whitney test for the LPS-treated wild type vs. knockout groups, n=10 per group. **Abbreviations:** WT – wild type, KO – knockout, CTX- dorsoparietal and PYR – pyriform cortex, HIP – hippocampus, STR – striatum, THL – thalamus, EXC – external capsule.

Discussion

Infection/inflammation and hypoxic-ischaemic insult are widely accepted as having a devastating impact on the neonatal brain. These types of injury can cause severe brain damage alone, but when combined, work in a synergistic manner augmenting significantly and irreversibly subsequent perinatal brain damage, thus greatly enhancing the risk of cerebral palsy and/or other neurological deficits such as cognitive impairment, schizophrenia and autism.

Previous work performed in this laboratory has shown that LPS endotoxin, a known inducer of inflammation requires the TNF cluster of pro-inflammatory cytokines in order to sensitize the immature brain to subsequent hypoxic-ischaemic insult (Kendall et al., 2011b). Therefore, the logical next step was to study the individual TNF cluster members - TNF α , LT α and LT β – to see which one or combination of is responsible for mediating the LPS sensitization. In order to achieve this, in this study it was employed the use of transgenic mice with individual global deletion of TNF α , LT α and LT β genes, as well cell-specific deletion of TNF α in myeloid cells using MLys::CRE recombinase.

Lack of LPS-mediated sensitization can be resolved through serial introduction of wild type C57BL/6 background into the breeding

Pilot study performed by Drs Hristova, Kendal and Lange have shown a variation in LPS-mediated sensitization within different mouse strains, where TNF α and LT β animals were non-responders to bacterial endotoxin pre-treatment, only showing a baseline reaction to hypoxic-ischaemic insult. In their initial experimental design, where TNF α and LT β were used as homozygous breeding, with the latter being uncovered as actually containing some heterozygous animals, it was discovered that, unlike the separate wild type C57BL/6 controls (non-littermates), the F1 offspring of these two strains, displayed no sensitization to LPS pre-treatment (0.3 μ g/g) followed by hypoxic-ischaemic insult. This lack of sensitization appears to be novel, as multiple animal studies have successfully demonstrated LPS sensitization prior to hypoxic-ischaemic injury (Hagberg et al., 2002; Mallard et al., 2003; Kendall et al., 2011b; van de Looij et al., 2012). Additionally, In order to exclude the possibility that the new batch of LPS bacterial endotoxin had lost its function, a test was performed in commercially acquired C57BL/6 mice (see figure 4.6). This test revealed a significant increase across all assessed brain regions, in microglial activation in the LPS-treated grouped when compared to their saline-treated littermate controls.

Interestingly, the strain study described in chapter 3, revealed successful LPS-mediated sensitization to subsequent hypoxic-ischaemic insult in five different genetic background strains, including C57BL/6, the background strain used in this study. However, the strain study also demonstrated, similarly to other research looking at effects of strain differences in response to different stimuli, that different genetic strains might respond differently to insult. Consequently, these new findings regarding a lack of successful LPS-mediated sensitization led to the generation of two hypotheses, where non-responsiveness to bacterial infection could be a result of either strain differences, or due to gene deletion. In this study, this issue was initially addressed from a strain difference perspective. The

two mutant strains (TNF α and LT β) were separately crossed with wild type C57BL/6 mice, which had already proven to be well-responders to LPS administration (see chapter 3; figure 4.6). This re-crossing allowed obtaining heterozygous breeding, which then permitted the use of wild type littermate controls. However, one re-crossing appeared to not be sufficient, indicating that if this newly identified non-responsiveness was caused by strain variations, this must be due to an unknown dominant trait. A second attempt at crossing heterozygotes for TNF α and LT β animals with wild type C57BL/6 mice respectively, thus introducing 75% of C57BL/6 background into the strain, finally generated positive results, where wild type LPS-treated littermates successfully revealed significant response to LPS pre-sensitization.

LT α , LT β and TNF α genes play dissimilar roles in the synergistic model of infection and neonatal hypoxic-ischemic insult

Although numerous molecules, including the TNF family of pro-inflammatory cytokines consisting of TNF α , LT α and LT β , are known to be up-regulated following antenatal, perinatal and early postnatal infection, the exact molecular mechanisms that take part in the aetiology of perinatal brain damage are only now beginning to be understood. Kendall et al., (2011b) demonstrated that deletion of the TNF cluster of pro-inflammatory cytokines leads to an abolishment in inflammation following LPS pre-sensitization of the neonatal mouse brain to subsequent hypoxic-ischaemic insult. The current study further investigated this effect, by looking at the individual role of each of the three cluster members, using murine models of global and cell-type specific gene deletion.

Removal of the LT α gene proved beneficial. Absence of LT α improved neuronal cell survival, with an overall 50% decrease in tissue infarction, microglia activation and neuronal cell loss. Deletion of this gene showed an overall reduction of 32% in TUNEL positive cells. However, astroglial activation was only minimally improved (8%) in the absence of LT α gene. A study performed by

Nelson et al., has also demonstrated $LT\alpha$ as a possible inducer of cerebral palsy, where it was revealed that single nucleotide polymorphism of the $LT\alpha$ gene in very preterm infants was associated with enhanced risk of CP (Nelson et al., 2005). However, the $LT\alpha$ results in this study did not reproduce the same abolishment effect seen in the TNF cluster knockout study (Kendall et al., 2011b). Here, one can see that homozygous $LT\alpha$ mutants had a more moderate effect, with an overall 35% reduction on brain damage across the four different assessments. These results therefore indicate that other genes or factors are at play in this synergistic model of neonatal brain injury.

Conversely to the $LT\alpha$ results, deletion of $LT\beta$ gene in animals that underwent LPS pre-sensitization had a detrimental effect across all four performed assessments. Tissue infarction assessment showed a clear and significant detrimental effect, with an overall 130% increase in tissue necrosis of animals that lacked $LT\beta$ gene. Overall injury score assessment once again demonstrated a significant increase in microglial activation and neuronal cell loss of around 42%. This toxic effect was most evident in TUNEL+ cell quantification where there was nearly a fourfold increase in DNA fragmentation-associated cell loss in the global $LT\beta$ knockouts. However, this gene deletion had a less pronounced effect in astrogliosis, where there was only a small increase of 8% in astroglial activation in LPS-treated $LT\beta$ deficient mice. These results show a clear different effect from what was observed in the TNF cluster deletion study (Kendall et al., 2011b). In this particular model of perinatal brain damage, there appears to be a clear significant neuroprotective role for the $LT\beta$ gene, where ablation of this gene leads to an overall twofold increase in brain damage when taking into account all four assessments performed.

Of the three cluster members, the role of $TNF\alpha$ as a mediator of HI injury has been the most extensively investigated. $TNF\alpha$ is produced by a variety of cell types, but are predominantly expressed by activated macrophages. Studies using

exogenous TNF α have shown its ability to increase permeability and to cross the blood brain barrier. Up-regulation of this protein activates NK cell toxicity and generates T-lymphocytes. There is also evidence of changes in endothelium, which promote not only inflammation but also thrombosis (Uno et al., 1997). However, in this particular study, the TNF α cytokine appears to have a beneficial role in the synergistic mouse model of LPS infection and neonatal hypoxia-ischemia. Similarly to what was observed in the LT β experiment, animals lacking both copies of the TNF α gene demonstrated more susceptibility to brain damage, with a 53% increase in tissue infarction, and over 60% increase in DNA fragmentation-associated cell death. Despite only a trend, there was also an increase of 8% in microglial activation and neuronal cell loss, with a further increase of 15% in astrogliosis in the brains of LPS-treated TNF α global knockouts.

Interestingly, not only individual global deletion of LT α , Lt β and TNF α did not reproduce the abolishment of LPS-mediated sensitization seen previously in the TNF cluster study (Kendall et al., 2011b), this study has shown that these cytokines have dissimilar roles in this combined form of injury, and that attempt at acute neutralization of the individual cytokines would not be successful in preventing and reducing perinatal brain damage following synergistic infection and hypoxia-ischaemic insults.

