UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

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Centre for Biological Sciences

Immune to Brain Communication in Allergic Lung Inflammation

by

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ABSTRACT

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES, INSTITUTE FOR LIFE SCIENCES, CENTRE FOR BIOLOGICAL SCIENCES
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Emelie Olivia Johanna Larsson BSc. (Hons)

Asthma, a chronic TH2-mediated inflammatory disease of the airways, is the most common form of allergy in the Western world, affecting 300 million people worldwide. Epidemiological studies have shown that asthma is associated with mood disorders, such as anxiety and depression, and numerous experiments have reported that asthma induces functional changes in neuronal fibres of the peripheral nervous system (PNS), which innervate the brain. It is unknown, however, how allergic lung inflammation impacts on the central nervous system (CNS). The ability for peripheral inflammation to impact on the brain, altering behaviour and neuronal activity in the CNS, is a well-recognised and physiological phenomenon, known as immune to brain communication, but has, until now, only focused on how innate pro-inflammatory and TH1, but not TH2, type immune responses impact on the brain. Critically, immunomodulatory therapeutics, which involve stimulation of an innate pro-inflammatory immune response, are currently being developed for the treatment of asthma, highlighting the importance of understanding the effect of allergic lung inflammation and its treatment on the brain. Consequently, using acute and chronic localised TH2 models of inflammation, we investigated how allergic lung inflammation impacted on the CNS and subsequently determined the secondary impact of immunomodulation with the Toll-like receptor 7 (TLR7) agonist resiquimod.

Acute TH2 inflammation in the peritoneum and lung was found to communicate with the brain, via a vagal route of communication. Crucially, it led to a distinct pattern of neuronal activity, with no changes in sickness behaviour or CNS inflammation, changes widely different to those known to occur following systemic TH1 inflammation. At chronic stages of lung inflammation, changes in genes associated with synaptic plasticity in the brainstem and altered expression of the $GABA_B$ receptor and brain-derived neurotrophic factor in the hippocampus were observed, firstly providing a CNS-dependent biological explanation for airway hyperresponsiveness, a critical pathological symptom of asthma, and secondly offering a biological justification for the prevalence of mood disorders in asthmatic patients. Resiquimod treatment in allergic animals was associated with attenuated central inflammatory responses, as compared to treatment in healthy animals, encouraging and reassuring in terms of patient well-being and, critically, also insinuating that safety of therapeutics differs in diseased, as opposed to healthy individuals. The results in this thesis are some of the first to identify that physiological inflammatory diseases impact on the CNS, highlighting the importance of immune to brain communication on pathological and psychopathological symptoms of a disease, and additionally demonstrating how inflammatory conditions can modify the off-target effects of a drug. Not only do these results provide a foundation for the future of immune to brain communication research, namely understanding how physiological inflammatory diseases impact on the CNS, but also have the potential to be translational and emulated in a clinical setting.

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Declaration of Authorship

I, **Emelie Olivia Johanna Larsson**,

declare that this thesis entitled

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and the work presented in this thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is clearly attributed;
- where I have quoted from work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where this thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission

Signed: …….

Date: …….

Dedication

To my family.

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List of Abbreviations

ACTH	Adrenocorticotrophic Hormone
AHR	Airway Hyperresponsiveness
AD	Alzheimer's Disease
AKT	Protein Kinase B
AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AP	Area Postrema
AP-1	Activator Protein 1
APC	Antigen Presenting Cells
APES	3-Aminopropyltriethoxysilane
ASM	Airway Smooth Muscle
BALF	Bronchoalveolar Lavage Fluid
BBB	Blood Brain Barrier
BCG	Bacillus Calmette-Guérin
BDNF	Brain Derived Neurotrophic Factor
BNST	Bed Nucleus of The Stria Terminalis
BRF	Biomedical Research Facility
BSA	Bovine Serum Albumin
CAMKII	Ca^{2+}/Calmodulin-Dependent Protein Kinase
CeA	Central Nucleus of the Amygdala
CGRP	Calcitonin Gene Related Peptide
CLR	C-Type Lectin Receptor
cmNTS	Caudomedial NTS
CNS	Central Nervous System
COX	Cyclooxygenase
CpG ODN	CpG Oligodeoxynucleotides
CRF	Corticotrophin-Releasing Factor
CVO	Circumventricular Organ
Cys-LT	Cysteinyl Leukotriene
DAB	3,3'-Diaminobenzidine
DAMP	Danger-Associated Molecular Pattern

DC	Dendritic Cell
DMV	Dorsal Motor Nucleus of the Vagus
DPBS	Dulbecco's Phosphate Buffered Saline
DRG	Dorsal Root Ganglion
ECM	Extracellular Matrix
ELISA	Enzyme Linked Immunosorbant Assay
EP	PGE_2 Receptor
EPM	Elevated Plus Maze
ETS	Extended Tobacco Smoke
FcR	Fc Receptor
FEV1	Forced Expiratory Volume 1
FSC	Forward Scatter
FST	Forced Swim Test
GABA	γ-amino butyric acid
GABAB(1)R	GABA B(1) Receptor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GC	Glucocorticoids
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPCR	G-protein Coupled Receptor
GR	Glucocorticoid Receptor
HBSS	Hank's Based Salt Solution
HDAC2	Histone Deacetylase 2
HDM	House Dust Mite
HMGB1	High Mobility Group Box 1
Hp	Hippocampus
HPA	Hypothalamic Pituitary Adrenal
HPRT	Hypoxanthine-Guanine Phosphoribosyltransferase
i.c.v.	Intracerebroventricular
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
ICS	Inhaled Corticosteroids
IDO	Indoleamine 2,3-Dioxygenase

IEG	Intermediate Early Gene
IFN	Interferon
IFNR	Interferon Receptor
Ig	Immunoglobulin
IKK	IκB Kinase
IL	Interleukin
IL-1R1	IL-1 Receptor 1
IL-1ra	IL-1 Receptor Antagonist
IL-4R	IL-4 Receptor
IL-6R	IL-6 Receptor
i-NANC	Inhibitory Non-Adrenergic Non-Cholinergic
iNOS	Inducible Nitric Oxide Synthase
IRAK	Interleukin Receptor Associated Kinase
IRF	Interferon Regulatory Factor
IκB	Inhibitor of NFκB
JNK	c-Jun N-terminal Kinase
KO	Knock-Out
LABA	Long-Acting β_2 Adrenoceptor Agonists
LBP	LPS Binding Protein
LBPN	Lateral Parabrachial Nucleus
LC	Locus Coeruleus
LPS	Lipopolysaccharide
LRt	Lateral Reticular Nucleus
LTP	Long Term Potentiation
MAPK	Mitogen-Activated Protein Kinase
MBP	Major Basic Protein
MCP	Monocyte Chemoattractant Protein
ME	Median Eminence
mGluR1	Metabotropic Glutamate Receptor 1
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MnPO	Median Preoptic Nucleus
mPGES	Microsomal Prostaglandin E Synthase

mPVN	Magnocellular PVN
MSD	Mesoscale Discovery
MyD88	Myeloid Differentiation Primary Response Gene 88
NBD	NEMO-Binding Peptide
NEMO	NFκB Essential Modulator
NF-IL6	Nuclear Factor IL-6
NFκB	Nuclear Factor κB
NGF	Nerve Growth Factor
NK	Natural Killer
NK1	Neurokinin Receptor
NKA	Neurokinin A
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
NR1	NMDA Receptor Subunit ζ-1
NR2d	NMDA Receptor Subunit ε-4
NTS	Nucleus of the Solitary Tract
OCT	Optimum Cutting Temperature
OF	Open Field
OVA	Ovalbumin
OVLT	Organum Vasculosum of the Lamina Terminalis
PLF	Peritoneal Lavage Fluid
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
PGD_2	Prostaglandin D_2
pPVN	Parvicellular PVN
PRR	Pathogen Recognition Receptor
PVA	Paraventricular Nucleus of the Thalamus
PVN	Paraventricular Nucleus of the Hypothalamus
qPCR	Quantitative Polymerase Chain Reaction
RAR	Rapidly Adapting Receptor
RN	Raphe Nucleus

ROS	Reactive Oxygen Species
RR	Ruthenium Red
RT	Room Temperature
RW	Ragweed
SABA	Short-Acting β_2 Adrenoceptor Agonists
SAR	Slowly Adapting Receptor
SFO	Subfornical Organ
SIT	Allergen-Specific Immunotherapy
SOCS	Suppressor of Cytokine Signalling
SON	Supraoptic Nucleus
SP	Substance P
SSC	Side Scatter
STAT	Signal Transducers and Activators of Transcription
TAK	Transforming Growth Factor β-activated Kinase
TCA-3	T-cell Activation-3
TCR	T-cell receptor
TGFβ	Transforming Growth Factor β
TIR	Toll/Interleukin-1 Receptor
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like Receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour Necrosis Factor
TRAF	TNF Receptor Associated Factor
TRAM	TRIF-Related Adaptor Molecule
TREM	Triggering Receptor Expressed on Myeloid Cells
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon β
TRP	Transient Receptor Potential
TRPA1	TRP Ankyrin 1
TRPV1	TRP Vanilloid 1
TSLP	Thymic Stromal Lymphopoietin
VCAM	Vascular Cell Adhesion Molecule
VLM	Ventrolateral Medulla
VMPO	Ventromedial Preoptic Area

VNS	Vagus Nerve Stimulation

Chapter 1:

Introduction

1.1 General Introduction

It has been well established that systemic infections trigger a wide range of responses in the mammalian body. Entry of foreign or infectious substances leads to activation of the innate immune system and inflammation, a process involving the recruitment of immune cells to the entry site and the release of inflammatory mediators, such as cytokines, chemokines and growth factors, which collectively act to eliminate the substance[1]. An equally important, but often overlooked event following inflammation is the cross-talk between the immune system and the brain. Specific chemical mediators, released during an inflammatory event, trigger behavioural, metabolic and psychological changes through various routes of humoral and neuronal communication to and from the central nervous system (CNS)[2]. Not only do these changes aid the body's elimination of the foreign substance, such as by inducing fever to prevent pathogen growth[3], but also act to downregulate the immune responses to prevent its detrimental overactivation[4]. This bi-directional communication is well described for TH1 type immune responses, using bacterial and viral mimetics, but is less well described for acute or chronic TH2 type immune responses, the dominating immune response in allergic asthma.

Asthma, a chronic inflammatory disease of the airways, is the most common form of TH2-mediated inflammation, affecting over 300 million people worldwide[5]. Allergic asthma is characterised by high numbers of TH2 cells, eosinophils and mast cells, and high levels immunoglobulin E (IgE), interleukin (IL)-4, IL-5 and IL-13 in the airways and circulation [6]. Existing asthma treatments, including β_2-agonists (e.g. salbutamol) or glucocorticoids (e.g. budesonide) are beneficial, but are only temporary aids, and do not treat the fundamental cause of the disease. Current research instead focuses on preventing or eliminating the underlying inflammatory event through immunomodulation, a modification of the immune response. Immunomodulators act by dampening the dominant TH2 type immune response though induction of a TH1 type immune response via, among others, the administration of Toll-like receptor (TLR) agonists: analogues of bacterial or viral components.

Knowledge concerning the mechanisms of neuroimmune communication and the impact of inflammatory responses on the brain has long been focused on the effect bacterial and viral infections. Though this has undoubtedly been useful in providing insight into the

mechanisms or pathways by which the immune system can communicate with the brain, it has ignored a large proportion of the vast and complex immune response. Specifically, there is a gap in the literature on if, and how, TH2 immune responses such as asthma impact on the brain; further knowledge of this area is essential in providing a fuller picture on neuroimmune communication, particularly for physiological models of inflammation. It would additionally provide an insight into how and why asthma has been associated with panic and anxiety disorders, as well as if allergic lung inflammation, which negatively impacts the activity of peripheral neuronal fibres, leads to adverse changes in the activity of neurons in the brain. A comprehensive appreciation of how allergic lung inflammation impacts on the brain will form a foundation upon which to identify the further impact of immunomodulation on the CNS, through immune stimulation with analogues of viral components. Delineating the pathways involved would further provide a target for intervention, not only to prevent potential negative changes after lung inflammation, but also to hinder pathological and psychopathological features of asthma.

1.2 Divisions of the Immune System

In order to appreciate the role of the immune system in neuroimmune communication, in disease (e.g. asthma) and in disease treatment (e.g. immunomodulation), it is essential to give a detailed introduction into the vastness, complexity and incredible specificity of this mammalian system. The immune system moulds and adapts to specifically target dangerous invading agents, or pathogens, expertly forming particular inflammatory responses of cells and soluble mediators, to optimally clear the body of foreign particles. Depending on the type, concentration, structure and route of entry of the foreign agent, different inflammatory responses are raised, including innate, adaptive, cell-mediated and humoral, composed and dominated by various different immune cells and immune mediators. A subset of these will be covered below.

1.2.1. The Innate Immune Response

A key feature in the ability for the immune system to mount an effective immune response is the ability for it to recognise and discriminate "self" from "non-self". The innate immune response acts as the first line of defence in the recognition and defence against a non-self, or foreign, agent. With its relative non-specificity in tackling invading pathogens, it involves common cell-types, immune mediators and modes of action to rid the body of an infection. Initial entry of a foreign particle into the mucosal epithelium, i.e. in the lungs, is met by tissue resident macrophages and mast cells. Activation of these cells can lead to phagocytosis of the invading agent, as well as immune mediator production, causing inflammation and recruitment of further innate immune cells, such as neutrophils, eosinophils, basophils and natural killer (NK) cells[1]. These cells each have exclusive roles and functions involved in the initial defence against danger. Though the innate immune system is considered relatively non-specific, certain cell types and mechanisms involved, including macrophages, Toll-like receptors and granulocytes shape the innate immune system and target it for specific tasks, including the elimination of particular types of microorganisms and the restoration of tissue structure after destruction.

1.2.1.1 Toll-like receptors

The Toll-like receptors (TLRs) are key components of the innate immune response and offer a form of specificity in this more inexact inflammatory response. TLRs encompass a large group of cell-surface and intracellular transmembrane pattern recognition receptors (PRRs). Like all PRRs, TLRs recognise a variety of pathogen-associated molecular patterns (PAMPs), components derived from the cell-surface and nuclear space of bacteria and viruses, as well as danger-associated molecular patterns (DAMPs), such as heat-shock proteins, high mobility group box 1 (HMGB1), DNA and RNA heparin sulphate[7]. In response to these structures, TLRs employ discrete signalling pathways made up of specific components of a shared group of molecules, leading to unique and targeted inflammatory responses (Figure 1.1). TLRs trigger the expression of inteferons (interferon (IFN)-α, IFN-β), interferon response genes (monocyte chemoattractant protein (MCP)-5), inducible nitric oxide synthase (iNOS), pro-inflammatory cytokines (IL-1β, IL-6, tumour necrosis factor (TNF)-α, IL-12) and T-cell costimulatory molecules (major histocompatibility complex (MHC) Class II, CD80, CD86) from a distinct set of immune cells, including macrophages, dendritic cells (DCs), B-cells and mast cells[1,8]. For the purpose of this thesis, only TLR2, 4, 7 and 9 will be covered in detail.

TLR4 was the first TLR to be characterized in humans[9] and is the most well-described TLR. This cell-surface transmembrane receptor recognises bacterial lipoglycans, such as lipopolysaccharide (LPS)[10], and through the interaction with these components, as well as the co-receptors MD-2, LPS binding protein (LBP) and CD14, triggers two main intracellular signalling cascades[8]. The myeloid differentiation primary response gene 88 (MyD88)-dependent pathway results in the activation of two transcription factors: activator protein (AP)-1, via a mitogen-activated protein kinase (MAPK) pathway, as well as nuclear factor κB (NFκB), which together result in the production of TNF-α, IL-6 and IL-12 p40, as well as MHC Class II, CD80 and CD86. Activation of the MyD88-independent, TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway results in the activation of the transcription factor interferon regulatory factor (IRF) 3, resulting in the production of type I IFNs (IFN-α and IFN-β), which further triggers the expression of IFN-inducible genes, including MCP-1, MCP-5 and iNOS[11].

TLR2 is a cell-surface transmembrane receptor that recognises various bacterial components, including lipoproteins, peptidoglycans, lipoteichoic acid and glycoinositolphospholipids[8]. Through interaction with TLR1 or TLR6, TLR2 activates a MyD88-dependent signalling cascade leading to the AP-1- and NFκB-dependent production of TNF-α, IL-6, IL-12 and macrophage inflammatory protein (MIP)-2, as well as DC maturation by increase of MHC Class II and co-stimulatory molecules[12]. TLR2 may also interact with dectin-1, a C-type lectin receptor (CLR) enabling it to respond to components of yeast, such as zymosan, through a MyD88-dependent signalling pathway[13].

TLR7 and TLR9 are intracellular transmembrane receptors, located in the endosomal membrane. These receptors share downstream signalling cascades, which result in the MyD88-dependent, IRF7, AP-1 and NFκB dependent-production of pro-inflammatory cytokines, interferons and interferon inducible genes[14]. TLR7 recognises single stranded RNA (ssRNA), whereas TLR9 recognises unmethylated CpG DNA and CpG oligodeoxynucleotide. The signalling pathways employed by TLR2, 4, 7 and 9 are depicted in Figure 1.1.