Proximity of TNF α , LT α and LT β genes could explain LT α -/- neuroprotective effect

TNF α , LT α and Lt β genes are located within 12kb of genomic DNA inside the major histocompatibility complex locus (Muler et al., 1987; Kuprash et al., 2002; Kendall et al., 2011b). These cytokines not only have overlapping activity functions, but they are also involved in the functional development of peripheral lymphoid organs and immune response (Kuprash et al., 2002; Liepinsh et al., 2006). Therefore, it is possible that global deletion of one of these genes could affect the expression of one or both other TNF cluster members. In fact, a study

by Alexopoulou et al., 1998 has shown that the more severe phenotypic defects seen in $LT\alpha$ deficient mice when compared to $TNF\alpha$ and $LT\beta$ mutants is in fact a consequence of partial $TNF\alpha$ production deficiency (Alexopoulou et al., 1998; Kuprash et al., 2002). In this study, complementing $LT\alpha^{-/-}$ animals with TNF -expressing transgenes led to partial restoration of primary B cell follicles, follicular dendritic cells, germinal centers, as well as re-establishment of B/T cell segregation in a p55 TNF receptor dependent manner. Furthermore, this reduced $TNF\alpha$ function in $LT\alpha^{-/-}$ mice is further evidenced when peritoneal macrophage supernatants as well as serum of $LT\alpha$ knockout animals injected intraperitoneally with LPS bacterial endotoxin failed to demonstrate production of $TNF\alpha$ at physiological levels (Alexopoulou et al, 1998). Therefore, it is possible to assume, at least partially, that the neuroprotective effect seen in the current $LT\alpha^{-/-}$ results could be attributed to an addition of reduction in $TNF\alpha$ synthesis by peripheral macrophages as well as lack of the $LT\alpha$ pro-inflammatory cytokine. Considering how $TNF\alpha$, $LT\alpha$ and $Lt\beta$ are known to have overlapping functions, this added inhibition in inflammatory response could explain the different effect global deletion of $LT\alpha$ gene has, i.e. neuroprotective, when global deletion of either $TNF\alpha$ or $Lt\beta$ revealed a detrimental effect.

The current study attempted to verify this theory by looking at mRNA expression of $TNF\alpha$, $LT\alpha$ and $LT\beta$ in the spleen of the different knockout mice. At P7, knock out animals as well as littermate wild type controls for each of the 3 gene mutations were injected with either LPS (n=4) or saline (n=4) and 4h later the spleen, liver, cortex, striatum and hippocampus were collected and snap frozen before RNA was extracted. Following cDNA synthesis, the samples were run on a qPCR machine, where each sample was checked for its own deleted gene, as well as for the other 2 genes and a house keeping gene. Control samples were also used, where commercially available wild type C57BL/6 underwent the same procedure. Unfortunately, the beta-actin house keeping gene primers used appear to have lost activity following the trial test, and did not give any readouts,

thus impeding reading and comparison of expression levels of the three cytokines of interest. Incorporation of such findings in future studies will benefit not only the refinement of animal experimentation, but also the understanding of how deletion of such closely related genes may affect function of the remaining cluster members.

Dual role for the TNF α gene depending on which cell-type it is being expressed?

Several studies have shown differing effects associated with TNF α production. Treatments blocking TNF α has demonstrated to improve outcome in patients with autoimmune disorders such as Crohn's disease, rheumatoid arthritis and autoimmune psoriasis (Feldmann and Maini, 2001). Conversely, deletion of TNF α in mice has shown an increase in retention of T cells in secondary lymphoid organs, causing exacerbation of animal models of multiple sclerosis (MS) (Kassiotis and Kollias, 2001). Additionally, therapeutic administration of TNF α reduced MS severity in both TNF α knockout and wild type mice (Liu et al., 1998). Furthermore, the current study suggests a positive role for TNF α in the combined inflammation and neonatal hypoxia-ischaemia model, as its deletion appears to be detrimental.

However, these studies only looked at global effects of TNF α gene expression, not considering whether or not its source has an effect in this dual role. TNF α is expressed by a multitude of cells, including macrophages, T and B cells, NK cells, dendritic cells and neutrophils (Grivennikov et al., 2005). In this study we wanted to further characterize the role of TNF α by looking at specific cellular sources, i.e. granulocytes and macrophages - excluding microglia - and understand their role in the synergistic model of inflammation and neonatal hypoxic-ischaemic insult.

Cell-type specific deletion of TNF α in MLys-expressing cells revealed a clear neuroprotective effect, where deletion of TNF α gene in granulocytes and macrophages of myeloid origin led to a significant reduction of 70% in tissue

infarction, and 30% decrease in microglial activation and neuronal cell loss. The overall presence of TUNEL+ cells was halved and astrogliosis assessment also showed a trend towards decreased astroglial activation.

A study looking at different cell sources of TNF α in experimental autoimmune encephalomyelitis (EAE) has also shown a detrimental effect for this cytokine depending on which cell-type it is expressed. TNF α MLYs knockout mice demonstrated a later disease onset when compared to wild type controls. Additionally, animals that lacked TNF α in T cells demonstrated significantly milder symptoms than wild type controls (Kurglov et al., 2011). This study by Kurglov et al., suggested that TNF α pathogenic activity seen in the EAE CNS is a result of a distinct contribution of TNF α expression by T cells and myeloid cells. This research group also suggested that TNF α mediation of pathogenesis functions through an initial phase of recruitment of inflammatory cells into the CNS.

Several studies have suggested an increase in neonatal BBB permeability due to inflammation. In-vitro studies of the human BBB have shown both TNF α and LPS-associated increase in BBB permeability (Wong et al., 2004). Furthermore, it is widely suggested that inflammatory cytokines not only affect the CNS increasing inflammation after injury and infection, but may also induce a disruption in BBB function (de Vries et al., 1997; Hagberg and Mallard, 2005). Knowing that the neonatal BBB is leaky following infection/inflammation, and considering the possibility suggested by Kurglov et al., where MLYs+ cells expressing TNF α lead to the initial recruitment of inflammatory cells into the CNS, this could provide a possible explanation to the results seen in this study, where animals which TNF α was expressed in MLYs+ macrophages had substantially higher brain damage 48h post-HI, when pre-treated with LPS. This also coincides with the LT α results in the current study, where global deletion of LT α gene, which also causes a reduced ability of peripheral macrophages to produce TNF α , has demonstrated a

neuroprotective effect. However, it is important to note that Alexopoulou et al, 1998, did not investigate TNF α production within the brain, and therefore it is not known whether neuronal and/or glial TNF α synthesis is also affected in LT α knockout animals.

However, since global deletion of TNF α is detrimental in this particular injury model, this gives rise to the question of which cells are producing the protective TNF α effect that counteracts the MLYS-specific TNF α negative role. A study by Uno et al. has looked specifically at TNF α expression following a mouse model of transient forebrain ischaemia, where they have shown that TNF α expression is evident as early as 1h30min following 30min global ischaemia. Through double staining of TNF α with GFAP, they have also been able to suggest that TNF α is being expressed by microglial cells rather than astrocytes (Uno et al., 1997). Additionally, Kendall et al., has successfully shown using *in situ* hybridization TNF α mRNA in the P7 mouse forebrain following systemic LPS administration (Kendall et al., 2011b). Furthermore, a study by Hristova et al. has also used *in situ* hybridization to show that TNF α is normally expressed at very low levels in the P7 mouse brain. However, following LPS-treatment, TNF α immunoreactivity is strongly upregulated (Hristova et al., 2010). Combining evidences from these three studies demonstrating TNF α expression in microglia following either LPS-mediated inflammation or ischaemia, it is possible to suggest that brain microglial expression of TNF α may have the protective effect seen in this study of combined inflammation and hypoxic-ischaemic injury.

TNF α receptors within the brain are expressed in both glia and neurons. However receptor activation and distribution vary depending on stressor marker, cell activation level and downstream effectors. Therefore, this difference in activation pattern could determine whether TNF α will play a detrimental or protective role following insult. Microglia represent the CNS first and main immune response to insult. It is known that microglia have a dual role, where

alternative M2 differentiation is neuroprotective as M2 macrophages dampen the immune response and promote tissue repair. Classical M1 polarization is believed to exert cytotoxic effects through release of pro-inflammatory cytokines. However, it is now accepted that both M1 and M2 phenotypes co-exist simultaneously (Prinz and Priller, 2014) indicating a concept of co-existence where acute inflammation is required for production of pro-inflammatory cytokines (M1 polarization) leading to recruitment of immune cells and destruction/removal of pathogen (Huang and Feng, 2013) and promotion of regeneration (M2 polarization) (Huang and Feng, 2013; Prinz and Priller, 2014). Therefore it is entirely possible that microglial TNF α production, at least initially, is neuroprotective in this combined model in inflammation and hypoxia-ischaemic insult. However, depending on the degree of homeostatic imbalance caused by severity of insult combined with disruption of BBB, there can be an extensive infiltration of immune cells into the brain, thus exacerbating the inflammatory response and either greatly reducing the M2 neuroprotective effect or leading M2 macrophages to switch into the classical M1 detrimental phenotype.

TNF cluster deletion effect versus individual cytokines role in combined infection/inflammation and neonatal hypoxic-ischaemic insult

As aforementioned, individual deletion of TNF α , LT α and LT β produced not only moderate, but also diverse effects. In summary, global deletion of the LT α gene provides a degree of neuroprotection to the brain. Conversely, global deletion of LT β induced a significant increase in brain damage. TNF α global deletion also revealed a detrimental effect, albeit only moderately. The opposite effect was seen when TNF α was deleted in all MLYS-expressing peripheral macrophages, which proved to be rather beneficial.

Initial studies, considering only the existence of TNF α and LT α cytokines, which was widely known as lymphotoxin (LT), demonstrated that mice deficient in LT

have normal thymus development, however, no morphologically detectable lymph nodes or Peyer's patches. Additionally, white pulp of the spleen showed a failure of normal segregation of B and T cells, suggesting an essential role for LT in the normal development of peripheral lymphoid organs (De Togni et al., 1994). Additionally, $LT\alpha$ knockout animals have suboptimal physiological production of $TNF\alpha$ (Alexopoulou et al, 1998). Discovery of $LT\beta$ (Browning et al., 1993) has led to subsequent studies where all three cytokines $TNF\alpha$, $LT\alpha$ and $LT\beta$ were genetically removed. A study by Kuprash et al., looking at lymphoid tissue, has shown that individual inactivation of $TNF\alpha$, $LT\alpha$ and $LT\beta$ showed significant overlap in phenotypes, where splenic germinal centers were defective in these knockout animals. The same study also looked at deletion of the entire TNF cluster. This cluster deletion demonstrated an additive combination of the individual cytokine deletion defects, with greater impairment in lymphoid development and maintenance of lymphoid organs. Furthermore TNF cluster knockout mice had a much more severe structural spleen defect than individual $TNF\alpha$, $LT\alpha$ and $LT\beta$ knockout mice (see table 6.1). This more severe phenotype seen in the cluster mutants is highly suggestive of non-overlapping functions for each of the cytokines in vivo (Kuprash et al., 2002). This assumption correlates with the results obtained in this study, as each of the three individual cytokines demonstrated different roles in the combined infection and hypoxia-ischaemia model of injury.

Strain knockout	Structural architecture defect
TNFα -/-	<ul style="list-style-type: none"> Splenic defect leading to lack of primary B cell follicles, follicular dendritic cells and germinal centers (Pasparakis et al., 1996) .
LTα -/-	<ul style="list-style-type: none"> Disruption in development of lymph nodes and Peyer's patches and follicular dendritic cells (Kuprash et al., 2002); Reduced germinal center formation segregation (Matsumoto et al., 1996); Splenic white pulp defect causing reduced B and T cells segregation (de Togni et al., 1994); Impaired NK cell development and recruitment (Ito et al., 1999); Lack of mesenteric and cervical lymph nodes (de Togni et al., 1994).
LTβ -/-	<ul style="list-style-type: none"> Disruption in development of lymph nodes and Peyer's patches (Koni et al., 1997); Lack of germinal centers (Alimzhanov et al., 1997).
TNF cluster -/-	<ul style="list-style-type: none"> Combined defects in lymphoid organs development from all three individual cytokines: no Peyer's patch and no mesenteric, brachial, axillary, inguinal or popliteal lymph nodes (Kuprash et al., 2002); Quantitative defect of single TNFα, LTα and LTβ spleen structural defects: no germinal center formation, lack of follicular dendritic cells, reduced segregation of B and T cells (Kuprash et al., 2002).

Table 6.1: Structural defects in peripheral lymphoid organs and spleen of single TNF α , LT α , LT β knockouts as well as TNF cluster deletion.

Conclusion

In summary, it is known that there is a greater structural defect seen in the spleen and peripheral lymphoid organs of TNF cluster deletion, compared with individual TNF α , LT α and LT β cytokine knockouts (Kuprash et al., 2002). It has also been shown that deletion of the TNF cluster also abolished the endotoxin sensitization prior to neonatal hypoxic-ischaemic insult (Kendall et al., 2011b). Additionally, this study has demonstrated that deletion of the individual cluster members not only does not reproduce the same lack of LPS-mediated sensitization, but also shows dissimilar roles for the different cytokines with

strong suggestion that peripheral immune cells play a significant detrimental role in this particular model. Therefore, considering all these facts, it can be suggested that the phenotypic structural defect of peripheral lymphoid organs caused by the cluster deletion, rather than the cytokines functions is causal to the lack of LPS-mediated sensitization in the neonatal model of hypoxic-ischemic insult. This is a novel hypothesis where peripheral immunity response may play a bigger detrimental role in perinatal hypoxia-ischaemia encephalopathy associated with endotoxin inflammation, rather than inflammatory cytokine activation having a direct effect in neonatal brain damage.

CHAPTER 5: THE ROLE OF ACQUIRED CELLULAR IMMUNE SYSTEM IN LPS-MEDIATED NEONATAL HYPOXIC-ISCHAEMIC BRAIN DAMAGE

Abstract

Bacterial endotoxin LPS and hypoxic-ischaemic insults not only induce a robust inflammatory response in the immature brain, but are also known to cause strong activation of the immune system. The aim of this study was to use athymic nude mice to investigate the role of T cell lymphocytes in a synergistic model of neonatal inflammation and hypoxic-ischaemic injury.

P7 offspring of homozygous Nude (no T cell) versus Nude heterozygotes (controls) breeding underwent unilateral carotid occlusion followed by 30min 8% O₂ exposure. 12h prior, animals received a single intraperitoneal injection of 0.6µg/g LPS or saline as a control. Following 48h survival post-hypoxic-ischaemic insult, mice were sacrificed and their brains assessed histologically.

LPS pre-treatment resulted in significant microglial up regulation, neuronal injury and infarct, DNA fragmentation and astrogliosis in the heterozygotes. This LPS-mediated effect was abolished in the Nude mice, where LPS sensitization to subsequent hypoxic-ischaemic insult was not present. These results suggest that T cell function may be required for successful LPS-mediated sensitization to subsequent hypoxic-ischaemic insult.

Heterozygous versus homozygous Nude breeding

As described in chapter 2, genotyping of the recessive nude (nu) mutation is rather difficult. Therefore, males homozygous for the nude gene (nu/nu) were bred against females that were heterozygotes for this gene (nu/+). F1 offspring were either nu/+ (controls) or nu/nu (mutants) in a 50:50% ratio. All animals used in this study were raised in the BALB/c background. As demonstrated in

chapter 3, this particular strain background is moderately sensitive to mild 30min hypoxic-ischaemic insult alone or when combined with saline as a control.



Figure 5.1: Untyped athymic nude mice are macroscopically differentiated at P9 prior to transcatheter perfusion. Mice heterozygotes (nu/+) for the recessive nude mutation are physically larger and covered with white fur, whereas homozygous (nu/nu) mutants are smaller in size and hairless.