Table 1.1

Location, recognised PAMPs, signalling adaptors, transcription factors and effector cytokines of TLRs

TLR	Location of TLR	PAMPs recognised	Signalling Adaptor	Transcription Factor	Effector Cytokines
TLR1/2	Plasma membrane	Triacyl lipopeptides	TIRAP, MyD88	NFκB	Pro-inflammatory cytokines
TLR2	Plasma Membrane	Peptidoglycan, LAM	TIRAP, MyD88	NFκB	Pro-inflammatory cytokines
TLR3	Endosome	ssRNA, dsRNA (virus)	TRIF	NFκB, IRF3, 7	Pro-inflammatory cytokines, Type I IFNs
TLR4	Plasma Membrane	LPS, lipoteichoic acids,	TIRAP, MyD88, TRAM and TRIF	NFκB, IRF3, 7	Pro-inflammatory cytokines, Type I IFNs
TLR5	Plasma Membrane	Flagellin	MyD88	NFκB	Pro-inflammatory cytokines
TLR6/2	Plasma Membrane	Diacyl lipopeptides	TIRAP, MyD88	NFκB	Pro-inflammatory cytokines
TLR7	Endosome	ssRNA	MyD88	NFκB, IRF7	Pro-inflammatory cytokines, Type I IFNs
TLR8	Endosome	ssRNA (virus)	MyD88	NFκB, IRF7	Pro-inflammatory cytokines, Type I IFNs
TLR9	Endosome	dsDNA (virus)	MyD88	NFκB,IRF7	Pro-inflammatory cytokines, Type I IFNs

Adapted from Kumar et al [15]

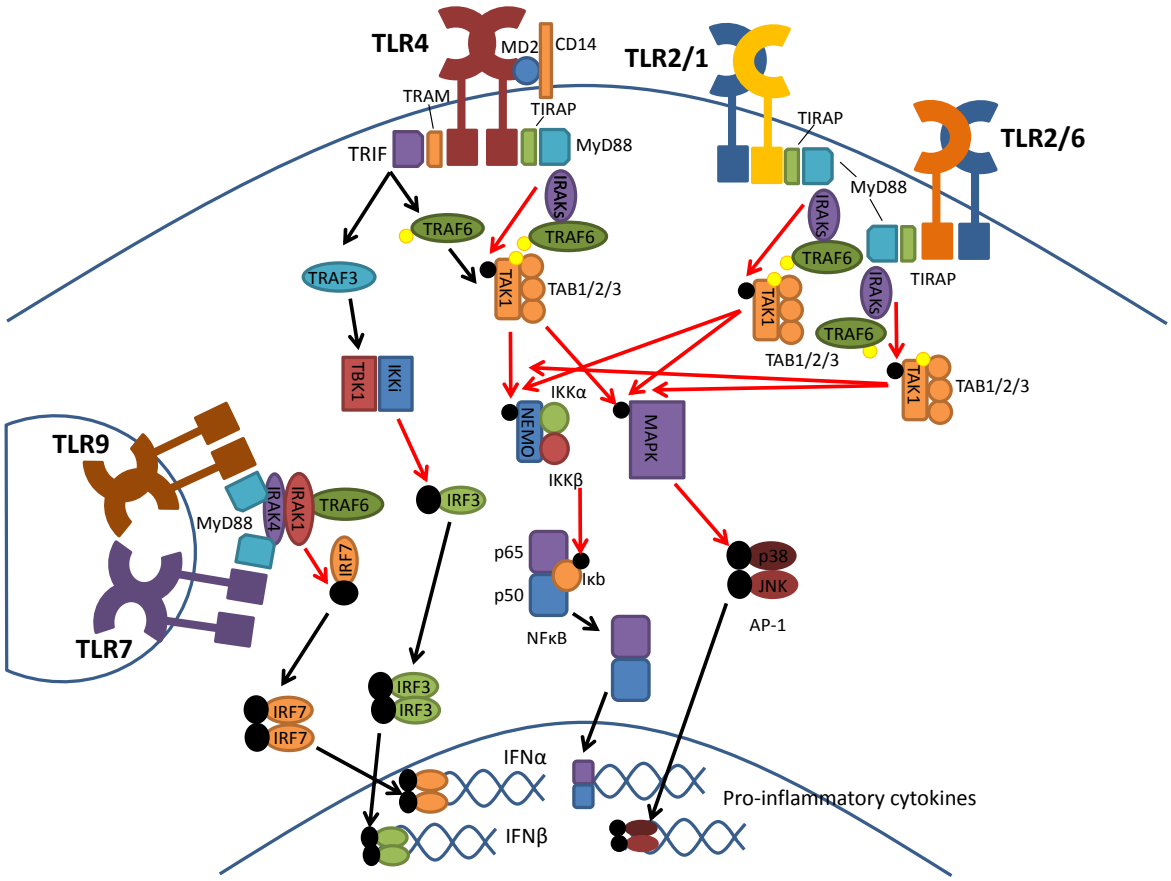


Figure 1.1 – TLR signalling. LPS and LBP binds TLR4, associating with coreceptors MD2 and CD14. TLR4 dimerises, triggering the activation of a MyD88-dependent and independent pathway. This pathway is also activated by TLR2/1 and TLR2/6 dimerization. It involves binding of TIRAP and MyD88 to the intracellular death domain of these TLR dimers resulting in the recruitment of IRAK proteins (IRAK4 and IRAK1) and subsequent recruitment of TRAF6. TRAF6 ubiquitinates TAK1, resulting in the formation of an ubiquitin bridge, to allow the phosphorylation of TAK1 by IRAK. Phosphorylated and activated TAK1 phosphorylates IKK complex, composed of NEMO, IKKα and IKKβ. Upon activation, this phosphorylates IκB, releasing p65 and p50 (which make up NFκB) to enter the nucleus and induce expression of pro-inflammatory cytokines. Phosphorylated TAK1 also phosphorylates MAPK, which phosphorylates and activates p38 and JNK, which combine to form AP-1. AP-1 enters the nucleus and induces the expression of pro-inflammatory cytokines. TLR4 activation additionally activates a MyD88-independent pathway, involving the recruitment of TRAM and TRIF. This activates TRAF3, IKK1 and TBK1, which phosphorylates IRF3. IRF3 dimerises and enters the nucleus to induce expression of type I interferons. Activation of TLR7/9 leads to the activation of a MyD88-dependent pathway, recruiting MyD88, IRAK4, IRAK1 and TRAF6 to the death domains of the receptors. IRAK1 phosphorylates IRF7, which dimerises and enters the nucleus to induce phosphorylation of Type I interferons.

IκB: Inhibitor of NFκB; IKK: IκB Kinase; IRAK: Interleukin receptor associated kinase; JNK: c-Jun N-terminal kinase; TAK: Transforming growth factor β-activated kinase 1; TBK1: TANK-binding kinase 1; TIRAP: TIR domain containing adaptor protein; TRAF: TNF receptor associated factor; TRAM: TRIF-related adaptor molecule. Adapted from Kawai and Akira[16] and Murphy[1]

1.2.1.2 Macrophages

Macrophages are key players of the innate immune response. Their multiple roles within the immune system, from initial recognition of dangerous exogenous or endogenous agents, to antigen presentation and activation of the adaptive immune response, as well as phagocytosis and clearance of both necrotic and apoptotic cellular debris allow these cells to be highly heterogenic, expressing a multitude of interchangeable phenotypes[1]. Macrophages are often broadly, and rather simply, subdivided into two main phenotypes: the M1 and the M2 phenotype. The M1 or classically activated macrophage has enhanced microbicidal activity, expressing high levels of MHC Class II, producing large amounts of oxygen- (O_2^-) and nitrogen (NO^-) radicals and secreting increased levels of pro-inflammatory cytokines and mediators, such as IL-1β, IL-6 and TNF-α. M1 macrophages play an important role in the defence against intracellular pathogens[17]. In contrast, M2 macrophages, also known as alternatively activated macrophages, play an optimal role in the fight against parasitic and fungal infections as well as, crucially, wound-healing[17]. Differentiation of macrophages into alternatively activated macrophages is partially dependent on the production of IL-4, released by basophils and mast cells in response to tissue injury or chitin, a biopolymer found in fungi and parasites. M2 macrophages are known to express high levels of arginase, aiding reconstruction after tissue injury, as well as chitinase and chitinase-like molecules, aiding the breakdown of parasites and fungi, but also promoting matrix reorganisation and wound healing[17,18]. Macrophages are highly plastic, constantly altering their phenotype. Therefore, the division of macrophages in purely M1 and M2 phenotypes is rather oversimplified, as many intermediate phenotypes exist, exclusive to the multitude of roles macrophages may have[17,18] (Table 1.2).

Table 1.2

Inducers, phenotypical characteristics and functions of macrophage phenotypes.

	M1	**M2a**	**M2b**	**M2c**
Inducers	IFN-γ LPS TNF	IL-4 IL-13	Immune complex + TLR/IL-1R Ligands	IL-10
Secretory Products	IL-12^{high} IL-10^{low} IL-1 TNF IL-6 iNOS	IL-10 IL-1ra	IL-10^{high} IL-12^{low} TNF IL-1 IL-6	IL-10 TGFβ
Biological Markers	MHC II, CD16	MR MHC II Arginase I Relm-α	MHC II CD16	MR Arginase I Relm-α
Functions	Induces TH1 Type Immune Responses; Killing of intracellular pathogens	Induces TH2 Type Immune Responses; Allergy; Killing of parasites	Induces TH2 Type Immune Responses; Immunoregulation	Immunoregulation; Matrix Deposition; Tissue Remodelling

M1, or classically activated macrophages, are induced by IFN-γ, LPS or TNF-α and have high microbicidal activity and contribute to the induction of TH1 type immune responses. M2 macrophages can be subdivided into M2a, M2b and M2c phenotypes. M2a macrophages are induced by IL-4 and IL-13 and have a role in allergy, contribute to the development of TH2 responses and are involved in the killing of parasites. M2b macrophages are induced by immune complex and TLR ligands such as LPS, contributing to the development of TH2 responses, but also having a role in immunoregulation. M2c macrophages are induced by IL-10 and, similarly, have a role in immunoregulation but also contribute to tissue remodelling. Adapted from Mosser and Edwards (2008)[17] and Mantovani et al (2004)[18].

1.2.1.3 Granulocytes

Granulocytes, also termed polymorphonuclear leukocytes, include neutrophils, eosinophils and basophils and are, like macrophages, key players in the innate immune response. Their name derives from the large number of granules present in their cytoplasm, which contain a variety of enzymes and toxic proteins important in the destruction of foreign agents. Neutrophils are the most abundant of granulocytes, and are vital in the defence against microorganisms such as bacteria and viruses. They phagocytose microorganisms and destroy them in intracellular vesicles, which contain various antimicrobial proteins such as α- and β-defensins and cathelicidin, as well as reactive oxygen- and nitrogen species. Eosinophils, on the other hand, target foreign agents by secreting toxic proteins and inflammatory mediators through a process termed degranulation. Upon activation, through interaction with a variety of cell-surface receptors (e.g. IL-5R, Fc Receptors (FcRs) and C3), eosinophils release various toxic enzymes (e.g. eosinophil peroxidase), proteins (e.g. major basic protein (MBP)), cytokines (e.g. IL-5) and lipid mediators (e.g. leukotrienes), which target microorganisms (particularly extracellular microorganisms such as parasites), but also induce bystander tissue damage. Eosinophils play a central role in the allergic airway inflammatory response, amplifying inflammation, and contributing to airway tissue remodelling. Basophils, like eosinophils, are involved in allergy and parasitic infection, though their role has been less well-characterised. Basophils degranulate in response to the interaction between IgE and FcεR, releasing, among others, histamine, heparin, lipid mediators and proteolytic enzymes[1].

1.2.2 The Adaptive Immune Response

The complexity of the immune system arises with the activation of the adaptive immune response. This immune response is a concoction of specifically designed subtypes, composed of purposely selected cells and immune mediators that exclusively and fiercely combat invading pathogens. Through an interaction with an antigen presenting cell (APC) of the innate immune system, and the surrounding micro-environment, T-lymphocytes, or T-cells, differentiate into specially selected phenotypes, consequently determining development, manifestation and functionality of the adaptive immune system[1]. Numerous

phenotypes of T-cells exist, including the TH1, TH2, TH17, Treg, TH3, TH9 and TH22[19] subtypes. For the purpose of this thesis, only TH1 and TH2 subtypes will be covered.

1.2.2.1 T_H1 vs. T_H2-type immune responses

The key players in TH1 and TH2-type immune responses are the $CD4^+$ T helper (T_H) cells (Figure 1.2). These immune cells aid in moulding and establishing the adaptive immune response, helping B-cells produce antibodies and undergo class-switching, recruiting and activating numerous other cells, including macrophages, $CD8^+$ cells, neutrophils, eosinophils and basophils, as well as acting directly on an affected tissue or organ. Depending on the concentration, affinity and nature of the pathogen and consequent cytokine milieu established by the innate immune response, non-differentiated TH0 cells will polarise, leading to the production of specific immune mediators, recruitment of particular immune cells and the establishment of a unique and focused inflammatory response[1].

Development of the TH1 type immune response is initially dependent on the production and proliferation of TH1 cells, phenomena which occur in the presence of IFN-γ, mainly produced by NK cells of the innate immune system. IFN-γ binds to the type I IFN receptor (IFNR), leading to the dimerization and activation of signal transducers and activators of transcription (STAT) 1. This results in the activation of transcription factors Hlx4 and Runx4, as well as, crucially, T-bet and the consequent production of IFN-γ, as well as increased expression of IL-18R1 and IL-12Rβ on the surface of the TH1 cell[20]. Differentiated TH1 cells will produce IL-18 and further IFN-γ through self-amplification with IFN-γ and stimulation with IL-12, produced by DCs[21]. The production of IFN-γ by TH1 cells plays a critical role in the development and maturity of the TH1 type immune response, resulting in an increased microbicidal activity of macrophages, increased expression of MHC class I and II, promotion of NK cell activity and promotion of CD8+ T-cell activity[1]. Thus, TH1 type immune responses are optimally suited to the fight against and elimination of intracellular microorganisms, such as mycobacteria and viruses.

The development of the TH2 type immune response occurs with the differentiation of TH2 cells, dependent on the presence of IL-2, IL-7, thymic stromal lymphopoietin (TSLP) and,

crucially, IL-4. The production of these mediators is highly dependent on the route of entry and strength of T-cell receptor (TCR) signal by the foreign particle. Low concentrations and weak affinity antigens are strong promoters of TH2 cell differentiation. In addition, entry through epithelial cells of the mucosal barriers can result in the production of IL-4, IL-33 and TSLP[22]. IL-4 binds the IL-4 receptor (IL-4R), resulting in the dimerization and activation of STAT6, and the subsequent transcription of the transcription factor GATA3. Co-stimulation with IL-2 or IL-7 results in STAT5 phosphorylation and activation, leading to the activation of GATA3 and transcription of TH2 cytokines including further IL-4 as well as IL-5, IL-9 and IL-13[20,21]. IL-4 acts as a self-amplifier resulting in further TH2-cell differentiation and further IL-4 production. In addition, IL-4 is key in the differentiation, proliferation and antibody class-switching to IgE in B-cells, activating the humoral immune system[1]. Production of IgE leads to the activation of such innate immune cells as mast cells and basophils, and TH2-cell-production of IL-5 and IL-9 leads to recruitment of eosinophils and mast cells, respectively[23]. In contrast to TH1 type immune responses, TH2 type immune responses are optimally suited to fighting parasitic infections. They are also, however, key in the development of an allergic reaction.

TH1 and TH2 cell differentiation is counter-regulatory and self-reinforcing. The production of IFN-γ by TH1 cells and IL-4 by TH2 cells enhances the differentiation of the respective cell types. Furthermore, IFN-γ suppresses the differentiation of TH2 cells by a Hlx4-dependent inhibition of IL-4 production, whereas IL-4 suppresses the differentiation of TH1 cells via a GATA3-mediated inhibition of Runx3, responsible for IFN-γ expression[20]. Historically, it was assumed that cross-regulation and self-reinforcement of these differentiated $CD4^+$ cell types fixed the cells in their specified phenotypes. However, more recent data, though sparse, has suggested that there is some amount of plasticity within TH1 and TH2 cell subsets. The finding that TH2 cells can transform into cells with both TH1 and TH2 functions[24,25], as well as completely alter phenotype to a TH1 cell *in vivo* when exposed to appropriate innate immune and cytokine environments suggests a large degree of plasticity. Some studies suggest such plasticity is only possible in early stages of differentiation[26,27], although this has also been disputed[24]. The control of TH1 and TH2 polarisation is a complex tightly regulated mechanism, as the balance of these two immune responses is critical for immune homeostasis. Imbalance of T helper cell responses is an underlying feature of numerous diseases.