Nude phenotype abolishes LPS-mediated brain infarction

Results on the effects of LPS-sensitization in tissue infarction showed that heterozygotes (controls) pre-treated with saline (n=11) had moderate total tissue infarction of $31\pm 8\%$. When compared to the nu/+ LPS (n=14), there was a significant increase in tissue infarction in the latter group to $72\pm 6\%$ ($p < 1\%$). This substantial cell death was evident in all six assessed forebrain regions. In the dorsoparietal cortex there was an increase in ipsilateral brain tissue loss from $42\pm 10\%$ to $82\pm 7\%$ ($p < 1\%$), pyriform cortex from $26\pm 11\%$ to $67\pm 11\%$ ($p < 1\%$), hippocampus from $26\pm 11\%$ to $67\pm 11\%$ (5%), striatum from $42\pm 10\%$ to $82\pm 7\%$ ($p < 5\%$), thalamus from $5\pm 6\%$ to $48\pm 11\%$ ($p < 1\%$) and external capsule white matter from $30\pm 8\%$ to $79\pm 4\%$ ($p < 0.1\%$). Nude animals treated with saline (n=11) showed no difference when compared with heterozygotes saline group ($38\pm 11\%$, $31\pm 8\%$ respectively). Additionally, Nude LPS-treated animals (n=13) demonstrated no LPS-sensitization to HI insult. When compared with heterozygotes (nu/+) LPS-administered animals, this lack of LPS-mediated sensitization was statistically significant, showing a decrease in total tissue loss from $72\pm 6\%$ to $39\pm 9\%$ ($p < 5\%$). Individual assessment of the same ipsilateral forebrain regions confirmed an abolition of LPS-mediated sensitization, where infarct volume in the dorsoparietal cortex decreased from $82\pm 7\%$ to $53\pm 11\%$ ($p < 5\%$), pyriform cortex from $67\pm 11\%$ to $37\pm 12\%$ ($p < 5\%$), hippocampus from

67±11% to 37±12% (p<5%), striatum from 82±7% to 53±11% (p<5%), thalamus from 48±11% to 17±10% (p<5%) and external capsule from 79±4% to 42±10% (p<1%) (see figure 5.2.A).

Animals homozygous for the nude gene (nu/nu) do not show an increase in brain injury when treated with LPS prior to HI-injury

Assessment of brain injury using the combined injury score system described in chapter 2 demonstrated that heterozygotes injected with saline had considerable microglial activation and neuronal cell loss (3.8±1.0). This is consistent to what was demonstrated in the assessment of the BALB/c strain in chapter 3 (3.8±1.0). However, when compared with the nu/+ LPS group, the latter showed a maximal increase in combined injury score by a factor of 2, to 7.6±0.3 (p<1%). Similarly to what was shown in the infarct assessment, this increase in brain damage also evident in most ipsilateral brain regions assessed, with most regions almost reaching the maximal combined injury score of 8: dorsoparietal (3.9±1.1 to 7.9±0.1, p<1%) and pyriform cortex (2.7±1.1 to 7.2±0.5, p<1%), hippocampus (3.3±1.0 to 7.9±0.1, p<1%), striatum (4.6±0.9 to 7.8±0.2, p<5%) and thalamus (2.2±1.0 to 6.7±0.7, p<1%). Although not statistically significant, there was also a visible trend in increased brain injury in the external capsule of nu/+ LPS from 6.1±0.8 (nu/+ saline) to 8.0±0.0. Again, observation of the Nude saline and Nude LPS groups showed no difference in alphaM activation and Nissl cell death when compared to each other and to nu/+ saline (4.0±1.1, 4.5±1.1 and 3.8±1.0 respectively). Consequently, comparison between both LPS groups – nu/+ and nu/nu – demonstrated once again that not only athymic nude animals are non-responsive to LPS sensitization, but also, this lack of sensitization is statistically significant when compared to nu/+ LPS littermates in terms of brain injury (4.5±1.1 vs. 7.6±0.3, p<5%). Furthermore, individual scoring of the assessed forebrain regions showed that this decrease was almost by a factor of 2. Dorsoparietal cortex of nu/nu LPS animals showed a decrease in injury score to 4.9±1.1 (p<5%), pyriform cortex to 3.8±1.1 (p<5%), hippocampus to 4.4±1.1

($p < 1\%$), striatum to 5.0 ± 1.0 ($p < 5\%$), thalamus to 3.9 ± 1.0 ($p < 5\%$) and external capsule to 5.2 ± 1.0 ($p < 5\%$) (see figure 5.2.B).

Nude mice have considerably less DNA fragmentation than heterozygotes littermates

Quantification of TUNEL+ cells showed minimal DNA fragmentation cell death in the heterozygote saline, Nude saline and Nude LPS groups (9.5 ± 5.2 , 27.0 ± 13.8 and 38.7 ± 16.1 respectively). Conversely, heterozygote animals treated with LPS prior to HI insult demonstrated a significant increase in TUNEL+ cells (189.6 ± 48.7) not only when compared to heterozygotes saline controls ($p < 0.1\%$), but also when compared to Nude LPS animals ($p < 0.1\%$). Furthermore, this substantial increase in DNA fragmentation-associated cell death was significant in all assessed forebrain regions of the ipsilateral brain hemisphere. The dorsoparietal cortex of nu/+ LPS animals had an average TUNEL+ cell count of 40.1 ± 92.9 whereas nu/+ saline had an average count of 46.3 ± 23.8 ($p < 0.1\%$) and nu/nu LPS animals 114.4 ± 38.1 ($p < 1\%$). In the pyriform cortex average TUNEL+ counts of 148.6 ± 38.9 were reduced to 1.2 ± 1.0 and 13.5 ± 9.7 (0.1%), hippocampus from 142 ± 28.7 to 5.9 ± 4.7 and 14.8 ± 8.4 ($p < 0.1\%$), striatum from 180.6 ± 46.1 to 0.5 ± 0.4 and 68.2 ± 32.8 ($p < 1\%$, $p < 5\%$), thalamus from 172.6 ± 52.4 to 0.2 ± 0.1 and 0.0 ± 0.0 ($p < 0.1\%$), and external capsule from 91.7 ± 33.4 to 2.8 ± 1.3 and 21.1 ± 7.8 ($p < 1\%$, $p < 5\%$) respectively (see figure 5.2.C).

GFAP immunoreactivity is significantly reduced in nu/nu LPS treated animals

Assessment of astrocytic activation demonstrated once again that LPS treatment does not increase GFAP immunoreactivity in athymic nude mice. Optical luminosity values for nu/nu LPS animals (53.5 ± 7.1) showed no difference when compared to nu/+ saline (58.2 ± 6.6) and nu/nu saline (55.8 ± 8.3) animals. However, GFAP synthesis was consistently and significantly higher in the nu/+ LPS group (91.0 ± 9.3) when compared to nu/+ saline controls ($p < 1\%$) and to nu/nu LPS ($p < 0.1\%$) littermates. Closer observation of the individual forebrain

regions showed the same significant up regulation in astrogliosis in the nu/+ LPS group when compared to nu/+ saline and nu/nu LPS respectively. Assessment of the dorsoparietal cortex showed a significant increase in astrocyte immunoreactivity from 53.9 ± 6.5 in the nu/+ saline and from 43.9 ± 6.1 in the nu/nu LPS animals to 94.8 ± 10.9 in the nu/+ LPS group ($p < 0.1\%$). In the pyriform cortex from 51.3 ± 8.0 and 47.4 ± 6.7 to 82.2 ± 11.7 ($p < 5\%$), hippocampus from 60.4 ± 5.9 and 64.0 ± 5.7 to 97.5 ± 7.0 ($p < 1\%$), striatum from 68.6 ± 5.8 and 58.6 ± 7.2 to 90.0 ± 7.7 ($p < 5\%$ and $p < 1\%$), thalamus from 35.9 ± 7.1 and 41.0 ± 7.9 to 70.9 ± 11.3 ($p < 5\%$), and external capsule from 78.8 ± 6.4 and 66.2 ± 8.8 to 110.7 ± 7.4 ($p < 5\%$, $p < 1\%$) respectively (see figure 5.2.D).

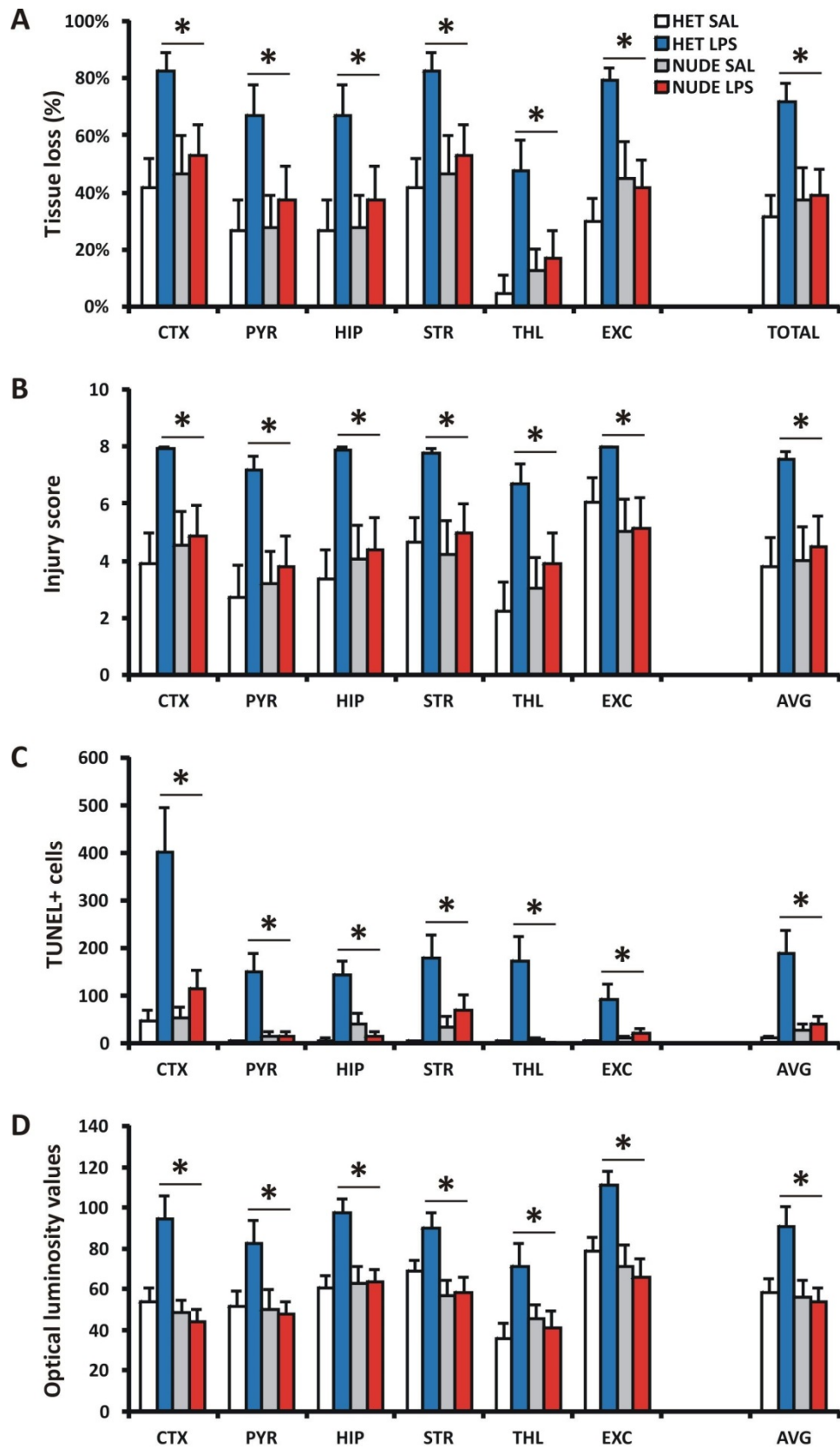


Figure 5.2: Effects of the nude gene (*nu/nu*) on neonatal hypoxic-ischemic brain injury after pre-exposure to 0.6µg/g LPS endotoxin (LPS). Animals injected with saline (SAL) served as controls. **A:** Infarct volume assessment of individual forebrain regions and total ipsilateral hemisphere

infarction as percent of contralateral hemisphere showed that LPS-mediated tissue loss was abolished in the nu/nu animals. **B:** Brain injury assessment of nu/nu mice revealed again an abolishment of LPS-mediated increase in inflammatory response and neuronal cell loss. **C:** DNA fragmentation cell death assessment using TUNEL+ cell quantification demonstrated a significant increase in TUNEL+ cells in the LPS heterozygotes. However, LPS pre-treatment in the athymic nude mice failed to elicit this type of cell death beyond the baseline hypoxic-ischemic saline response. **D:** Astroglial immunoreactivity assessment using optical luminosity values showed a significant increase in GFAP synthesis in the LPS heterozygotes. This LPS-mediated effect was absent in the LPS nu/nu mice. Contralateral hemisphere not shown. * p<5% in Mann Whitney test for the LPS-treated wild type vs. knockout groups, n=11 for HET SAL, n=14 for HET LPS, n=11 for NUDE SAL and n=13 for NUDE LPS. **Abbreviations:** HET – heterozygotes, CTX- dorsoparietal and PYR – pyriform cortex, HIP – hippocampus, STR – striatum, THL – thalamus, EXC – external capsule.

Discussion

The results obtained in the cytokines chapter (chapter 4) of the current study demonstrated moderate and differing roles for the TNF cluster members, TNF α , LT α and LT β , thus suggesting that endotoxin-mediated sensitization seen on the TNF cluster study (Kendall et al., 2011b) was not a result of a single action of one of these three cytokines. Consequently, we were unable to answer, so far, the original question of this study: Which, or combination of, these three cytokines is responsible for mediating the LPS sensitization to subsequent neonatal hypoxia-ischaemia?

Interestingly, studies looking at global deletion of these genes have also shown that global deletion of TNF α , LT α or LT β produce a defect in the acquired immune system (Kuprash et al., 2002). Furthermore, deletion of the entire TNF gene cluster causes more severe phenotypic defects, which appeared to be both a combination and quantitative sum of the individual TNF cluster members' defects, indicating these cytokines play an important role in the immunological development (Kuprash et al., 2002). Therefore, in this study we wanted to investigate the role of the acquired immune system in this combined model of injury using the athymic nude mouse model of T cell deficiency.

The athymic nude mouse was first described by Flanagan in 1966, and was characterized macroscopically by a lack of fur, increased postnatal mortality and general susceptibility to systemic toxoplasmosis (Flanagan, 1966). It was later found that homozygous nude mice (nu/nu) also lack a functional thymus (Pantelouris, 1968), thus leading to a profound T cell deficiency, with a severely compromised cell-mediated immune response and humoral immunity (Romano et al., 2011). However, disturbed development of hair follicles and thymus are independent phenotypic effects of nude mice, indicating a pleiotropic mutation (Eaton, 1976; Nehls et al., 1996). The 'nude' phenotype is a spontaneous autosomal recessive mutation of the *Foxn1* transcription factor gene of the conserved winged-helix domain family, and *Foxn1* gene is known to regulate keratin gene expression (Nehls et al., 1994). *Foxn1* transcription in mammals occurs mainly in the skin and thymus. However, there have been some cases of expression in developing nails, tongue, teeth, palate and nasal passages (Nehls et al., 1996; Lee et al., 1999). Foxn1 protein is expressed exclusively in epithelial cells, suggesting that *Foxn1* activity is specific to these cells, including thymus epithelial cells (Brisette et al., 1996). Therefore, functional loss of *Foxn1* leads to athymia where thymic epithelial cells cannot mature and attract hematopoietic precursor cells. T cell development is dependent on these two events. However, the exact signaling pathways that regulate *Foxn1* expression and the microenvironment necessary for T cell development still remain unknown (Balciunaite et al., 2002).