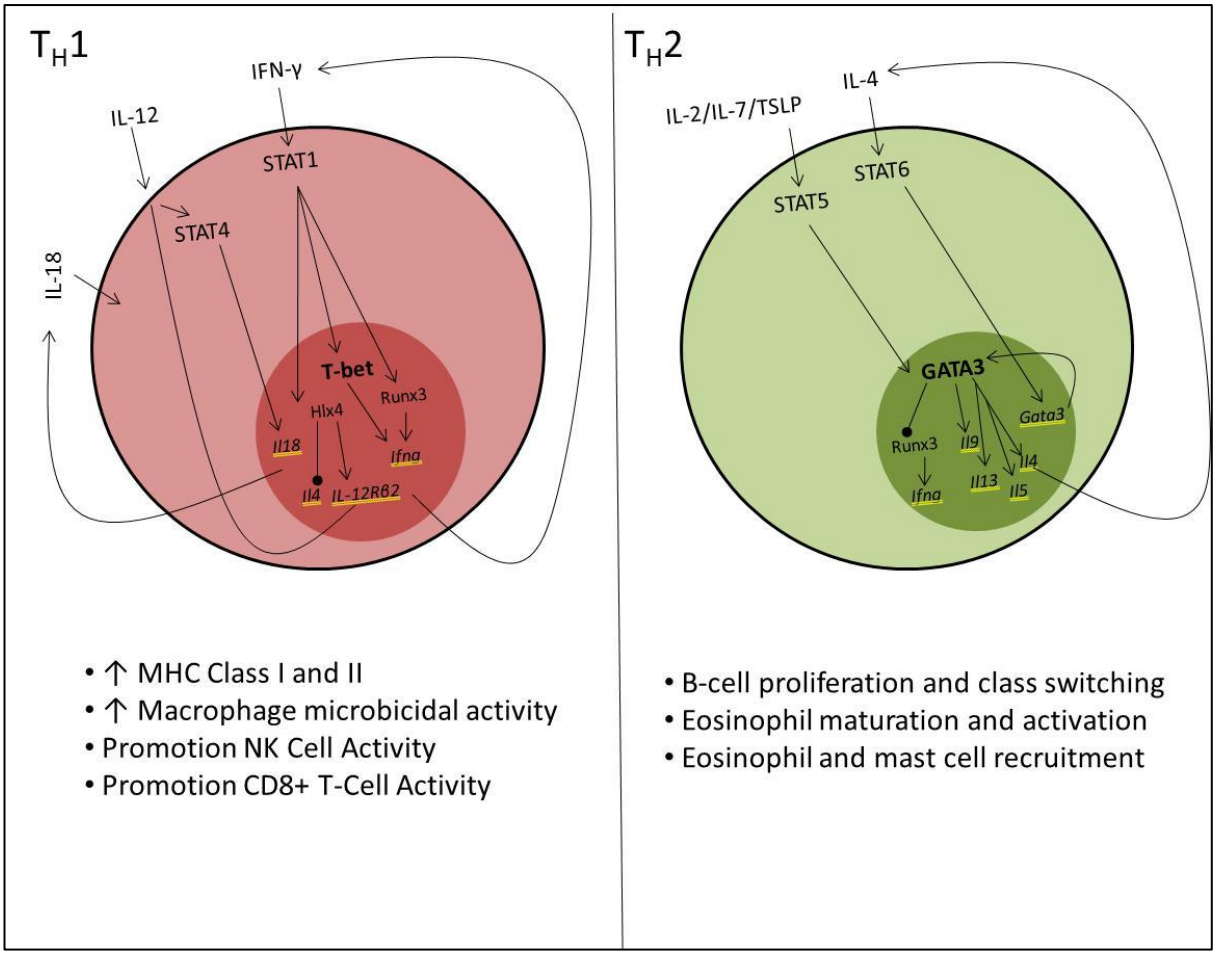


Figure 1.2 – Differentiation of TH1 and TH2 cells. Differentiation of TH1 cells is triggered by IFN-γ, which activates the transcription factor T-bet and instigates the expression of IL-18, IL-12R and further IFN-γ. TH1 cells increase MHC Class I and II expression, heighten macrophage microbicidal activity and promote NK and $CD8^+$ T-cell activity, making them highly suitable to target bacterial and viral infections. Differentiation of TH2 cells is triggered by IL-4, as well as IL-2, IL-7 and TSLP. This results in activation of the transcription factor GATA3, which instigates the expression of IL-4, IL-5, IL-13 and IL-9. TH2 cells enhance B-cell proliferation and class switching, induce eosinophil maturation and activation and recruit eosinophils and mast cells, making them highly suitable to target extracellular parasitic infections.

Understanding the roles and functions of TH1 and TH2 cells is becoming increasingly important in the world of asthma. Allergic or atopic asthma is prototypically a TH2-based disease, with the presence of large numbers of TH2 cells and eosinophils, as well as cytokines IL-4, IL-5 and IL-13 and the immunoglobulin IgE. Current research into the treatment of asthma involves the prevention and/or reversal of this TH2-based immune response by restoring the TH1/TH2 balance through inducers of the TH1 type immune response[28]. The success of these new drugs is highly dependent on the understanding of the cellular and molecular pathways involved in the differentiation and plasticity of these immune responses.

A general understanding of the immune response provides a necessary introduction in the comprehension and appreciation of numerous physiological and pathological events. It provides a foundation by which we can understand processes in infection and disease, allowing us to more accurately treat or prevent them. In addition, and significantly, understanding immunity is necessary in appreciating the indirect effect that systemic and localised inflammation can have on other organs, such as the brain. In this thesis, we will delve into the impact of inflammation in infection and allergic disease on the brain, and establish how modulating the immune system through disease treatment affects this process.

1.3 Neuroimmune Communication

At the start of the 20th century, research in the fields of neuroscience and immunology was growing. With Camillo Golgi and Santiago Ramón y Cajal's work on the world of synapses in the CNS[29] and the identification of the basics of cellular and humoral immunity pioneered by Ilya Mechnikov and Paul Ehrlich[29], the understanding of the workings of the nervous and immune systems was rapidly advancing. Just over 20 years later, it was discovered that the neurotransmitter acetylcholine was endogenously produced in immune organs such as the spleen[30], and that immune reactions could be conditioned by Pavlovian means, involving the brain[31]. However, the progress of research into the immune system and the CNS continued to occur on parallel, non-intersecting pathways and few acknowledged that these two systems were intricately intertwined. This didn't occur until 1949, when the Nobel Laureate Phillip Hench recognised that centrally produced molecules, namely corticosteroids, could regulate inflammation[32], in a process which has since been termed the hypothalamic-pituitary-adrenal (HPA) axis. It is now widely appreciated that there is a large degree of bi-directional communication between the immune system and the CNS. Peripheral infections and inflammatory events are known to elicit various behaviours typical of illness, collectively termed sickness behaviours, which are centred in the brain, as well as direct a CNS-mediated downregulation of the peripheral immune response through hormonal (HPA axis) and neuronal (cholinergic anti-inflammatory reflex) pathways[4,33].

1.3.1 Immune to Brain Communication

When the body is challenged with a peripheral infection, the immune system responds by orchestrating a defensive attack composed of a plethora of immune mediators, including cytokines, chemokines, prostaglandins and complement. Though these mediators are key in coordinating the immune response and directly thwarting the invading pathogen, as discussed in section 1.2, they play an equally important role in the body's defence against infections through their ability to communicate with the brain. IL-1β, TNF-α, IL-6, prostaglandin E $(PGE)_2$ and complement C5a are known to signal to the brain to induce changes in neuronal and glial activity, leading to metabolic changes (e.g. fever) and sickness behaviours (e.g. anhedonia, anorexia, reduced social interaction and cognitive

decline)[34]. Collectively, these changes are physiological and key in the maintenance of bodily homeostasis. Where elevation of the body's temperature through fever reduces pathogen growth, reduced social interaction prevents the spread of infection[3]. In addition, activation of brain areas through peripheral inflammation instigates a reciprocal feed-back mechanism (see Section 1.2.2), which downregulates the immune response, maintaining immune homeostasis. However, most inflammatory mediators are large (15-20kDa) and are therefore unable to cross the tightly sealed blood-brain barrier (BBB)[35] and directly enter the brain parenchyma. As a result, the body employs three specially developed humoral and neuronal pathways to interact with the brain[2]. By interacting with cells of the circumventricular organs (CVOs), cells of the BBB (endothelial cells, perivascular cells and pericytes), as well as peripheral sensory fibres of the vagus nerve (Figure 1.3), these mediators can alter the activity of neurons and glia of the CNS, and consequently induce the metabolic and behavioural changes associated with peripheral infection.

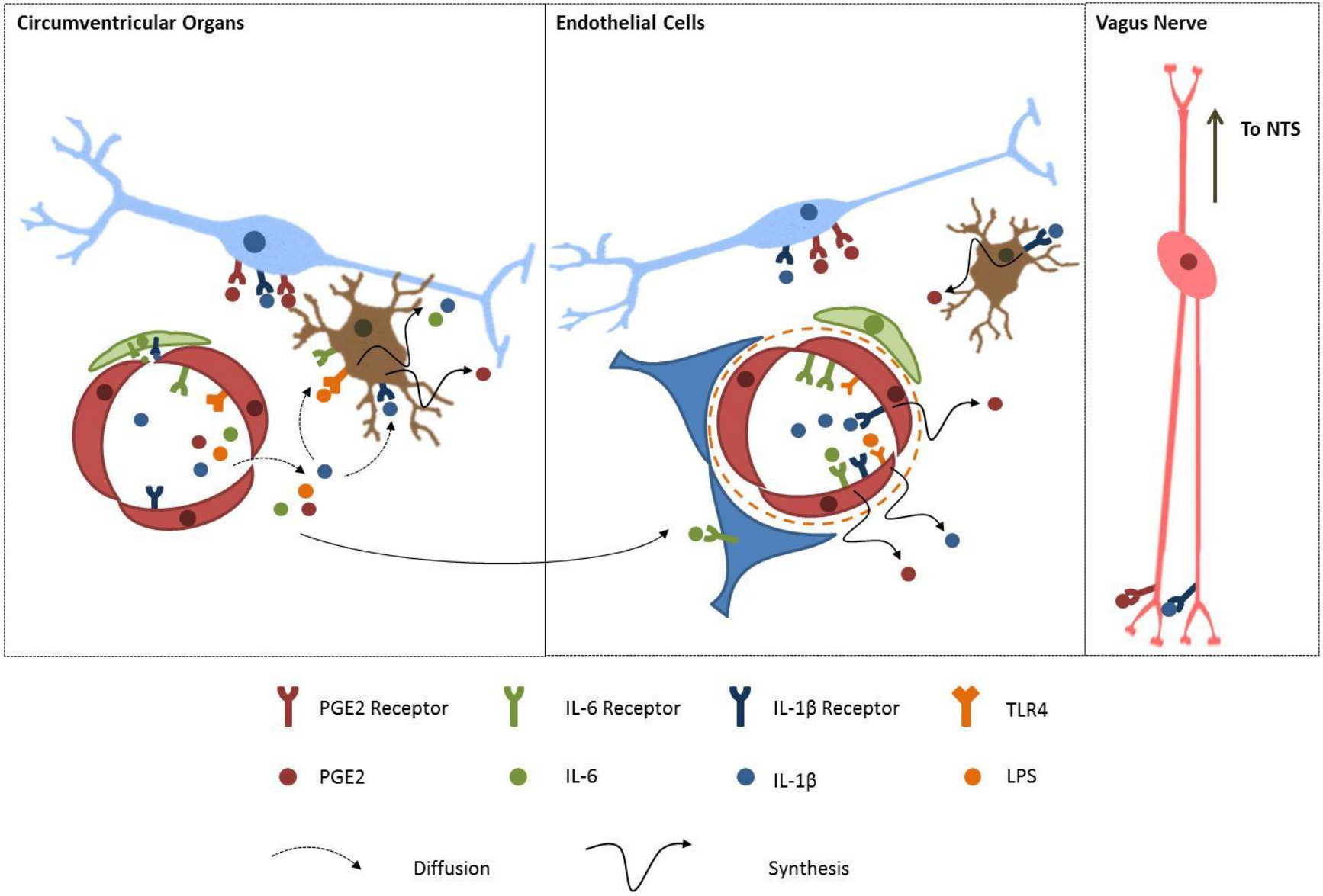

Circumventricular Organs
Endothelial Cells
Vagus Nerve
To NTS
PGE2 Receptor
IL-6 Receptor
IL-1β Receptor
TLR4
PGE2
IL-6
IL-1β
LPS
Diffusion
Synthesis

Figure 1.3 Pathways of immune to brain communication. The peripheral immune system communicates with the brain via three key pathways: via the CVOs, the cerebral endothelium or the vagus nerve. CVOs have an incomplete BBB, which allows the free diffusion of circulating PAMPs (e.g. LPS) or immune mediators (e.g. IL-1β, IL-6, PGE_2) into the brain. Endothelial cells express PRRs (e.g. TLR4) or cytokine receptors (e.g. IL-1R or IL-6R) resulting in *de novo* synthesis of cytokines or prostaglandins by the endothelium and release into the brain parenchyma. Immune mediators released into or produced in the brain can interact with glia or neurons to stimulate neuronal activation. Peripheral immune mediators such as IL-1β or PGE_2 can also interact with peripheral fibres of the vagus nerve, resulting in a rapid neuronal signal from the periphery to nucleus of the solitary tract (NTS) in the brain, which projects to numerous other regions in the brain. Together these pathways result in activation of various brain nuclei leading to metabolic (fever, activation of the HPA-axis) and behavioural (anhedonia, anorexia, reduced social exploration) changes associated with inflammation.

1.3.1.1 Humoral immune to brain communication – the circumventricular organs and the endothelium

CVOs are often referred to as the "windows of the brain". These areas, which include the subfornical organ (SFO), the median eminence (ME), the organum vasculosum of the lamina terminalis (OVLT) and the area postrema (AP) are characterised by an incomplete BBB, the presence of fenestrated capillaries and large perivascular spaces. Scattered throughout the brain, adjacent to regions implicated in the sickness syndrome (Figure 1.4), neurons within the CVOs can accurately monitor and interact with circulating compounds and/or pathogens and transfer these signals to the wider brain parenchyma. Cells within the CVOs have the inherent ability to respond to peripheral immune signals, as demonstrated by the constitutive expression of inflammation-associated cell-surface and intracellular proteins on endothelial cells in this area, including TLR4, CD14[36], IL-6 receptor (IL-6R)[37], IL-1 receptor 1 (IL-1R1)[38], IκBα[39] and suppressor of cytokine signalling (SOCS)3[40], determined through *in situ* hybridisation studies. Upon peripheral immune stimulation with LPS, cells within the CVOs respond, through the upregulation of cell-surface cytokine receptors and PRR co-receptors, such as IL-6R[37] and CD14[41], increased expression of associated downstream signalling molecules and transcription factors, including gp130[37], IκBα[39], STAT3[42], SOCS3[40] and nuclear factor-IL6 (NF-IL6)[43] and *de novo* expression of cytokines IL-6[37], TNF-α[44] and IL-1β[45]. Double-labelling immunohistochemistry as well as *ex vivo* microculture studies suggest these events occur primarily in perivascular macrophages and glia (microglia and astrocytes) in the CVOs[46,47]. Peripheral immune stimulation with IL-1β[48] or LPS[49] is further associated with an upregulation of c-fos in the CVOs, an intermediate early gene (IEG), that acts as a marker for neuronal activity, suggesting stimulation of the cerebral innate immune system impacts

on neuronal signalling in these areas[49]. Indeed, elegant immunohistochemical experiments by Goehler and colleagues[50] reveal that responsive immune cells in the AP are apposed to neuronal elements, providing a means by which immune cells can directly impact on neuronal signalling. However, cytokines which are produced in CVOs do not diffuse into other areas of the brain, due to the presence of tanycytic barrier[51] and therefore do not directly impact neurons that do not lie in close proximity. Instead, cytokines contribute to neuronal activity in other regions of the brain (e.g. the Nucleus of the Solitary Tract (NTS), the lateral parabrachial nucleus (LPBN), the bed nucleus of the stria terminalis (BNST) and the paraventricular nucleus of the hypothalamus (PVN), through a multitude of neuronal projections to these areas.

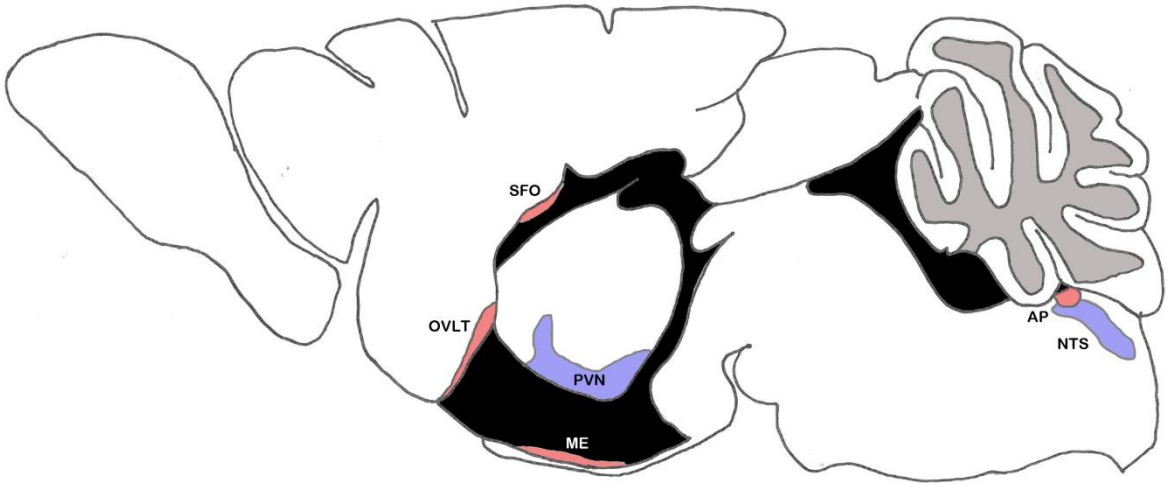


Figure 1.4 – **The location of CVOs in the mouse brain.** CVOs (red) are areas in the brain that lack a complete BBB. They are scattered throughout the brain and often lie adjacent to nuclei that have importance in immune to brain communication (blue), such as the PVN and NTS. They also lie adjacent to ventricles (black). Approximate coordinates: lateral – 0.04mm.

With the exclusion of the CVOs, the majority of the brain parenchyma is protected from infiltration of circulating plasma proteins by the presence of the BBB. However, the BBB, with particular focus on endothelial cells, also plays an active role in the transduction of peripheral immune signals to the brain. The BBB has, firstly, been shown to allow active transport of murine IL-1α, IL-1β[52] and TNF-α[53] from the circulation to the brain parenchyma. Endothelial cells, more significantly, also constitutively express cytokine receptors, including IL-1R1[54,55] and the p55 subunit of the TNF-α receptor[56] as well as pathogen recognition receptors TLR2, TLR3, TLR4 and TLR6[57,58], suggesting the inherent

ability to respond to blood-borne immune signals. Indeed, primary endothelial cells have been shown to express pro-inflammatory cytokines, such as IL-1β and IL-6 *in vitro* in response to LPS[59,60] and *in vivo* studies have demonstrated increased levels of central pro-inflammatory cytokines independent of peripheral cytokine production following LPS challenge[61], suggesting direct action of LPS on the endothelium. In addition, upon peripheral immune challenge, numerous studies suggest that there is an upregulation of the prostaglandin synthesis pathway within endothelial cells, as exhibited by an upregulation of Iκbα following intraperitoneal (i.p.) LPS challenge[62], cyclooxygenase (COX)-2 following IL-1β, IL-6[63] or LPS challenge[64] and microsomal prostaglandin E synthase (mPGES) following LPS challenge within cells of the cerebral vasculature[65]. Challenge with peripheral cytokines and LPS has repeatedly been shown to induce central PGE_2 synthesis[66-70] *in vivo,* and i*n vitro* studies have identified that this induction is at least partially due to a direct interaction of cerebral endothelial cells with LPS, IL-1β, IL-6[71] or TNF-α[72]. Collectively, this suggests that endothelial cells act as a relay system between the peripheral and central immune system, depositing immune mediators in the brain parenchyma in response to peripheral immune activation.

1.3.1.2 Humoral immune to brain communication - the role of peripheral and central IL-1β and prostaglandins

Stimulation of cells within the CVOs and the endothelium leads to the central production of pro-inflammatory cytokines (e.g. IL-1β, TNF-α and IL-6) and prostaglandins, which have repeatedly been shown to be involved in the induction of the sickness syndrome following peripheral inflammation[73,74]. Specific inhibition of central NFκB expression, a transcription factor involved in the production of pro-inflammatory cytokines and prostaglandins, through intracerebroventricular (i.c.v.) injection of the NEMO binding domain (NBD) peptide inhibits central c-fos expression in the NTS and PVN[75] following i.p. administration of IL-1β, suggesting a clear role for these mediators in neuronal activation. In addition, a specific role for central IL-1β in inducing aspects of sickness has been validated by Dantzer and colleagues in rodent models of acute LPS- or cytokine-induced inflammation[76-78], as well as in more chronic inflammatory models, such as induced by Bacillus Calmette-Guérin[79]. Direct i.c.v. administration of recombinant IL-1β reduces social exploration and body weight[76], and i.c.v. administration of IL-1 receptor

antagonist (IL-1ra) attenuates peripheral LPS- or peripheral and central IL-1β-induced c-fos expression in the central nucleus of the amygdala (CeA) and BNST and prevents reductions in social exploration[77,78]. Though these studies do not distinguish whether IL-1ra is inhibiting the action of *de novo* synthesised central IL-1β or infiltrating circulating IL-1β in the CVOs, more recent studies have shown that behavioural changes and central IL-1β are induced in response to peripheral LPS administration in the absence of circulating cytokines, suggesting a strong role for central, as opposed to peripheral IL-1β[61]. In addition, expression of functionally active IL-1 receptors on neurons in the dentate gyrus of the hippocampus and Purkinje neurons in the cerebellum suggest the ability for central IL-1 to act directly on neurons to induce sickness[80].

In addition to IL-1β, central PGE_2 has been shown to significantly contribute to neuronal activation and metabolic and behavioural changes following peripheral immune activation. Central PGE_2 may derive from activation of endothelial cells by IL-1β (as described above) or through the action of central IL-1β on glial cells. In both cases, centrally produced PGE_2 can act on neuronal PGE_2 receptors (EP1-EP4) to induce metabolic and behavioural changes following peripheral inflammation. The EP3 receptor is highly expressed in areas involved in fever induction, namely the median preoptic nucleus (MnPO) and PVN[81], and is believed to be vital for the induction of this metabolic change. EP3 receptor knock-out (KO) mice have attenuated fever responses following peripheral injection or recombinant IL-1β and LPS, and site-specific deletion of EP3 in the MnPO inhibits LPS-induced fever[81]. In contrast, EP4, which is expressed highly in the PVN, is believed to play a role in anorexia, as i.c.v. administration of the specific EP4 agonist ONO-AE1-239 mimics the anorexic effect of i.c.v. PGE_2[82].

Depletion of the *Ptges* gene[83,84], which encodes mPGES1, or the *Ptgs2* gene[85,86], which encodes COX-2, blocks LPS-[83] and IL-1β-induced[87] fever, as well as IL-1β-induced anorexia. Concomitantly, depletion of the *Ptges* gene[88] or inhibition of COX-2 with the selective inhibitor SC-236[89] blocks LPS-induced c-fos expression in multiple brain nuclei, including the NTS, PBN, locus coeruleus (LC), PVN and ventromedial preoptic area (VMPO)[88]. More recent studies suggest that peripheral prostaglandin production, mediated by COX-1, may play a role in anxiety or exploration-associated behaviours, whereas COX-2, induced at the endothelium, plays a role in classical sickness behaviours[70]. However,

further cell-type specific inhibition of PGE_2 synthesis enzymes is necessary to distinguish the roles of central and peripheral prostaglandins in metabolic and behavioural changes.

1.3.1.3 Humoral immune to brain communication – two pathways, two disparate roles?

Though many studies have repeatedly shown the role for pro-inflammatory cytokines and prostaglandins in inducing the sickness syndrome, few studies have been able to distinguish the distinct roles of the two humoral pathways in induction of metabolic and behavioural changes. IL-1β can be induced centrally in CVOs and by endothelial cells. In addition, the presence of central prostaglandins may be the result of production by endothelial cells, by glia in the CVOs or direct infusion into the CVOs. However, a number of elegant experiments have discriminated the roles of the two pathways. *In situ* hybridisation studies reveal that cells within the CVOs do not express prostaglandin synthesis enzymes[64], suggesting the main contribution of prostaglandins to sickness is dependent on production via the endothelium. In addition, direct manipulation of CVOs has revealed key roles for this pathway, as opposed to the endothelium, in induction of fever and HPA axis activation. Intravenous administration of LPS provokes a dose-dependent rise in body temperature which can be attenuated by injection of IL-1ra or indomethacin into the OVLT[90]. Furthermore, lesioning studies have shown that removal of the AP leads to a considerable attenuation of c-fos expression in the NTS and PVN[91], a decrease in noradrenaline release from the PVN[92], as well as a blockage of adrenocorticotropic hormone (ACTH)[91] secretion, all indicators of HPA axis deactivation. The results from the latter studies are controversial, however, as separate studies have found that lesions in the AP do not significantly alter *c-fos* expression in the PVN[93]. The inconsistencies in these results may be explained by the discrepancies in doses of immune stimuli used and the various lesioning techniques employed. Though these studies have suggested distinct roles for CVOs and the endothelium in the induction of sickness, physiologically, these pathways undoubtedly work in concert, where pro-inflammatory cytokines and prostaglandins are induced centrally via TLR or cytokine receptor stimulation of the endothelium and glial cells of the CVOs.

1.3.1.4 Neuronal immune to brain communication – the vagus nerve

In addition to the direct interaction with cells of the brain or BBB, inflammatory mediators can communicate with the CNS through a third, neuronal pathway, via the vagus nerve. The vagus nerve is the tenth cranial nerve, extending from various viscera within the body, including the heart, lungs, spleen, gut, stomach and pancreas, to the NTS in the brainstem. Fibres within the vagus nerve carry signals between the organs of the body to the brain, being critical for maintenance of bodily homeostasis and, correspondingly, immune homeostasis. Vagal fibres, particularly within the ganglia and paraganglia, have been shown to express functional IL-1 and EP3 receptors[94,95], and respond to peripheral administration of IL-1β in a prostaglandin-dependent manner, as measured by increased c-fos expression and increased electrical activity of the afferent fibres[95]. Activation of these fibres, by LPS or IL-1β, sends a signal from the periphery to the brain, as evidenced by the release of neurotransmitters at the central synapse of vagal afferents in the NTS[96]. The NTS acts as a relay station in the CNS, receiving multiple afferent inputs from the fibres of the vagus nerve and projecting to a multitude of brain nuclei involved in the induction of the sickness syndrome, including the LPBN, CeA and PVN. Elegant studies involving severance of the vagus nerve, a process known as vagotomy, have identified key roles for the vagus nerve in inducing metabolic and behavioural changes following peripheral inflammation, including IL-1β-induced fever[97,98], behavioural depression[99], anorexia[100] and activation of the HPA-axis[101-104].

The role of the vagus nerve in the induction of sickness behaviours and metabolic changes is, however, highly dependent on the dose and route of administration of immune stimulus. In most cases, subdiaphragmatic vagotomy only blocks the behavioural and metabolic effects of an immune stimulus when the immune stimulus is injected i.p., not intravenously (i.v.)[105], and at very low doses. For example, subdiaphragmatic vagotomy completely blocks production of ACTH in response to 20μg/kg LPS (i.p.), but only attenuates this response after 250μg/kg LPS (i.p.)[106]. In addition, Hansen et al[98] showed that the hyperthermic effect of low doses of IL-1β (0.1 μg/kg rhIL-1β), administered i.p., is completely blocked by subdiaphragmatic vagotomy, whereas this procedure has progressively less effect as the dose increases (up to 1.0 μg/kg rhIL-1β). Subdiaphragmatic vagotomy has been shown to have no effect on hyperthermia induced by 25μg/kg IL-1β[99]. The effect of dose is disputable, however, as other studies have shown

that subdiaphragmatic vagotomy blocks the hyperthermic effect of up to 2.0μg/kg rhIL-1β[97]. This variation may, of course, be dependent on source of IL-1β and vagotomy procedure. On the other hand, subdiaphragmatic vagotomy completely blocks behavioural depression following sickness, even after high doses of stimuli (25μg/kg IL-1β or 250μg/kg LPS)[99]. However, vagotomy does not block burrowing behaviour, induced by low (1μg/kg) and high (100μg/kg) doses of LPS[61]. The impact of local inflammation on the CNS, such as induced by infection or immune complex formation in the gut, as well as localised subcutaneous pouch administration of LPS[107] has been shown to be solely vagally mediated, as inactivation of c-fibres through capsaicin administration blocks central affects, including central c-fos expression and sickness behaviours[108]. Together, this data suggests that the neuronal pathway is important in the induction of hyperthermia (but only after low levels of systemic immune mediators), influences a subset of sickness behaviours, including behavioural depression, and is vital in signal transduction to the brain following local inflammation.

1.3.1.5 Collaboration of humoral and neuronal immune to brain communication pathways

In the literature, immune to brain communication pathways are often subdivided into three distinct pathways, and experimental papers often attempt to elucidate the significance of an individual pathway. In reality, where infections and inflammatory disorders have both localised and systemic components, however, these pathways undoubtedly work in concert, where the systemic, circulating component of an inflammatory response signals to the brain via the circumventricular organs and the endothelial cells, whereas localised inflammation impacts on the CNS via the nearby vagal afferents. Collectively, these pathways induce the activation of a constellation of brain regions, located at every level of the neuraxis, including the NTS and ventrolateral medulla (VLM), the raphe nucleus (RN), the LC, the LPBN, the thalamic and hypothalamic nuclei, the MnPO, the BNST and the CeA[49]. The brainstem, predominantly the NTS and the VLM, acts as a particularly important relay station, integrating signals from the afferent vagal fibres, the adjacent CVO (AP) and the endothelium, and transducing signals to the BNST, PVN, CeA and LPBN, as elegantly demonstrated by Gaykema et al[109] through retrograde labelling. In addition, neurons within the CVOs project to adjacent brain nuclei, such as projections from the

OVLT or SFO to the PVN and from the AP to the NTS[110]. The discrete and patterned expression of EP3 receptors in hippocampus, medial and cortical amygdala, anterior and mediodorsal thalamus, PVN, MnPO, NTS, LPBN and RN[111], as well as IL-1 receptors in the dentate gyrus and Purkinje cells of the cerebellum[80] give insight into how endothelial stimulation by peripheral mediators can induce specific, patterned neuronal activation. The activation of this assemblage of brain nuclei by peripheral pathways, and the web of intricate efferent and afferent projections between them, results in a patterned, but scattered upsurge of activity within the CNS, leading to the metabolic changes, such as fever and HPA-axis activation and behavioural changes familiarly associated with peripheral inflammation (Figure 1.5). The distinct role of particular pathways becomes interesting when infections or inflammatory events are purely systemic or purely localised, such as during localised gut or lung inflammation. For example, localised gut infection by *Campylobacter jejuni*[112] and localised allergic inflammation in the gut[108], which are not associated with circulating IL-1β, TNF-α and IL-6, result in discrete neuronal activation in the NTS and PVN and anxiety-like behaviour, suggesting that a purely neuronal pathway of communication results in discrete neuronal and behavioural outcomes.

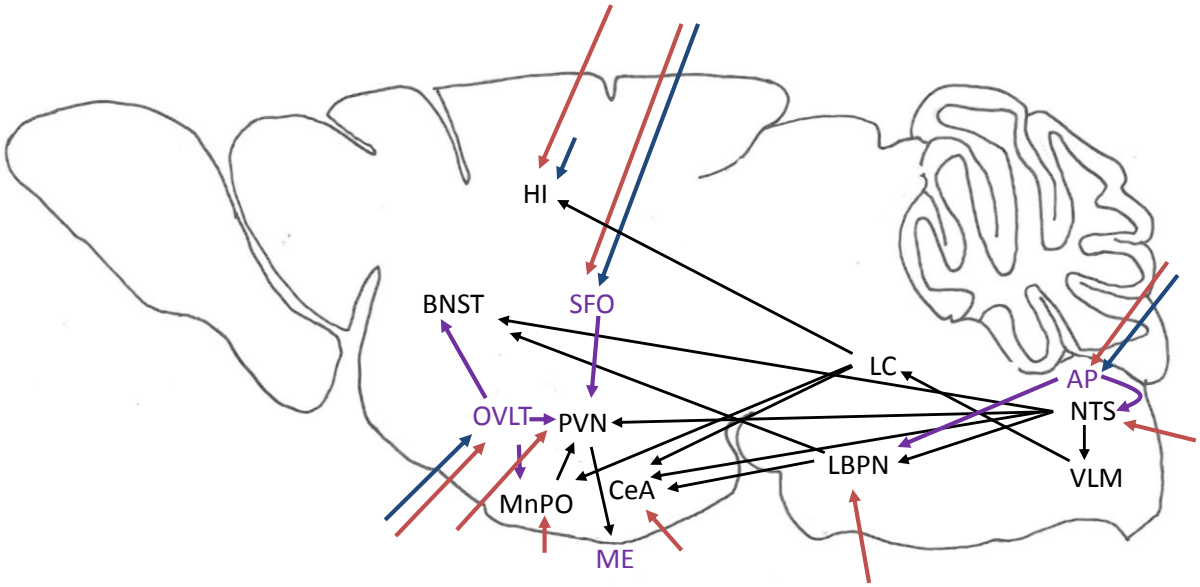


Figure 1.5 – Network of CNS activity following peripheral inflammation. Following inflammation, circulating mediators such as IL-1β or PGE_2 can enter CVOs (shown in purple), resulting in activation of CVO neurons, which project to nearby brain regions. IL-1β or PGE_2 may also be produced centrally by endothelial cells, perivascular macrophages or glia and subsequently act on areas that express IL-1R (blue arrow) or EP3 receptors (red arrow). Alternatively, peripheral IL-1β or PGE_2 may also act on the vagus nerve, leading to activation of the NTS. All regions are in intertwined through neuronal projections (black arrows) resulting in a patterned constellation of neuronal activity following systemic inflammation.

1.3.2 Brain to Immune Communication

The activation of various parts of the brain by peripheral inflammation or infections leads to a variety of changes in behaviour, metabolism and immune cell activity. A central feature of these alterations is the ability for the brain to reciprocally communicate with the body. By triggering the release of hormones via the activation of the HPA axis, as well as directly stimulating efferent fibres of the vagus nerve through the cholinergic anti-inflammatory pathway, the brain downregulates activity of peripheral immune cells and prevents dangerous overactivation of the immune system and aids the body's removal of the infection.