Athymic nude mice do not respond to LPS sensitization prior to neonatal hypoxic-ischaemic insult

In this study, LPS treatment resulted in a significant increase in tissue infarction, brain injury, DNA fragmentation-associated cell death and astrogliosis in the heterozygotes littermates (nu/+) of homozygous nude mice (nu/nu). However, nude phenotype abolished the LPS-sensitization effect to subsequent hypoxic-ischaemic insult.

Both *in vitro*, *in vivo* and clinical studies have demonstrated up-regulation of T cell activation following bacterial infection. Vogel et al., 1983 were the first to suggest that endotoxin can stimulate T cells directly. In this study, a cloned cytotoxic T cell line (IL-2-dependent CT 6) which was free of other contaminating lymphoid cell types, such as B lymphocytes and macrophage-derived products was used. The CT 6 cell line was shown to respond proliferative to LPS, and this response was optimal at 48h post-stimulation, thus suggesting the presence of an endotoxin-responsive T cell population. (Vogel et al., 1983). In a mouse study, LPS intravenous administration was assessed three days later in spleen tissue of wild type C57BL/6 mice. Results showed a substantial increase in CD8⁺ cells, even at minimal dose of 10ng (Tough et al., 1997). A separate study using bacterial *L. monocytogenes* as a means of infection, showed a substantial increase in T cells within 24h. Furthermore, this surge in T cell activation was not altered by use of different concentrations of live *L. monocytogenes* (1,000 to 25,000), thus indicating that even minimal doses of a pathogen can lead to an immediate substantial activation and proliferation of T cells (Mercado et al., 2013). Blood samples taken from patients with localized bacterial soft tissue infections showed an increase in the presence of CD8⁺, and to a smaller extent, also CD4⁺ in the site of infection (Wagner et al., 2008).

Furthermore, a study looking at acute hepatotoxicity using *Pseudomonas* exotoxin (exotoxin A) showed that athymic BALB/c nu/nu mice – the same strain as the one used in the current study – were resistant to endotoxin A-induced liver damage. Additionally there was a clear reduction in circulating cytokine levels, including TNF α , a known mediator of hepatocellular apoptosis. BALB/c control animals demonstrated TNF α to be locally produced within the liver by Kupffer cells within 3h after exotoxin A administration, and in the cytoplasm 12h after administration. These findings suggest that T cells play an essential role in TNF α production by liver macrophages, as well as having an involvement in

exotoxin A-induced hepatotoxicity. (Schümann et al., 1998). These results suggest a relevant sensitization mechanism by which T cells affect directly the production of cytotoxic TNF α , other than its production by direct LPS-mediated stimulation of monocyte/macrophages, as seen on the MLys results (chapter 4), where there was a partial neuroprotective effect in TNF α -deficient myeloid cells. However, it is important to note that the current study did not look at TNF α levels in the liver, as well as within the brain of nude mice in order to confirm whether the effect seen in the Schümann et al. study is applicable in a neonatal mouse model of combined inflammation and hypoxia-ischaemia injury.

T cell function may be required for LPS-mediated sensitization to neonatal hypoxic-ischaemic insult

The nude phenotype is caused by a defect in the *Foxn1* gene, which has shown to be essential for thymic epithelial cell to mature and support subsequent T cell generation (Zhang et al., 2012). Additionally, LPS administration leads to T cell activation and proliferation. Thus, the prevention of LPS-sensitization seen in the nude mice in this study could be caused by the absence of mature T cells. This might suggest that LPS-mediated sensitization of the neonatal brain to subsequent hypoxic-ischaemic insult requires T cell function. This could provide a link to the results seen by Kendall et al., looking at TNF cluster knockouts. Thus suggesting that it is the structural defects in the peripheral immune system that are causal to the lack of LPS-mediated sensitization in the TNF cluster-/- animals, rather than cytokine function.

However, alternative explanations are possible – for example *Foxn1* might be important for some aspect of LPS signal transduction directly involved in endotoxin-sensitization. Unfortunately, the precise target genes regulated by *Foxn1* still remain largely unknown (Zhang et al., 2012). Furthermore, it is important to note that the acquired cellular immune system is comprised of other cells as well, such as B cells and NK cells, and that their role in the

combined endotoxin and hypoxic-ischaemic insult also need to be investigated in order to further understand whether the *Foxn1* mutation is interfering with LPS-mediated sensitization through its immune defect, or if other factors could be at play.

Studies have looked at combined T and B cell deficiency using either Rag1 or Rag2 recombination activation genes. These genes are involved in the recognition and cleavage of signal specific sequences required for somatic rearrangement of T cell receptors and immunoglobulin (Mombaerts et al., 1992; Shinkai et al., 1992). A study looking at transient ischaemic stroke in adult mice has shown that Rag1^{-/-}, CD4⁺ T cell^{-/-}, CD8⁺ T cell^{-/-} and IFN- γ ^{-/-} animals demonstrated substantial reduction in ischaemia/reperfusion-induced platelet and leukocyte adhesion compared to wild type controls. Additionally, cerebral infarction was also reduced in the Rag1 knockout mice. When Rag1^{-/-} animals were reconstituted with wild type splenocytes, this protective effect was lost. Reconstitution with IFN- γ ^{-/-} splenocytes led only to a partial improvement, indicating that T lymphocytes may only be a small source for this cytokine. Furthermore, mature B cell negative animals did not show an altered response to ischaemic injury when compared to wild types. This study highly suggests that CD4⁺ together with CD8⁺ T lymphocytes, and not B cells, play a relevant role in inflammation and brain injury following an ischaemic episode (Yilmaz et al., 2006). This study corroborates the results found in the current study, where athymic nude mice demonstrated a lack of LPS-mediated sensitization, therefore, it is highly possible that *Foxn1* defect in the nude mice is in fact acting through its T cell immune defect.

Interestingly, there have been studies suggesting that nude mice may have an elevated peritoneal macrophage activity. These macrophages have been associated with releasing oxidising agents such as hydrogen peroxide and superoxide anions following a burst of oxidative metabolism. Peritoneal

macrophages from nude mice have shown a significantly higher release of these agents following exposure to phorbol myristate acetate, thus conferring a resistance to *L. monocytogenes* not seen in euthymic mice (Sharp and Colston, 1984). Also, T cell-deprived C57BL/6 mice have shown an increase in macrophage activation following *M. lepraemurium* infection (Brett and Butler, 1988). These findings suggest that athymic nude mice might be either compensating for their immunodefects by increased phagocytic activity and therefore systemic macrophage activation may occur via T cell-independent besides dependent mechanisms, or that peritoneal macrophage activity is suppressed in the presence of T cells. Either hypothesis highlights a beneficial macrophage activity effect that is either not present, or suppressed in the presence of mature T cells. These findings suggest a potential beneficial effect for suppression of these cells, which once again indicates that the beneficial effect seen in the current results may be a result of lack of T cell activity.

However, T cells are also an important component of the immune system. In a mouse study of *Coxiella burnetii* infection, SCID (no T and B cells), SCIDbg (no T, B and NK cells), nude (no T cells), muMT (no B cells), bg (no NK cells), TNF α -/- and IFN- γ -/- mice were infected with Nine Mile phase I (NM I) highly virulent bacteria containing a complete LPS, or avirulent NM II. This study showed that SCID, SCIDbg, nude and IFN- γ -/- animals showed the highest susceptibility to NM I infection. Furthermore, SCID, SCIDbg and nude mice showed the slowest disease progression. Additionally, muMT and bg mice did not demonstrate a discernible difference in bacterial clearance or disease progression, however, there was a visible increase in severity of histopathological changes. This study has shown an important role for T cell clearance of *C. burnetii*, whereas B cells appear to be important for prevention of tissue damage (Andoh et al., 2007).