1.3.2.1 The hypothalamic pituitary adrenal (HPA) axis

The HPA axis is a fundamental biological system by which centrally produced molecules, including corticotrophin-releasing factor (CRF) and ACTH, are released to maintain the

body's homeostasis, under basal and stress-related conditions. The HPA axis responds to a variety of changes in the body's internal environment, in relation to stressors including inflammation. It is, in consequence, able to regulate numerous bodily functions, including metabolism, appetite, motor reflexes, arousal, attention, pain tolerance and inflammation[4].

As explained above, peripheral inflammatory mediators released during inflammation signal to the brain via humoral and neuronal pathways, to trigger the activation of the PVN, the location of central activity of the HPA-axis. The PVN is composed of magnocellular (mPVN) and parvicellular (pPVN) sub-divisions, each with their own contribution to the HPA-axis. Upon activation, the mPVN works alongside the supraoptic nucleus (SON), secreting vasopressin and oxytocin into the circulation via the posterior pituitary. The pPVN, on the other hand, projects to the ME via CRF-secreting neurons, releasing CRF into the capillary beds the join the hypophyisal portal vessels of the anterior pituitary. CRF acts on receptors in the anterior pituitary, leading to the release of ACTH from the pituitary gland. ACTH acts on the adrenal cortex, which subsequently results in the production of glucocorticoids, which are released into the general circulation[113] (Figure 1.6).

Glucocorticoids are vital in the regulation of the immune system, due to their immunosuppressive properties. Glucocorticoids bind the glucocorticoid receptor (GR) in the cytoplasm of immune cells, resulting in transactivation of anti-inflammatory genes and transrepression of inflammatory genes through interaction with transcription factors AP-1 and NFκB. This results in the inhibition of various inflammatory processes, including the activation and mobilisation of immune cells, as well as the production of AP-1 and NFκB-dependent cytokines[114]. Glucocorticoids also induce apoptosis of thymocytes, lymphocytes, monocytes, eosinophils and endothelial cells[115]. As a result, the release of glucocorticoids into the circulation following a systemic infection is a critical feature in maintaining immunological homeostasis, preventing potentially destructive overactivation of the immune system.

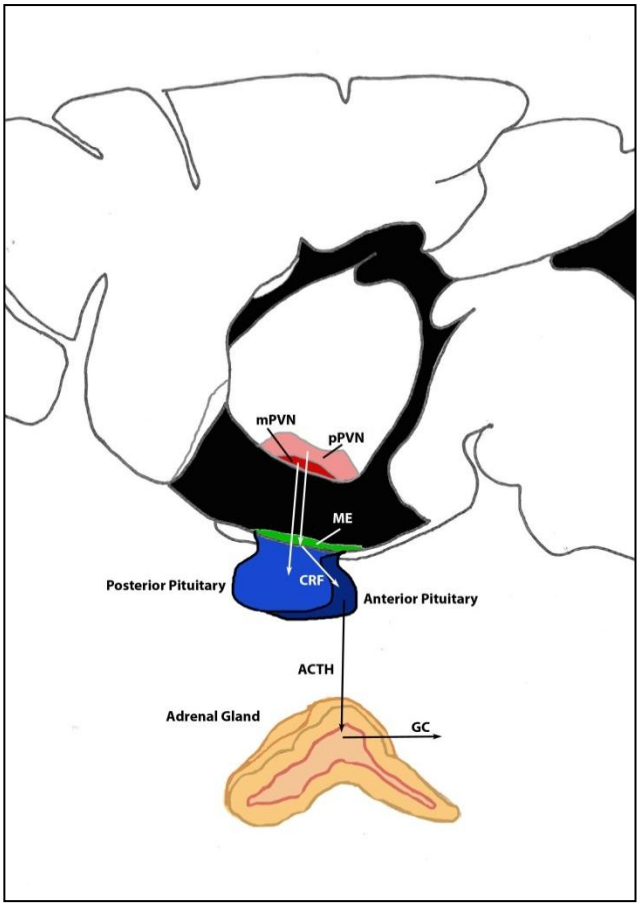


Figure 1.6 – **The HPA axis**. Upon activation, neurons of the pPVN (which project to the ME) trigger the release of CRF into the capillary beds which join the hypophyisal portal vessels of the anterior pituitary. Acting on CRF receptors in the anterior pituitary, this leads to the release of ACTH into the general circulation. Acting at the adrenal gland, this leads to the release of glucocorticoids (GC) into the circulation.

1.3.2.2 The cholinergic anti-inflammatory pathway

More recent studies have revealed that neuronal pathways also have the ability to downregulate the immune system. This occurs via a reflex that passes through the afferent and efferent components of the vagus nerve, termed the cholinergic anti-inflammatory reflex (Figure 1.7). Electrical[116], pharmacological[117], or immune stimulation of afferent vagal fibres leads to the release of glutamate at their synaptic terminals in the NTS[96]. The signal is transmitted to the dorsal motor nucleus of the vagus (DMV), the origin of the preganglionic efferent fibres of the vagus, resulting in the activation of efferent coeliac and splenic fibres, particularly the catecholaminergic (noradrenaline-producing) splenic nerve[118]. Noradrenaline release by the splenic nerve is believed to bind to α and β-adrenoceptors on the surface of T-lymphocytes, resulting in the production and release of

acetylcholine[119]. Key to this pathway is the expression of the α7 nicotinic acetylcholine receptors on the surface of macrophages[120]. Binding of lymphocyte-derived acetylcholine to these receptors results in the inhibition of post-transcriptional synthesis of pro-inflammatory cytokines TNF-α, IL-6, IL-1β and IL-18, but not the anti-inflammatory cytokine IL-10[116]. The cholinergic anti-inflammatory reflex is a relatively new concept, and the intricate mechanism is still being elucidated. However, this reflex has been shown to play a contributing role in a variety of different inflammatory disorders, including sepsis[116], post-operative ileus[121], and pancreatitis[122]. In comparison to the HPA axis, where released glucocorticoids circulate throughout the body, acetylcholine is released in discrete areas of inflammation, resulting in targeted immunosuppression.

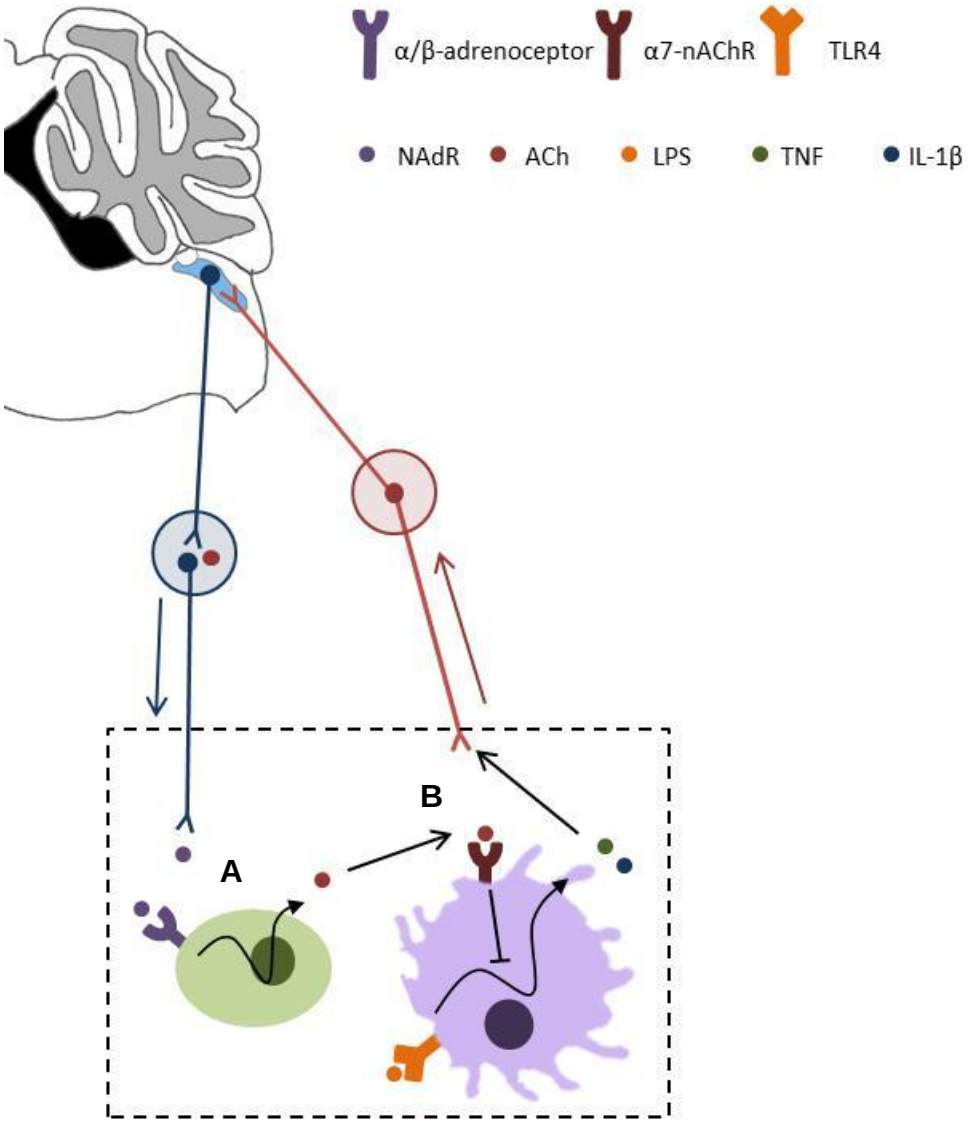


Figure 1.7 – The cholinergic anti-inflammatory reflex. Release of peripheral cytokines such as IL-1β from stimulated macrophages results in the activation of the afferent fibres of the vagus nerve, carrying signals up to the brainstem. This leads to the activation of peripheral efferent coeliac branches of the vagus nerve, which release acetylcholine (ACh) at the celiac ganglion. Post-ganglionic splenic vagal fibres release noradrenaline (NAdr) which act directly on β_2-adrenergic receptors on macrophages, inhibiting pro-inflammatory cytokine release (A) or act on β_2-adrenergic receptors on the surface of lymphocytes, which release ACh that acts on α7 nicotinic acetylcholine receptors on the surface of macrophages, resulting in post-translational inhibition of cytokines production (B)

1.3.3 Implications of Neuroimmune Communication

Acknowledging and delineating the mechanisms of neuroimmune communication has provided a new understanding of numerous chronic conditions, specifically the relationship and interaction between peripheral inflammation and the CNS in disease. It is now well-

established that negative mood and stress play key roles in the progression of peripheral inflammatory diseases and, conversely, peripheral inflammation can impact on CNS disease. It is also now understood that several disease treatments, which involve an interference with the immune system, can have significant impact on brain neurochemistry and inflammatory status, as well as mood.

It is widely accepted that peripheral inflammation is a key contributing factor in CNS-based diseases. A plethora of studies have identified strong correlations between peripheral inflammation and Major Depression (as reviewed by Krishnadas and Cavanagh[123]), specifically identifying elevations in circulating IL-6 and C reactive protein in depressed patients. In addition, it has long been known that following treatment with the immune mediator IFN-α, cancer (e.g. melanoma)[124] and hepatitis C patients[125] show a decline in mood. Recent research has also shown that peripheral immune stimulation (with LPS or Bacillus Calmette-Geurin (BCG)) triggers depressive-like behaviour in mice accompanied by an upregulation of indoleamine 2,3-dioxygenase (IDO) in microglia and endothelial cells. Immune stimulation of IFN-γ KO mice and pharmacological inhibition of IDO abolishes the depressive behaviour, suggesting a crucial role for IFN-γ and central IDO in depression following peripheral immune activation[126-128].

Peripheral inflammation has additionally been shown to be a key contributing factor in the development and progression of neurodegenerative disease. It has been shown that neurodegenerative disease leads to a priming of the normally quiescent microglia in the CNS, a process by which these cells alter their phenotype and become more sensitive to secondary stimuli[129]. Upon peripheral inflammation, primed microglia become active, resulting in the release of cytokines and consequent neuronal damage, accelerating progression of neurodegeneration[130]. This phenomenon has also been attributed to ageing, where ageing, like neurodegeneration, can lead to microglial priming, making the aged brain more sensitive to peripheral inflammation[131]. It has additionally been shown that continuous peripheral inflammation can prime microglia or endothelial cells, resulting in a heightened response to a secondary central insult[132]. These observations clearly underpin the importance of the pathways of immune to brain communication.

Outlining the key mechanisms of neuroimmune communication as well as acknowledging the importance of these processes in disease and mood disorders, has progressed research

in this field further. Initial studies used simple immune stimuli, such as recombinant cytokines, LPS and poly I:C, at relatively high doses (i.e. >10μg/kg IL-1β or >200μg/kg LPS). Though these innate immune stimuli helped to outline key pathways by which the immune system can communicate with the brain, they are not physiological. The immune system is a vast, intricate and heterogenic, consisting of multiple sub-systems, numerous cell types and hundreds of immune mediators; the understanding of how inflammation affects the CNS should not simply involve looking at the impact of a single cytokine or a single component of a bacterium to mimic infection. This notion has allowed research to progress further, where studies are now investigating the central impact of other TLR and non-TLR agonists, such as TLR7 agonists[133] and Dectin-1/TLR2 agonists[134], investigating the impact of sub-pyrogenic TLR agonism[61], as well as the effect of real-live infections with Salmonella[132], BCG[127,135,136] and influenza[137] on the brain and additionally looking at the role of other immune mediators and enzymes, such as COX-1 and prostaglandins[61,70,86,134].

It is equally important to note that studies on immune to brain communication have only focused on TH1-type immune responses. Little is known on how TH2-type immune responses, which are the preliminary characteristic of allergies and asthma, communicate with the brain. Because allergies and asthma are becoming increasingly prevalent in the Western world, with 7% of the UK population being affected directly[138], it is important to understand how this critical type of chronic immune activation affects the brain, especially considering the exacerbating effect peripheral inflammation has on the ageing or neurodegenerative brain. Understanding how chronic inflammatory disorders, such as asthma, affect the brain may not only bring us closer to elucidating the full and complex network of neuroimmune communication, but may also allow us to appreciate the role it plays in disease pathology, ultimately leading to novel therapeutic interventions.

1.4 Asthma

Asthma is a chronic inflammatory disorder of the airways, affecting over 235 million people worldwide[139] and becoming considerably more prevalent in the Western world[140]. It is generally characterised by persistent inflammation, airway hyperresponsiveness (AHR), airway remodelling and symptoms of wheezing, cough and shortness of breath[5]. Asthma is a complex, heterogenic and multicausal disease, with numerous diverse phenotypes; this thesis will focus on the most common and well understood type of asthma: atopic or allergic asthma. Asthma is currently treated with bronchodilating and anti-inflammatory drugs, therapies that, though widely beneficial, have numerous side-effects and, in some cases, only abolish symptoms of the disorder. Prospective treatments, such as immunomodulators, act instead by altering the disease course and preventing disease development. It is unknown, however, if asthma alone, or the prospective treatments, have an influence on other organ systems, such as the CNS.

1.4.1 Immunobiology of Allergic Asthma

Allergic asthma is dominated by an on-going TH2-type inflammatory event in the airways. The immune response is often described as an intricate interplay between epithelial cells, innate and adaptive immune cells and immune mediators. Over time, these induce structural and functional changes in the airways, and ultimately contribute to the symptoms of asthma. The allergic cascade is often sub-divided into three main stages: the initial sensitisation to the allergen, followed by the early phase and late phase of inflammation, upon reencounter with allergen. Through an elaborate and systematic chain of events, these stages collectively induce chronic airway inflammation.

1.4.1.1 Sensitisation

Initiation of the allergic cascade occurs with the instigation of a dampened immune response against an allergen (e.g. house dust mite (HDM) or pollen) in the airways, a phenomenon known as sensitisation. DCs, which have previously migrated and found residence in the airways in response to prior insults[141], take up and process the allergen, migrate to regional lymph nodes and present the processed peptides in the context of MHC

Class II to naïve T-cells. In the presence of IL-4, the naïve T-cells differentiate and mature into TH2 cells and subsequently interact with B-cells, which undergo class switching to produce allergen-specific IgE, in the presence of TH2-cell-derived IL-4 and IL-13. IgE is consequently distributed systemically[142].

The ability for an allergen to sensitise the immune system, that it is to say induce a TH2 type immune response, is still poorly understood, but is believed to be highly dependent on the nature of the allergen (e.g. low concentration), as well as the genotype and phenotype of the host[22]. The epithelium plays a particularly important role, as development of asthma has repeatedly been shown to be associated with the breakdown or disruption of the epithelial barrier[143]. Early-life viral infections as well as environmental pollutants, which disrupt tight junctions in the epithelium, are known risk factors for the development of asthma[144,145]. In addition, products produced by the damaged epithelium, such as IL-33, IL-25 and TSLP, have been shown to enhance TH2 polarisation[146]. Allergens themselves also play an active role in enhancing their ability to sensitise the immune system, as they contain protease activity, which additionally reduces epithelial barrier function[147]. Furthermore, allergens may also have activity as PAMPs or DAMPs on epithelial or resident immune cells, having an adjuvant effect on the allergic cascade[22]. The distinct changes in the epithelial barrier combined with the precise nature of the allergen results in the development of an army of resting immune cells, prepared to react in response to allergen re-exposure.

1.4.1.2 The early phase

Once an individual is sensitised, reencounter with allergen results in a two-phase, vigorous inflammatory response in the airways. The initial phase, termed the early phase, occurs within minutes of allergen exposure and is dominated by resident innate immune cells in the lungs, specifically the mast cells[148]. Mast cells express the high affinity IgE receptor, FcεRI, which binds to allergen-specific IgE. Subsequent binding of allergen to IgE results in cross-linking of the FcεRI, activation of the mast cell, degranulation, phospholipid metabolism and *de novo* synthesis of pro-inflammatory cytokines[23,142,148]. The release of pre-formed mediators (e.g. biogenic amines, serglycin proteoglycans, proteases and cytokines) through degranulation, as well as the production of phospholipid-derived mediators (prostaglandins and leukotrienes), results in an early phase reaction in the

surrounding tissue. Acting on the vascular tissue, smooth muscle, epithelial and goblet cells, innervating sensory fibres and resident immune cells, mast cell-derived mediators induce vasodilation, increased vascular permeability, smooth muscle contraction, increased mucous secretion and cough, and initiate the final phase of the allergic cascade (Figure 1.8) [23,142,148].

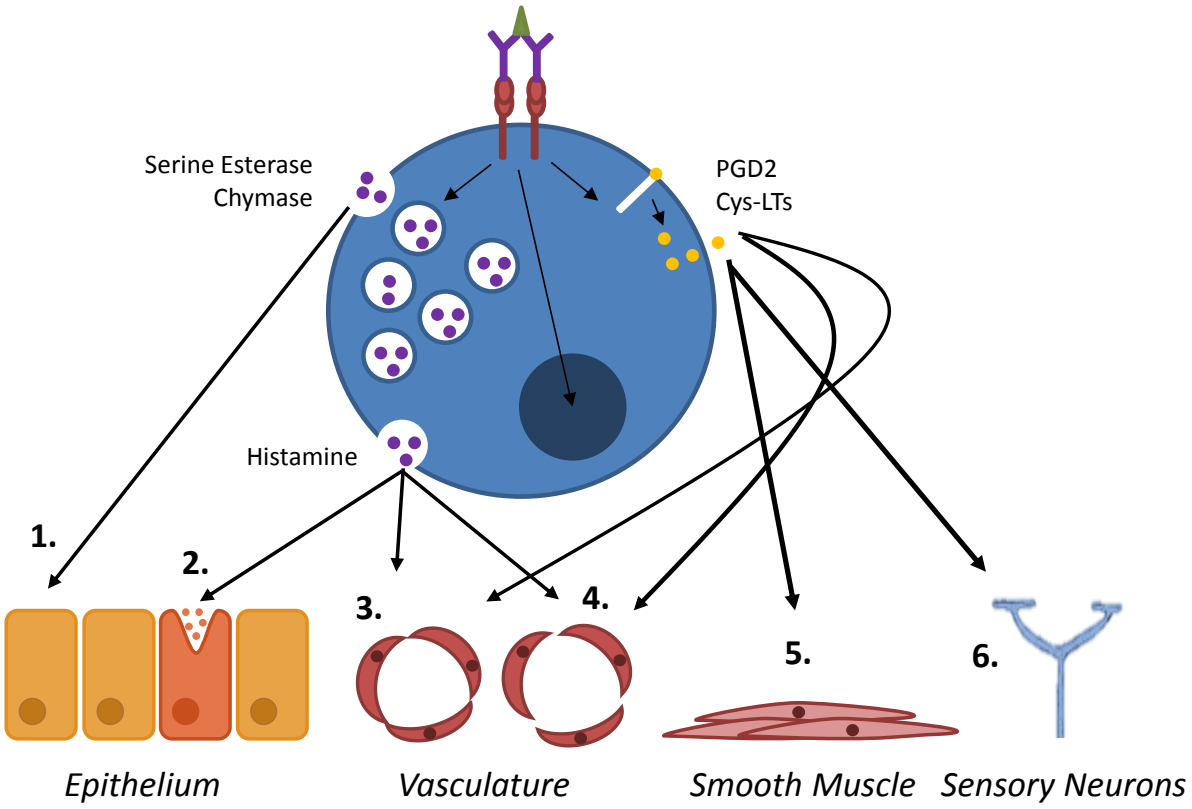


Figure 1.8 – The early phase of allergic inflammation in the airways. Allergen binds to IgE attached to FcεRI on the surface of mast cells, causing a cross-linking of the FcεRI. Consequently, mast cells degranulate, releasing histamine, serine esterases, and chymase. In addition, through metabolism of phospholipids, the mast cells produce and release eicosanoids, including prostaglandins (PGD_2) and cysteinyl leukotrienes (Cys-LTs). The release of these mediators leads to epithelial damage (1.), mucous secretion (2.), vasodilation (3.), increased vascular permeability (4.), bronchoconstriction (5.) and stimulation of sensory neurons (6.), inducing cough.

1.4.1.3 The late phase

Following resolution of the early phase, respiratory function is temporarily restored, but the immune system is covertly mounting a second elaborate response in the airways. This ultimately results in a secondary decline in lung function 6-9 hours following allergen

exposure[142]. This phase, termed the late phase, is an orchestrated interplay of epithelial cells, resident and newly infiltrating innate and adaptive immune cells and a multitude of immune mediators, which cooperate to expand the inflammatory response and impact on the surrounding airway tissue. Release of mediators (e.g. granulocyte macrophage colony-stimulating factor (GM-CSF)) from mast cells and damaged epithelial cells results in an initial maturation of airway-resident DCs, which capture allergen and present it to memory $CD4^+$ T-cells in the lung mucosa, in regional lymphoid organs and in bronchus-associated lymphoid tissue. This results in the expansion of a TH2-cell subset and rapid infiltration into the airways[6]. In collaboration with active resident mast cells, the newly infiltrated TH2 cell population establishes an intricate inflammatory milieu in the airways, consisting of cytokines (IL-4, IL-5, IL-9, IL-13, GM-CSF), chemokines (IL-8, CCL5, CCL2, eotaxin) and other immune mediators (e.g. prostaglandins, cysteinyl leukotrienes)[142]. Together, these trigger the infiltration and maturation of granulocytes (e.g. eosinophils, neutrophils) and additional lymphocytes, and impact on the surrounding airway tissue (Figure 1.9)[6,23,142]. The late phase of allergic inflammation in the lung is, at least in part, dependent on central neuronal reflexes, as the inflammatory event is attenuated by anti-cholinergics[149]. The assemblage of immune cells in the cytokine- and chemokine-rich environment results in a rapid expansion of inflammation in the airways and chronically impacts on vasculature, airway smooth muscle, epithelium and mucosa, mesenchyme and sensory neuronal fibres[142]. This will ultimately contribute to the structural and functional decline of the lungs, and result in the acute and chronic symptoms of asthma.

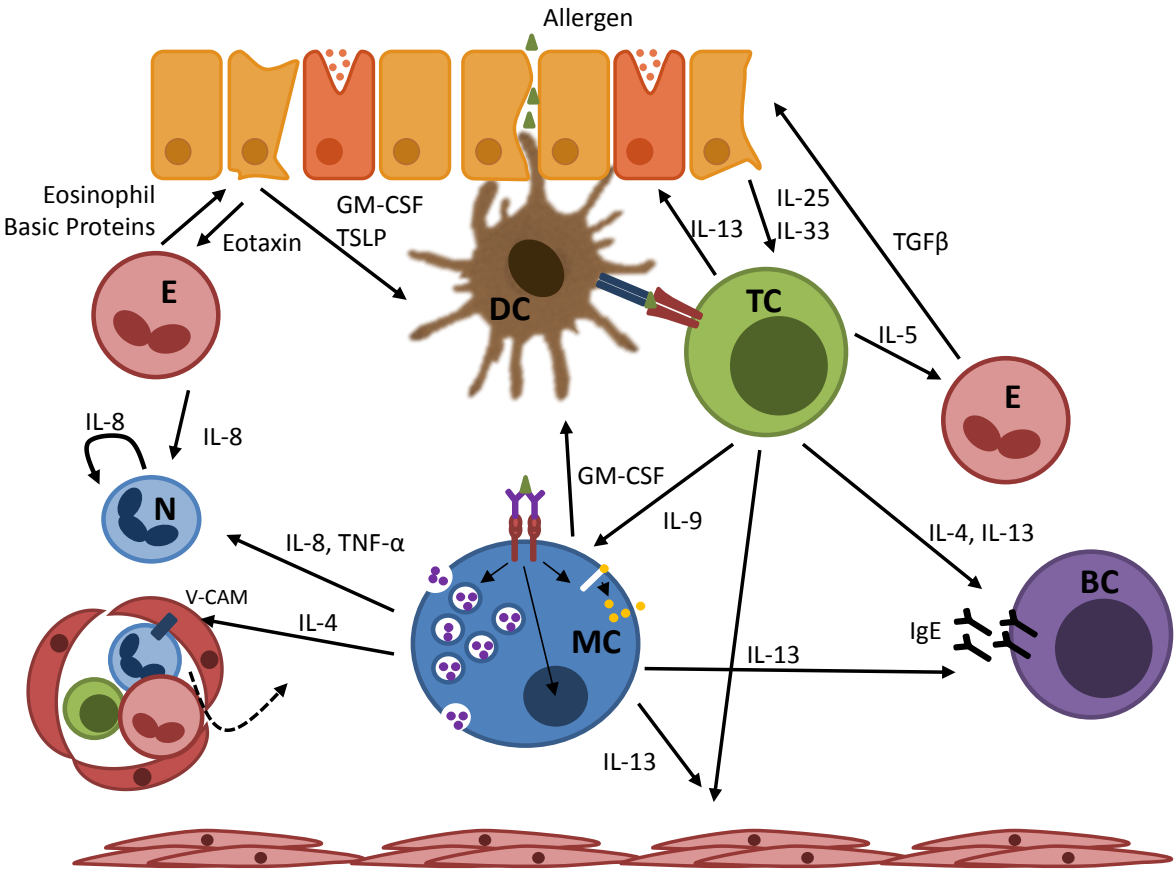


Figure 1.9 – **The late phase of allergic inflammation in the airways.** Epithelial damage, as well as the degranulation and slow release of immune mediators by mast cells results in a secondary late phase inflammatory response in the airways. Mast cells (**MC)** and epithelial cells release GM-CSF[148], promoting the maturation of dendritic cells (**DC**), which extend their processes between epithelial cells to take up allergen[150]. Through interaction with memory $CD4^+$ T-cells in the lung mucosa and regional lymphoid organs (not shown), under the influence of MC-derived IL-4, and epithelial derived IL-25, IL-33 and TSLP, they promote the differentiation and proliferation of a population of TH2 cells (**TC**)[146]. Reciprocally, TC-derived IL-9 promotes proteolytic activity of mast cells and enhances FcεRI expression[23]. Release of IL-4 from mast cells promotes the infiltration of TCs and other leukocytes into the airways, via upregulation of cell adhesion molecules such as vascular cell adhesion molecule (VCAM) on endothelial cells[23]. MC- and TC-derived IL-4 and IL-13 additionally promote maturation of and production of IgE by B-cells (**BC**). MCs, TCs and epithelial cells further induce the infiltration of granulocytes through secretion of IL-5 and eotaxin, promoting eosinophil (**E**) differentiation and proliferation, and IL-8 and TNF-α, promoting neutrophil (**N**) recruitment. Newly recruited cells release cytokines and other immune mediators which impact on the surrounding airway tissue: IL-13 induces bronchoconstriction and mucous secretion, and eosinophil basic proteins and TGFβ induce epithelial damage and fibrosis[142] .

1.4.2 Structural and Functional Changes in Asthma

Allergic TH2-type lung inflammation chronically results in detrimental changes to lung structure and function, two clinical hallmarks of asthma pathogenesis. Epithelial injury in combination with the distinct cytokine milieu in the lungs propagates structural changes to the cells of airway, leading to thickening of bronchial wall and narrowing of the airway lumen, a process termed airway remodelling. Concurrently, the airways become overresponsive to exogenous and endogenous chemical and mechanical stimuli, a functional change termed airway hyperresponsiveness (AHR). Together, these clinical changes contribute to the progression of asthma and consequent deterioration of lung function.

1.4.2.1 Airway remodelling

The structural changes in the airway lumen, termed airway remodelling, are a critical hallmark of asthma, playing a key role in the development, progression and symptoms of the disease. The phenomenon collectively refers to hypertrophic and hyperplastic changes in the airway smooth muscle (ASM), epithelial cell shedding, angiogenesis, extracellular matrix (ECM) deposition (termed subepithelial fibrosis) and goblet cell hyperplasia[151]. The precise causes and mechanisms that lead to airway remodelling are unclear, though epithelial injury and inflammation are thought to be crucial players. Eosinophil-derived transforming growth factor (TGF)-β is pro-fibrotic, contributing to ECM deposition and enhancing ASM migration[152]. In addition, TH2 cytokines have been shown to enhance mucous gene expression, subepithelial fibrosis and epithelial hypertrophy, and chemokines have been shown to enhance migration of ASM[148]. Airway remodelling collectively results in the thickening of the bronchial wall and a narrowing of the airway lumen, leading to airflow obstruction, decreased forced expiratory volume (FEV1) and AHR, ultimately contributing to the symptoms of asthma[153].

1.4.2.2 Airway hyperresponsiveness

AHR can be defined as the exaggerated bronchomotor response to endogenous or exogenous chemical and mechanical stimuli, such as histamine, metacholine, adenosine monophosphate, SO_2, fog and cold air[154]. It is believed to be made up of two main components: baseline or persistent AHR and variable or episodic AHR[155]. Baseline AHR is present in the majority of patients and is thought to be the result of airway remodelling, changes in airway smooth muscle gene expression and increased expression of contractile proteins[156]. Variable AHR, on the other hand, is thought to be induced by inflammatory events and inflammatory mediators in the lungs (e.g. nitric oxide (NO), substance P (SP), PGD_2, $PGF_{2\alpha}$, CCL28, IL-5, IL-13 and complement)[154]. It is still unclear precisely why inflammatory infiltrate causes variable AHR, but some suggest that innervating neuronal fibres play a role. For example, SP, PGD_2, $PGF_{2\alpha}$ and TXA_2, have been shown to lead to an increased production of the stimulatory neurotransmitter acetylcholine from innervating parasympathetic fibres[157]. AHR underlies some of the main symptoms of asthma, such as bronchoconstriction and dyspnoea, and is associated with lung function decline.

1.4.3 Established Asthma Treatments

Asthma is a multifaceted, heterogenic disease with widespread inflammatory, muscular, vascular and neuronal components. The complexity of the disease makes targeted and all-encompassing treatment difficult. However, current asthma treatment has proved widely beneficial in the relief of symptoms (by "reliever" drugs) and control of inflammation (by "controller" drugs") of the disease. β_2-adrenoceptor agonists (reliever) are the most commonly used fast acting drugs, inducing bronchodilation and consequently relieving asthma symptoms. Corticosteroids and cysteinyl leukotriene receptor antagonists (controller) are anti-inflammatory drugs that provide longer term control of the disease.

β_2-adrenoceptor agonists, particularly the short-acting agonists (SABAs) such as salbutamol, are the most effective treatment in providing rapid relief of asthma symptoms. They act by binding the β_2-adrenoceptor on airway smooth muscle to induce relaxation and, in consequence, bronchodilation[28]. Long-acting agonists (LABAs), such as formoteral and salmeterol, induce bronchodilation for at least 12 hours. However, they are often used as a

combination therapy with corticosteroids to reduce inflammation in poorly controlled asthma. Both SABAs and LABAs have proved widely beneficial in improving symptoms of asthma and reducing exacerbations[158].

Inhaled corticosteroids (ICS) are a group of anti-inflammatory molecules that successfully supress airway inflammation in asthma. Historically, ICS were believed to attenuate inflammation by binding to the cytosolic GR, resulting in translocation to the nucleus and transrepression of transcription factors such NFκB and AP-1, which are responsible for the transcription of various cytokines, chemokines and adhesion molecules[114]. However, new data suggests that the binding of ICS to GR recruits the histone deacetylase-2 (HDAC2), reversing acetylation of activated inflammatory genes and thus silencing transcription[159]. The use of ICS, such as budesonide, has markedly reduced morbidity and mortality in asthma, effectively controlling the symptoms of airway disease and increasing patient quality of life[158].

More recent established asthma therapy involves the direct inhibition of specific mediators involved in allergic asthmatic inflammation. Cysteinyl leukotrienes (CysLTs) are known to affect many diverse features of asthma, including smooth muscle contraction, eosinophil infiltration and neuronal activity, but they are not inhibited by corticosteroids. CysLT receptor 1 antagonists, such as montelukast, have proved beneficial in treating mild and moderate forms of asthma and have additionally shown efficacy as a combination therapy with corticosteroids[28].

1.4.4 Prospective Asthma Treatments

Though current treatments for asthma are widely prescribed and have proved greatly beneficial, new avenues are being explored in the search for a superior therapy. The unacceptable side-effects associated with long-term ICS use (e.g. adrenal suppression and decreased bone mineral density), the small, but costly proportion (5-10%) of "steroid-resistant" asthma patients, as well as the "phobia" or unwillingness for many patients to use steroids, has led research to investigate alternative therapies[28]. Asthma treatment today additionally relies heavily on temporarily alleviating symptoms or attenuating airway inflammation. New therapies are instead focusing on modifying the underlying asthmatic

inflammation and consequently halting the progression of the disease. One approach involves the use of immunomodulators.