Conclusion

This study has demonstrated a robust LPS-mediated sensitization in the heterozygote littermates of athymic nude mice. Conversely, nu/nu animals failed to respond to LPS-sensitization. These results strongly suggest that the TNF cluster abolishment of LPS sensitization seen by Kendall et al., 2011b, may be a result of its severe effect on the structure of peripheral immune system, which leads to a lack of mature T cells, rather than being a result of direct cytokine function. These results are very novel and suggest that manipulation of T cell function may be a useful strategy to block LPS-mediated neonatal hypoxic-ischaemic injury. This novel hypothesis is highly attractive as T cell immunosuppressants are already pharmacologically available. However, it is important to note that T cells have an important physiological role in cell-mediated immunity. Therefore, inhibition of T cell activity should be carefully monitored. Furthermore, it is still possible that the protective effect seen in this study using nude mice may be caused by a *Foxn1* effect direct on LPS signal transduction that is irrespective of T cell function, and therefore one should not exclude further characterization of this combined model of inflammation and hypoxia-ischaemia injury by looking at the other components of the acquired immune system, such as B and NK cells.

CHAPTER 6: GENERAL DISCUSSION

Infection/inflammation and hypoxic-ischaemic insult are widely accepted as having a devastating impact on the neonatal brain. These types of injury can cause severe brain damage alone, but when combined, work in a synergistic manner augmenting significantly and irreversibly subsequent perinatal brain damage, thus greatly enhancing the risk of cerebral palsy and/or other neurological deficits such as cognitive impairment, schizophrenia and autism (Bax et al., 2005). Despite continuous advance in science, the mechanisms involved in this synergistic form of brain damage remain largely unknown. To better understand detrimental factors leading to impairment to the developing brain, clinicians and scientists have not only relied on retrospective data, but also on both large and small experimental animal models. Classic studies by Dobbing and Sands, 1979, and later Clancy et al., 2001, have further established the relevance of using neonatal animal models to understand perinatal brain damage. Dobbing and Sands have demonstrated the correlation of brain growth across species (Dobbing and Sands, 1979), whereas Clancy et al. have used multivariate analysis from histological and functional maturity from multiple brain regions and neural systems across multiple mammalian species and provided comparison to both prenatal and early postnatal human brain development. These findings have given credit to the continual use of the P7 mouse model of hypoxic-ischaemic insult as they have demonstrated the P7 mouse to have an overall brain maturity equivalent to that of an early third trimester human baby (Clancy et al., 2001; Clancy et al., 2007). Furthermore, the availability of transgenic mice models represents a promising furthering step in elucidating pathways and mechanisms of brain injury. Therefore, the use of wild type and transgenic P7 mouse model of combined infection/inflammation and hypoxic-ischaemic insult could provide invaluable new knowledge in attempting to understand the mechanisms involved in perinatal brain damage.

However, despite the fact that the mouse is the most commonly used mammalian model due to its close genetic relationship to humans and availability for genome modification, the introduction of different mouse backgrounds and genetically modified animals has also given rise to variability in response to either insult or treatment (Beckmann, 2000; Anisman et al., 2008). As the current study required multiple genetically modified strains in order to address the original question set out by this project – to determine which of the three cytokines, TNF α , LT α and LT β is responsible for the endotoxin-mediated sensitization seen in the TNF cluster study by Kendall et al. – and knowing that there is a strain-related response to neonatal hypoxia-ischaemia insult (Sheldon et al., 1998), we decided to first investigate the response to combined inflammation and neonatal hypoxia-ischaemia in five commonly used background strains: 129SVJ, C57BL/6, BALB/c, CD1 and FVB.

The results in this study are in line not only with Sheldon et al, showing a greater sensitivity from the CD1 strain, followed by C57BL/6 and 129SVJ, they have additionally shown that BALB/c has a moderate response to hypoxic-ischaemic insult, and FVB appears to be the most sensitive of all five strains. Addition of LPS bacterial endotoxin pre-treatment successfully sensitized all five strains, revealing a substantial increase in tissue infarction, inflammation and cell death in the already hypoxic-ischaemic sensitive BALB/c, CD1 and FVB strains. Furthermore, both 129SVJ and C57BL/6 strains, which appeared resistant and moderately resistant respectively, to 30min hypoxic-ischaemic insult, also showed an extensive increase in damage in the presence of LPS-induced inflammation 12h prior to hypoxic-ischaemic surgery. This is a clear indication that despite known anatomical and immunological variations between different strains (Beckmann 2000; Hoover-Plow et al., 2008), immunomodulatory role of LPS is still present within all five strains. However, it is important to note that despite successful LPS-mediated sensitization, there is still noticeable variation in brain damage severity followed combined injury across all five strains.

Interestingly, this study has shown a novel effect for the use of saline treatment as a control, as some of the strains revealed a saline-associated stress. FVB mice demonstrated a saline-associated increase in microglial activation and neuronal cell loss. C57BL/6 mice showed an increase in astrogliosis upon saline administration 12h prior to hypoxia-ischaemia injury; this effect was also present in the contralateral brain hemisphere. This is of great relevance, as this effect may mask actual results, as different strains may have an altered immune response, where they could be either more sensitive or resistant, and therefore hinder more accurate interpretation of results.

The results obtained in this strain study highlight the importance of careful planning when choosing a background strain for use of genetically modified mice in research. This study has shown that, what is considered a moderate hypoxic-ischaemic event (30 min 8% O₂) with negligible response in 129SVJ mice, and minimal response in C57BL/6 animals, is in fact severe for CD1 animals, and even more so for FVB mice. This is interesting, as studies have shown that the longer the duration of transient hypoxic insult, the higher the mortality rate (Kendall et al., 2006). Also, studies using both C57BL/6 and CD1 mice have employed 10% O₂ for longer periods of 60-90min in order to reproduce ipsilateral brain damage (Hagberg et al., 2002; Kichev et al., 2014). In the current study, the 30min 8% O₂ experimental design did not cause lethality during either insult, or throughout the 48h window before transcardial perfusion. Therefore, it is possible to suggest using CD1 and FVB animals for 30 min 8% O₂ neonatal hypoxia-ischaemia alone as a preferential approach. Furthermore, both CD1 and FVB mice are known to produce large litters and are not known to cannibalise first litters, unlike C57BL/6 mice. This provides not only a quicker experimental approach, with less stress to animals due to reduced hypoxic period (30 min), but also a refinement and reduction in the requirement of animals per study.

This study has also identified that the preferential strain for combined LPS and hypoxic-ischaemic insult is the C57BL/6 inbred strain. C57BL/6 animals responded only mildly to hypoxia-ischaemia alone, but damage was drastically and significantly increased following LPS pre-treatment. This effect was consistent throughout all four different assessments. This provides an ideal experimental approach, as a large number of transgenic mice are provided with the C57BL/6 background. Using the inbred C57BL/6 background strain helps avoid the need to re-cross and backcross strains, once again providing experimental refinement. Furthermore, this is a very well characterized inbred strain, thus facilitating interpretation of results in the presence of variability.

All animals obtained for the cytokine part of this study came in a C57BL/6 background, thus facilitating progression of this project. However, after successful LPS-sensitization on the $LT\alpha$ experiment, this response to endotoxin pre-treatment was lost in both $TNF\alpha$ and $LT\beta$ experiments. This was a novel result, as both the strains experiments aforementioned, as well as multiple other studies have shown successful LPS-mediated sensitization (Hagberg et al., 2002; Mallard et al., 2003; van de Looij et al., 2012). As a result, we decided to test the LPS for loss of function, which proved to not be the case, as microglial activation scoring following alphaM immunohistochemistry revealed a significant increase in microglial activation. Subsequently, we hypothesised that this lack of LPS sensitivity could be a result of either a gene mutation or due to strain variation. As gene mutation would require a more complex approach, we decided to cross each of the non-responder strains, $TNF\alpha$ and $LT\beta$, with wild type C57BL/6 animals. However, one re-crossing was not sufficient to successfully induce a LPS-mediated response, and a second crossing against wild type C57BL/6 mice was required in order to successfully induce a robust endotoxin-mediated sensitization. This resulted in a 75% genetic introduction of the new C57BL/6 animals, which suggests that whatever the cause of this reduced response to endotoxin, this must be due to a dominant trait.