1.4.4.1 Immunomodulators

Immunomodulators modify the immune response with the aim of restoring the natural TH1-TH2 balance. Current immunmodulatory approaches typically target the overactive TH2 arm or act to mimic a TH1 response. These include IL-4, IL-5 and IL-13 receptor antagonists, IgE and mast cell degranulation inhibitors, recombinant IFN-γ and IL-12, or the TLR agonists, including agonists for TLR2, 4, 7 and 9.

Using TLR agonists to treat allergic lung inflammation may seem surprising and counterintuitive. It is widely believed that some of the main causes of complications and exacerbations of asthmatic inflammation are bacterial and/or viral infections[160], pathogens known to interact with TLRs. Respiratory viral infections, induced by influenza (which interacts with TLR7) or rhinovirus (which interacts with TLR3) account for a great majority (80%) of asthma exacerbations in both adults and children, insinuating that the use of TLR agonists in asthma may have deleterious consequences[160]. However, respiratory viruses are also known to interact with other PRRs, such as retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5), instigating larger and more complex inflammatory responses in the lung[161]. Activating the immune system with simply TLR agonists has proved promising pre-clinically and clinically, both when used alone or in combination with specific allergen, as a form of allergen-specific immunotherapy (SIT).

Numerous studies using animal models have provided evidence demonstrating the efficacy of TLR4 and TLR2 agonists in preventing and reversing allergic lung inflammation. TLR4 agonists (LPS or lipid A) have been shown to prevent inflammatory components of allergic lung inflammation (TH2 cytokine production, rise in serum IgE and airway eosinophilia) in an ovalbumin (OVA) immune complex model of asthma, when administered prior to OVA immunisation or challenge[10,162], as well as prevent functional changes (e.g. AHR) when administered prior to immunisation[10]. In a model of SIT, where LPS and OVA are co-administered, components of late-phase asthma, including late-phase AHR, cellular

influx and microvascular leakage are prevented[163]. Similarly, TLR2 agonists (e.g. peptidoglycan, Pam3Csk4 or LP40) have been shown to prevent inflammatory components of allergic lung inflammation when given prior to OVA immunisation or challenge in an OVA immune complex model of asthma[10,164]. TLR2 agonists have also been shown to reverse established inflammatory and functional (AHR) changes in an OVA immune complex model of asthma if given after OVA challenge[165,166]. However, the efficacy of TLR4 and TLR2 agonists is somewhat controversial. Low doses of LPS have been shown to aid and increase TH2-type inflammation induced by OVA[167,168]. In addition, high doses of peptidoglycan have been shown to increase TH2-type inflammation, in terms of TH2 cytokine production, eosinophilia, anti-OVA IgE and AHR, when administered in combination with OVA during immunisation[169,170].

TLR7/8 ligands (e.g. R-848/resiquimod) have also shown promise as candidate drugs for treatment of allergic asthma, having already passed Phase II clinical trials[171]. Pre-clinical studies with animal models have demonstrated that resiquimod can prevent airway inflammation when administered as SIT in conjunction with OVA[172,173], as well as prevent structural (goblet cell hyperplasia and smooth muscle hypertrophy) and functional (AHR) changes associated with airway inflammation when administered prior to OVA challenge, in a chronic OVA immune complex model[172-174]. This suppressive effect is most commonly long-lived[175] and is thought to occur in an IL-12[176] and IFN-γ[175,177] dependent manner. The TLR7 ligand imiquimod (commonly used in the treatment of atopic dermatitis) has also been shown to suppress AHR, inhibit eosinophillic inflammation, TH2 cytokine production via blocking GATA-3 production and increasing T-bet production when administered prior to OVA challenge[178,179]. However, imiquimod has been shown to induce extracellular matrix deposition in airway smooth muscle in vitro, suggesting that some TLR7 ligands may contribute to airway remodelling[180]. More recently, TLR7 and TLR8 agonists have proved beneficial in Phase II clinical trials for the treatment of allergic rhinitis[181,182] (Table 1.3).

TLR9 agonists (e.g. CpG oligodeoxynucleotides (CpG ODNs)) are the most widely studied TLR agonists, with numerous candidate drugs having entered clinical trials. When studied pre-clinically in the OVA immune complex model of asthma, they have been shown to prevent AHR and TH2 type inflammation when given before OVA challenge[183-186] or in combination with OVA challenge, as SIT[184,186]. CpG ODNs also reverse established

eosinophilia when given after OVA challenge[183]. In a ragweed (RW) pollen model of asthma, CpG ODNs work alone and as an SIT, preventing eosinophilia when administered before RW challenge[187], and preventing AHR and TH2 cytokine production when administered in combination with RW[188]. In a schistosoma egg model of asthma, CpG ODNs prevent eosinophilia, AHR and TH2 cytokine production when administered in combination with schistosoma immunisation, and prevent eosinophilia when given in combination with schistosoma challenge[189], as a form of SIT. CpG ODNs have additionally shown efficacy in a fungal model of asthma, reversing established AHR, circulating IgE production, increased mucous production and TH2 cytokine production when given after fungal challenge[190]. TLR9 agonists have shown promising results in pre-clinical trials in rodents and primates[191] and have entered clinical trials for allergic rhinitis and asthma[171](Table 1.3).

Table 1.3

Clinical phase of TLR agonists in treatment of allergy

Compound	Company	Target	Indications	Clinical Phase
AZD1419	Astra Zeneca	TLR9	Asthma	Phase II
VTX1463	VentiRx Pharmaceuticals	TLR8	Allergic rhinitis	Phase I
AVE0675	Sanofi Aventis/Coley Pharmaceuticals	TLR9	Asthma, allergic rhinitis	Phase I
SAR21609	Sanofi Aventis/Coley Pharmaceuticals	TLR9	Asthma	Phase I
QAX935	Idera Pharmaceuticals	TLR9	Allergy, asthma	Phase I
AZD8848	Astra Zeneca	TLR7	Allergy, asthma	Phase II
Pollinex Quattro	Allergy therapeutics	TLR4	Allergic rhinitis	Phase III

Adapted from Connolly and O'Neill[171]

Though immunomodulators have shown very promising results, their use as a treatment is still controversial. Despite showing great promise in the treatment of allergic rhinitis, clinical trials with CpG DNA in lower respiratory allergic inflammation, such as asthma, have been less successful[192]. Significantly, immunomodulators induce the production of TH2 and pro-inflammatory cytokines, which may have harmful, as opposed to remedying, effects on the lung environment and rest of the body. Administration of CpG DNA via intratracheal instillation can lead to neutrophil recruitment and synthesis of TNF-α, IL-6,

MIP-2 and KC (CXCL1 chemokine) in the lungs[193] as well as recruitment of DCs and NK cells[194]. TLR2 and 4 can trigger robust increases neutrophil and macrophage populations in the lungs of allergic mice and TLR3, 4 and 9 agonists trigger significant increases in IL-6, TNF-α and IL-1β[195]. Not only would this lead to a continuous, detrimental inflammatory response in the lung, but, with the knowledge that peripheral pro-inflammatory cytokines (such as IL-1β and TNF-α) and prostaglandins communicate with the brain, treatment with TLR agonists may impact on neuronal activation and neurochemistry, inducing sickness behaviour and glial priming or activation. Before further clinical trials of immunomodulators continue, it is vital to understand how TH2 immune responses communicate with and impact on the brain and how these drugs may affect this form of immune to brain communication.

1.5 The Role of the Peripheral and Central Nervous Systems in TH2-mediated Lung Inflammation

Like all visceral organs, the lungs are innervated by a variety of neuronal fibres, which are part of the autonomic nervous system and play a vital role in the physiology of the airways. Not only do these neurons regulate our breathing pattern, but also, through sensing mechanical and chemical changes in the airways, maintain homeostasis within the lung environment[196]. They augment inflammation in response to foreign agents, trigger the cough reflex to eliminate disturbances from the lung[197] and terminate inspiration upon lung inflation in a phenomenon known as the Hering-Breuer reflex[198]. In asthma, however, the neuronal innervations of the lungs play a detrimental role. Not only do the neurons exacerbate inflammation, but through phenotypical and genotypical changes of the neurons themselves, they become hypersensitive to changes in the airway environment, resulting in many of the symptoms of asthma, including increased bronchoconstriction and dyspnoea[199]. Understanding the role of neuronal changes in the airways during asthma may be critical in the treatment of this disease.

1.5.1 Neuronal Airway Innervation and Central Circuitry

1.5.1.1 Peripheral neuronal airway innervation

The lungs are innervated by both efferent motor fibres and afferent sensory fibres, which play important roles in various physiological lung functions (Figure 1.10). The efferent fibres are part of the autonomic nervous system and include the parasympathetic (cholinergic fibres and inhibitory non-adrenergic non-cholinergic (i-NANC) fibres), as well as the sympathetic fibres[200]. The parasympathetic fibres, which have their ganglia in the bronchial wall, innervate the airway smooth muscles, vascular smooth muscle and glands, mediating mucous secretion, as well as smooth muscle tone, through increased bronchoconstriction. The sympathetic fibres, on the other hand, innervate tracheobronchial blood vessels and glands. Unlike sympathetic fibres, parasympathetic fibres are part of the vagus nerve[201].

The afferent sensory neuronal fibres that innervate the airways are all part of the vagus nerve. These include the rapidly adapting receptors (RARs), the slowly adapting receptors

(SARs), the unmyelinated c-fibres, the myelinated Aδ-fibres and the newly discovered "cough receptor"[197]. The different fibres can be distinguished by their discrete sensitivity to various stimuli, their origin, their neurochemistry and their conduction velocity. RARs are myelinated fibres with a conduction velocity of about 15 m/s. They are highly sensitive to mechanical stimulation, such as changes in lung volume or smooth muscle contraction, and their signals are sent to cell bodies located in the nodose ganglion. SARs have similar properties to RARs, but are instead sensitive to sustained lung inflation. They are thought to be involved in the physiological Hering-Breuer reflex. Aδ fibres, too, are myelinated and have a similar conduction velocity to RAR and SARs, but differ in that they are more sensitive to chemical stimuli than mechanical stimuli and have their cell bodies in the jugular ganglia[202].

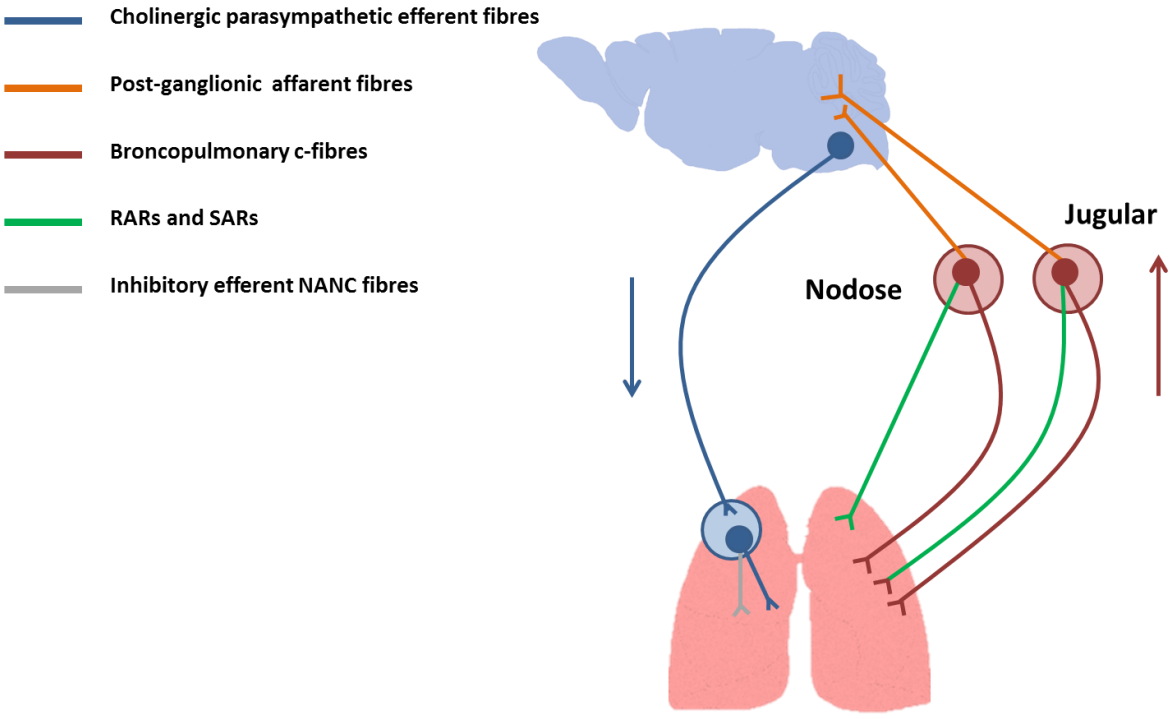


Figure 1.10 – Vagal innervation of the airways. The airway is innervated by a number of different types of neuronal fibres, which are all part of the vagus nerve. Efferent parasympathetic fibres include the cholinergic fibres (blue) and the inhibitory non-adrenergic non-cholinergic fibres (i-NANC) (grey), which mediate smooth muscle tone through constriction (cholinergic) and relaxation (i-NANC). The afferent vagal fibres include the c-fibres (red), the slow-acting (SAR) and rapid-acting (RAR) receptors (green), the Aδ fibres the cough receptors (not shown). These have their cell bodies in the nodose or jugular ganglia, from which neuronal fibres project to the brainstem.

1.5.1.2 The afferent bronchopulmonary c-fibres

Afferent c-fibres make up about 75% of the afferent fibres innervating the airways[203,204]. They play a vital role in regulating the airway environment in both physiological and pathological conditions. These fibres are unmyelinated and slow conducting (1 m/s), having their cell bodies in both the nodose and jugular ganglia. The bronchial c-fibres, which innervate the extrapulmonary airways have their cell bodies in the jugular ganglion, whereas the pulmonary c-fibres, instead, innervate the lungs and airways and have their cell bodies in the nodose ganglion[205]. Collectively airway c-fibres are referred to as bronchopulmonary c-fibres and are highly sensitive to exogenous and endogenous chemical stimuli. These include the exogenous products capsaicin (a product from chilli peppers), phenylbiguanide (a serotonin analogue), ozone, sulphur dioxide, ammonia, volatile anaesthetics and tobacco smoke, as well as endogenous inflammatory products (histamine, bradykinin, prostaglandins), adenosine, acidic solutions (e.g. lactic acid) and changes in osmolarity[203]. These various chemicals act to stimulate the c-fibres or increase their excitability. Bronchopulmonary c-fibres play a critical role in the symptoms and pathology of asthma[203].

1.5.1.3 Central circuitry of projections from the lungs

Upon activation (by mechanical and/or chemical stimuli) in the lung, afferent sensory fibres send signals to the nodose or jugular ganglia and onto the NTS in the brainstem. The different subtypes of afferent fibres terminate in different areas of the NTS and project to specific second order neurons. SARs project to the ipsilateral intermediate, interstitial, ventral and ventrolateral NTS. RARs project to ipsilateral and caudal regions of the NTS, including, primarily, the central and medial NTS, but also the dorsal, dorsolateral and intermediate NTS[198] (Figure 1.11). Vagal c-fibres, on the other hand, terminate predominantly in the caudal medial NTS[198,206]. From the brainstem, neuronal signals can either be further projected into the brain, to the LPBN, thalamus and granular insular cortex, where the information is processed, or projected back down to the lungs, through a central reflex, via the efferent parasympathetic fibres[207]. This reflex plays an important role physiologically, being involved in the cough reflex, ridding potentially harmful substances from the lungs, as well as the Hering-Breuer reflex[208].

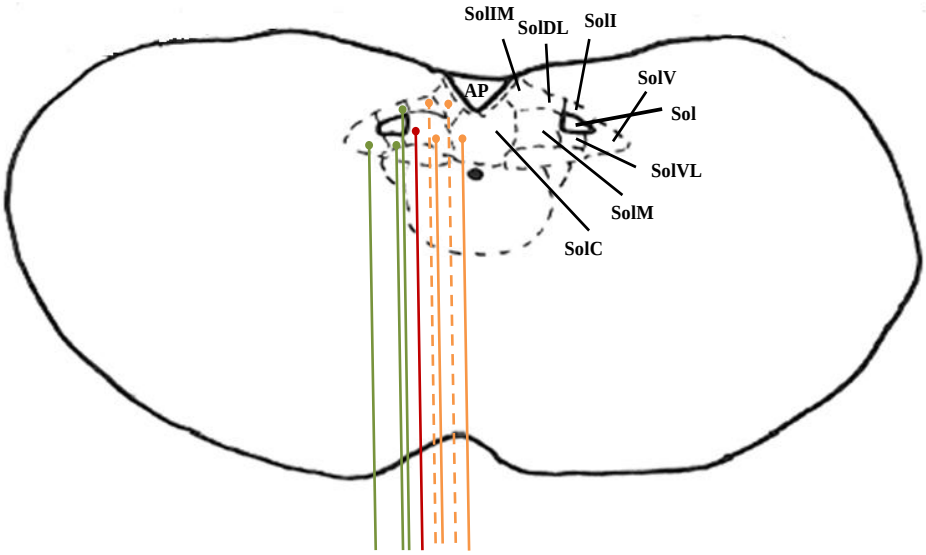


Figure 1.11 – Innervation of the NTS by afferent neurons of the airway. Slowly-adapting receptors (SARs, green) project to the ipsilateral interstitial (SolI), ventral (SolV) and ventrolateral (SolVL) nucleus of the solitary tract (NTS). Rapidly adapting receptors (RARs, orange) project predominantly to the ipsilateral medial (SolM) and central (SolC) NTS, but also the intermediate (SolIM) and dorsolateral (SolDL) NTS. The bronchopulmonary c-fibres (red) project mainly to the SolM. AP: Area postrema

1.5.2 Influence of Asthmatic Inflammation on Airway Afferent Neurons

Stimulation of afferent vagal fibres, specifically the bronchopulmonary c-fibres, by endogenous and exogenous chemicals in the airways results in bronchoconstriction of the airways, increased mucous secretion and increased inflammation, through axonal and central reflexes. These phenomena are, under physiological conditions, protective mechanisms. However, in diseased conditions, such as during asthma, excessive inflammation and remodelling in the airways can contribute to increased receptor stimulation and expression and genotypical changes in innervating neurons, resulting in heightened sensitivity of the neurons and an exaggeration of these reflexes, leading to an amplification of airway inflammation, bronchoconstriction and mucous secretion. This positive feedback ultimately contributes to the symptoms of asthma, namely breathlessness, wheezing, dyspnoea and cough[199].