Previous research has demonstrated that deletion of the TNF cluster of pro-inflammatory cytokines abolishes LPS-mediated sensitization to subsequent neonatal hypoxia-ischaemia (Kendall et al., 2011b). This suggests that endotoxin sensitization, in this particular model, occurs through a mechanism dependent on the TNF cluster of pro-inflammatory cytokines consisting of TNF α , LT α and LT β . The original aim of this study was to identify which of the three cytokines is responsible for this sensitization, using single gene deletion models for each of these cytokines, or cell-specific deletion of TNF α gene under Mlys::CRE promoter. Unexpectedly, the results obtained in chapter 4 of the current study demonstrated a diverging role for the TNF α , LT α and LT β cytokines members of the cluster, where only global deletion of the LT α gene was neuroprotective, albeit only moderately when compared to the TNF cluster knockout results (Kendall et al., 2011b). Furthermore, global deletion of the LT β gene provided a substantial increase in brain damage, whereas global TN α knockouts revealed only a trend towards increased damage following combined inflammation and hypoxia-ischaemia injury. As TNF α is expressed by different cell-types, we were also able to look at animals that did not produce TNF α in myeloid cells (Mlys::CRE TNF α flox/flox). These animals revealed a neuroprotective effect for this cell-type specific deletion of the TNF α gene, thus indicating a possible dual role for TNF α depending on which cell-type it is being expressed. Interestingly, these results were not able to successfully address and answer the original question set by this project: which, or combination of, these three cytokines is responsible for LPS-mediated sensitization to subsequent hypoxic-ischaemic insult. Furthermore, these results suggest that individual pharmacological inhibition of these cytokines would not be a desired approach for reduction of perinatal brain injury following combined inflammation and hypoxic-ischaemic insult.

A possible explanation for the opposing roles between $LT\alpha$ and $TNF\alpha$ and $LT\beta$ could be their close proximity within 12kb of genomic DNA within the major histocompatibility complex locus (Kendall et al., 2011b). These cytokines are known to have overlapping function, and to play an essential role in the development of secondary lymphoid organs and immune response. Therefore, it is entirely possible that deletion of one of these closely related genes could affect expression of one or both other TNF cluster members. In fact, a study by Alexopoulou et al. has demonstrated that $LT\alpha$ knockouts have a more severe lymphoid organs phenotypic defect when compared to the other two cluster members. Furthermore, this same study revealed that $LT\alpha$ mutants also had reduced $TNF\alpha$ production by peritoneal macrophages (Alexopoulou et al., 1998). These findings could explain the positive results seen both in the $LT\alpha$ and $MLys^{+/-} TNF\alpha$ flox/flox ($MLys$) experiments. $LT\alpha$ knockout animals cause a more severe defect on lymphoid organs, thus leading to a reduced immune response; this is coupled with a reduction in $TNF\alpha$ synthesis by peripheral macrophage cells. Additionally, it has been shown in the current study, that myeloid cells have a detrimental effect when normally expressing $TNF\alpha$ cytokine ($MLys$ experiment). The protective deletion of $TNF\alpha$ gene in $MLys^{+}$ cells seen in the current study is also in concordance with another study that showed that these same knockout animals have a later onset of experimental autoimmune encephalomyelitis. The same study also suggested that $TNF\alpha$'s detrimental effect in EAE could be a result of initial recruitment of immune cells into the brain (Kurglov et al., 2011).

A limitation of the current study is the fact that we were unable to verify whether deletion of one of the TNF cluster members has an effect on the expression of the other two members. qRT-PCR was performed on tissue spleen tissue from animals that had either $TNF\alpha$, $LT\alpha$ or $LT\beta$ gene globally removed, as well as wild-type mice. Unfortunately, the housekeeping gene β actin, which was successfully used for a trial experiment for this mRNA expression protocol failed

to work in this experiment, thus rendering impossible to do a correct readout and comparison of expression levels of each of the three cytokines in the different mutants. However, this experiment would be an invaluable future addition to the understanding of these cytokines' role in this combined form of injury.

As previously mentioned, TNF α , LT α and LT β global gene deletions leads to a structural defect in the peripheral immune system, with a more severe effect seen in the LT α mutants. Additionally, entire TNF cluster deletion leads to an additive phenotype defect equivalent of a combination of all three individual deletions in terms of impairment of lymphoid development and function (Kuprash et al., 2002). Therefore, knowing that individual cytokine deletion did not reproduce the abolition effect seen in Kendall et al, 2011b, and that impairment of TNF α expression in myeloid cells is rather beneficial, we decided to approach this study from a new perspective. We now decided to investigate if it was this defect in the acquired immune system that was causing the non-response to LPS sensitization seen in the TNF cluster study. To address this new hypothesis we obtained BALB/c athymic nude mice.

Bacterial administration, including LPS, lead to a substantial activation in T cells. However, the current nude experiment showed an abolishment of LPS-mediated sensitization to subsequent hypoxic-ischaemic injury in the nude group. This was significant and consistent across all forebrain regions, as well as all four assessments performed, thus suggesting the involvement of T cell in the LPS-mediated inflammation-induction. An ischaemic study using Rag1 animals (no T and B cells) has shown similar effects, where Rag1 animals demonstrated a substantial decrease in tissue infarction. Furthermore, this protective effect disappeared in animals that had been reconstituted with wild type splenocytes. Additionally, the same study showed that lack of mature B cells did not alter response to ischaemic injury (Yilmaz et al., 2006). These findings corroborate the

theory generated in the current study, where it is the immune defect generated by the TNF cluster mutants, which is causing the lack of LPS-mediated sensitization, rather than cytokine function. This hypothesis is strengthened by the fact that the single gene knockout animals provided varying and differing effects. The use of nude animals in this study, which further strengthens the theory that the acquired immune system plays a bigger role than previously expected in this combined form of injury, and that T cell physiological response to LPS is what is causing the sensitization to subsequent hypoxic-ischaemic injury. These findings could also indicate that the stress affecting the immune system, as seen in the C57BL/6 and FVB strains response to saline administration 12h prior to hypoxia-ischaemia could also occur through the same pathway, as peritoneal systemic saline injection led to a moderate increase in immune response.

However, the athymic nude mouse is a result of a spontaneous recessive mutation on the *Foxn1* transcription factor gene. This transcription factor is responsible for regulating keratin gene expression and occurs mainly in skin and thymus epithelial cells (Brisette et al., 1996). The exact pathways involved in *Foxn1* expression still remain unknown (Zhang et al., 2012), and therefore, one cannot exclude the possibility that *Foxn1* may be involved in LPS signalling in a T cell independent manner. Further clarification on whether it is the defective acquired immune system that is causing the nude non-responsiveness to LPS sensitization or if this is a result of another function of *Foxn1* gene could possibly be achieved by looking specifically at CD4+ and CD8+ subsets, as well as other members of the acquired immune system, such as B cells and NK cells.

Conclusion

In summary, this study has demonstrated a rather robust LPS-mediated sensitization of the neonatal brain to subsequent hypoxic-ischaemic insult across several inbred and outbred mouse strains. Even in cases where strains appeared

to be non-responsive to bacterial endotoxin, this could be reverted through serial crossing with C57BL/6 mice, shown to be high responders. This study has also raised the question of whether or not saline administration as a control is in fact representative of unchallenged animals, as some strains have shown a saline-associated increase in inflammatory markers. Another novel hypothesis raised by this study is the suggestion that the peripheral immunity response following combined endotoxin and neonatal hypoxia-ischaemia injury may play a bigger detrimental role in neonatal encephalopathy associated with bacterial infection, rather than inflammatory cytokine activation having a direct effect in neonatal brain damage. Furthermore, this study suggests that LPS-mediated sensitization may require T cell function. If correct, these findings provide an easier and more readily available approach for therapeutic intervention, as there are currently commercially available immunosuppressants that target T cell activation, such as cyclosporine A.

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