1.5.2.1 Neurogenic Inflammation

Physiologically, neurons, particularly c-fibres, undergo a phenomenon known as an axonal reflex, a local neuronal mechanism in which sensory neurons are stimulated to release peptides, without a signal being transmitted centrally to the brainstem and without neuronal depolarisation[209]. Exaggerated asthmatic inflammation leads to the heightened activation of bronchopulmonary c-fibres, ultimately resulting in the amplification of the axonal reflex. As a result, stimulated neurons release numerous neuropeptides into the lung compartments, establishing a phenomenon known as neurogenic inflammation. Neuropeptides, such as neurokinin A (NKA), SP and calcitonin-gene related peptide (CGRP), are pro-inflammatory mediators, which have numerous effects on the inflammatory environment in the lung. By binding to G-protein coupled receptors (GPCRs) on epithelial cells, vasculature and immune cells, neuropeptides may trigger vasodilation, plasma exudation, angiogenesis, eosinophil attraction, macrophage activation, mucous secretion and bronchoconstriction[210]. Neurogenic inflammation is a critical feature of asthma, largely contributing the inflammatory process in the lung (Figure 1.12).

1.5.2.2 Receptor expression – activation and excitability of neurons

Neurons innervating the lung, particularly the bronchopulmonary c-fibres, express numerous receptors that respond to inflammatory events in the lung. Binding to these receptors can lead to excitation of the neuron directly, activating central reflexes (e.g. cough) and triggering neurogenic inflammation, or otherwise change the excitability of the neuron.

The transient receptor potential (TRP) channels, including transient receptor potential vanilloid 1 (TRPV1)[211] and transient receptor potential ankyrin 1 (TRPA1)[212], are co-expressed on bronchopulmonary c-fibres and play a vital role in asthma pathology. TRPV1 is a capsaicin sensitive receptor known to be predominately responsible for burning sensations, but also sneezing, itching and cough. TRPV1 is activated by high temperature, as well as exogenous and endogenous compounds, including protons (present in acidic solutions)[213], certain lipid mediators[214] and bradykinin[215]. In humans and guinea pigs, activation of TRPV1 instigates the cough reflex[216] and may also play a role in neurogenic inflammation, as they have been colocliased with neuropeptides[217], although this has recently been disputed[218]. TRPA1, similarly, plays a vital role in asthma. It too

leads to burning sensations, but is instead activated by certain exogenous chemicals, including cinnamaldehyde (in cinnamon), allyl-isothiocynate (mustard oil), acrolein, isocyanate (in tobacco smoke), ozone and formaldehyde[219], as well as endogenous chemicals including prostaglandin D_2 (PGD_2)[220] and reactive oxygen species (ROS)[221,222]. Like TRPV1, TRPA1 induces a cough response in humans and guinea pigs[223]. In addition, the TRPA1 channel has been shown to play an important role in neurogenic inflammation. TRPA1 KO mice or mice treated with a TRPA1 antagonist have a reduced late-asthmatic response following OVA challenge, as compared to wild-type or untreated mice[149,224]. Paracetamol, which activates the TRPA1 channel, causes neurogenic inflammation in rodents[225].

Bronchopulmonary c-fibres also express P2X purinoreceptors, which are activated by ATP and other purines, 5-hydroxytryptamine (5-HT) receptors, acetylcholine receptors, acid-sensing ion channels, the A1 GPCR (activated by adenosine) and the B2 GPCR (activated by bradykinin) all of which can be directly activated by products of inflammation. Other receptors, particularly GPCRs, are involved in increasing the excitability of the neuronal fibres, such as the PGE_2 receptor[199].

1.5.2.3 Phenotypical and genotypical changes

Several studies have also examined the effect of lung inflammation on the genotype and phenotype of the neuronal fibres that innervate the lungs. These changes encompass alterations in transmitter production, receptor expression and ultimately excitability and sensitivity of these neurons. Inflammatory cells of the lung are known to produce neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which interact with receptors (p75NTR and tyrosine kinase linked receptors). Upon interaction, these receptors can become internalised, transported back to the cell nucleus and affect gene transcription. Through alteration of promoter activity, neurotrophins can alter gene expression of TRPV1[226], acid-sensitive ion channels[227] and voltage gated sodium channels[228]. Neurotrophins, specifically NGF, have additionally been shown to stimulate trafficking of TRPV1 to the plasma membrane via a PI3 kinase[229], and protein kinase A[230] dependent mechanism, resulting in hypersensitivity of the neurons to capsaicin[231]. In addition, bradykinin and adenosine A1, which act through GPCRs, have

been shown to gate TRP channels through interaction with phospholipase signalling molecules[196,232,233] (Figure 1.12). Together, this will result in an increased excitability of the vagal neuronal fibres innervating the lung.

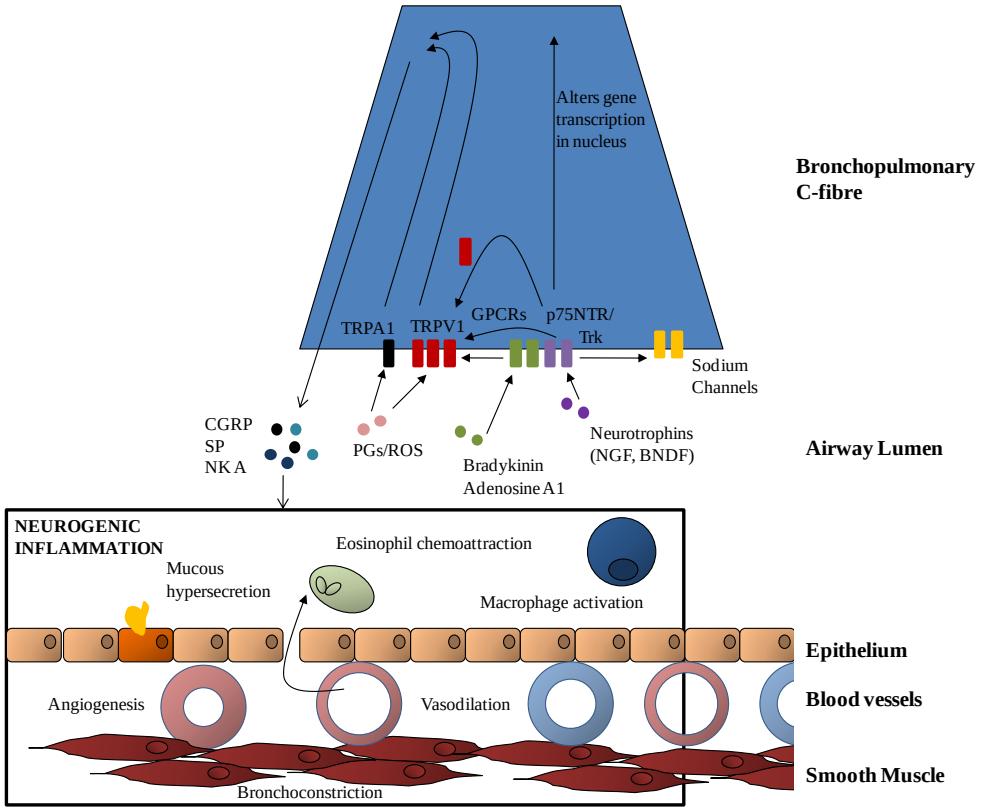


Figure 1.12 – Changes to the neuronal environment in the lung during allergic inflammation. Inflammatory mediators such as prostaglandins and reactive oxygen species act on receptors expressed on neurons such as TRPV1 and TRPA1, inducing the production of neuropeptides, including CGRP, SP and NKA. These neuropeptides have numerous effects on the airway environment, including eosinophil chemoattraction, macrophage activation, mucous hypersecretion, angiogenesis, vasodilation and bronchoconstriction. This process is termed neurogenic inflammation. Inflammatory mediators also have a large effect on the phenotype and genotype of bronchopulmonary c-fibres that innervate the airway. Bradykinin and adenosine A1 can act on their respective GPCRs, resulting in gating of the TRPV1 and TRPA1 channels. Neurotrophins, such as NGF and BDNF act via their respective receptors (p75NTR and TrkA, B and C) to alter gene expression of a variety of neuronal receptors by altering promoter activity, facilitate the trafficking of cation channels like TRPV1 and potentiate channel activity. Neurotrophins can also directly gate sodium channels as well as modulate their function.

1.5.3 Pathophysiology of Central Neuronal Circuitry in Asthma

In more recent years, it has been shown that inflammation in the lung, which acts upon peripheral neuronal fibres, results in changes to central neuronal fibres, specifically in the

NTS in the brainstem. Wilson et al.[234] showed that upon repeated allergen exposure, there is decreased expression of the α_{2A} adrenergic receptor on the cell bodies of the airway-related pre-ganglionic neurons in the brainstem, a receptor responsible for sending inhibitory signals to the parasympathetic cholinergic outflow in ferrets. Other studies have indicated a change in the excitability of NTS neurons in response to extended tobacco smoke (ETS) (which can predispose children to asthma) and extended allergen exposure in guinea pig[235,236] and primate models[207], a phenomenon believed to be dependent on the increased production of SP in afferent vagal neurons[237]. Indeed microinjection of SP into the NTS leads to sensitisation of the cough reflex[238]. This plasticity of NTS neurons is thought to play a key role in altering reflex outflow to the airways. Further experiments have shown that ETS alters the function of potassium channels in the NTS[239]. Primate and rodent models of asthma have also shown to have increased c-fos[240] and decreased histamine H3 receptor expression[241] in the NTS.

It has been suggested that the changes which occur in the brainstem play a role in augmenting sensitivity of peripheral neuronal fibres, in a process known as central sensitisation. This is a well-described phenomenon in the somatosensory system, playing a key role in inappropriate or neuropathic pain. Central sensitisation involves the heightened sensitivity of second order neurons in the CNS, due to the release of neurotransmitters and neuropeptides in this area, and activation of glial cells. These second order neurons not only respond to signals from c-fibres, but also from mechanosensory fibres. Thus, heightened sensitivity of the second order neurons in the NTS results in a misinterpreted, but heightened, sensitivity of the mechanosensory fibres. Indeed, activation of bronchopulmonary c-fibres leads to the hypersensitivity of Aδ-fibres to touch, resulting in a heightened cough reflex[242]. The specific changes which may lead to central sensitisation in asthma are, as of yet, unknown. Nevertheless, pathological changes in peripheral and central neuronal fibres play a vital role in asthma pathology.

1.6 Neuroimmune Communication is Asthma and Allergy

Communication between the immune system and the CNS is, as described above, an important phenomenon, triggering a range of metabolic, behavioural and psychological changes to rid the body of the infection and simultaneously downregulate the immune

response. Though much work has been put into understanding how TH1 type immune responses communicate with the CNS, little focus has been put into the effect of allergic inflammation on the brain and on behaviour. It has long been acknowledged that allergy has an impact on behaviour. As early as the 19th century, scientists were aware of what was termed the "rose effect", in which the sight of an artificial rose to which one was allergic could trigger thoughts of anxiety[108,243]. Understanding how peripheral TH2 immune responses communicate with and impact on the brain is not only important in providing a fuller picture of immune to brain communication, but may also be of pathological importance, since, as described above, changes in central and peripheral neurons contribute highly to the symptoms and pathology of asthma.

A variety of studies have provided some insight into the relationship between allergic reactions and the brain. A group in Brazil have explored the effect of OVA-induced lung inflammation in the mouse on brain activity and behaviour[108,244,245]. Allergic lung inflammation is shown to lead to avoidance behaviour, where mice avoid compartments that were associated with a negative experience, such as being challenged with OVA. The study showed an increase in *c-fos* activity in the PVN and CeA and an increase in CRF and serum corticosterone, indicating an activation of the HPA axis. Changes in brain activity and behaviour were found to be dependent on levels of IgE and mast cell degranulation, but not inflammatory cell-infiltrates, as depletion of IgE with anti-IgE antibodies and inhibition of mast cell degranulation by cromolyn abolished changes in behaviour and neuronal activity[245]. More recently it has been shown that tau phosphorylation, a hallmark of Alzheimer's disease, is increased after chronic allergic lung inflammation[246].

Other studies have shown how other forms of allergy, such as allergic rhinitis (hay fever) and food allergy, impact on the brain and on behaviour. Allergic rhinitis in mice and rats, induced by sensitisation and challenge with OVA or pollen, produces anxiety-like behaviour and reduces social interaction. In addition, allergic rhinitis increases the production of T_H2 cytokines IL-4, IL-5 and IL-13 in the olfactory bulbs and prefrontal cortex in mice and rats[247]. Furthermore, food allergy has been shown to result in avoidance behaviour, anxiety and c-fos expression in the PVN[248]. These studies provide the first convincing evidence that allergic inflammation affects the brain.

Bi-directional immune to brain communication is an important phenomenon, both physiologically and pathologically. Physiologically, it is important in alerting the brain to the presence of a peripheral inflammatory event, resultantly changing behaviour and metabolism to rid the body of its cause and downregulate the immune response. Pathologically, it plays a role in exacerbating neurodegeneration in age-related diseases such as Alzheimer's disease. Understanding how peripheral inflammation communicates with and affects the brain is limited and most studies have focused on TH1-mediated inflammatory events. TH2 inflammatory events are becoming increasingly prevalent, with asthma affecting over 300 million people worldwide, making their impact on the brain equally important. The involvement of vagal afferent and efferent neurons in the pathology of asthma makes their communication with and profound effect on the brain more significant. With the development of new treatments for asthma, namely the TLR agonist immunomodulators, immune to brain communication, specifically involving the vagus nerve, becomes that much more of a key issue. Switching the peripheral immune response to a TH1-medatied immune response, which is already known to have central effects, could not only alter already unknown central effects but also alter the signals passing through the vagus nerve. It is therefore necessary to understand the possible effects of TH2-mediated allergic lung inflammation on the brain, and possible role the vagus nerve plays and consequently discover if and how immunomodulators may affect this.

1.7 Aims

The general aim of this research project is to investigate the impact of localised lung-based TH2-type immune responses on the central nervous system.

- To achieve this, the initial aim is to compare the effects of systemic innate TH1-like (dominated by pro-inflammatory and TH1 cytokines) and localised innate TH2-like (dominated by TH2 cytokines) immune responses on the brain, in terms of alterations in behaviour, central cytokine production (as a determinant of central innate immune system activation) and neuronal activity.
- The subsequent aim is to determine the impact of an acute lung-based TH2 type immune response on the brain, using a well-established rodent model of allergic lung inflammation and investigating the effect on behaviour and neuronal activity.
- Consequently, the effect of a chronic lung-based TH2 type immune response on the brain will be determined, by developing a rodent model of chronic allergic lung inflammation and performing a broad investigation of alterations in neuronal activity and central immune activation.
- In achieving an understanding of the impact of acute and chronic TH2-mediated lung inflammation on the brain, the next aim is to determine the effect of peripheral immunomodulation of lung inflammation (using TLR7 agonists) on the central nervous system, through investigation of behavioural changes and central cytokine expression, as a marker of central innate immune activation.

Chapter 2: General Methodologies

2.1. In Vivo Methods

2.1.1 Animals – Southampton

Four- to six-month old female C57BL/6 (Biomedical Research Facility (BRF), Southampton, UK) or BALB/C (BRF, Southampton, UK) mice were used. They were housed in plastic cages with sawdust bedding in groups of 5 to 10. The housing and experimentation room was temperature controlled (19-23°C) with a controlled light-dark cycle (12h:12h with light on at 0700 h). Food (standard chow, RM1, SDS, UK) and water were available at all times during housing and experimentation. All procedures were performed in accordance with the United Kingdom Home Office Licensing Inspectorate.

2.1.2 Animals – GlaxoSmithKline (GSK), Stevenage, UK

Six to eight week old female BALB/C (Charles River, UK) mice were used. They were housed in plastic cages with sawdust bedding in groups of 6 to 10. The housing and experimentation room was temperature controlled (19-23°C) with a controlled light-dark cycle (12h:12h with light on at 0700h). Food (standard chow, RM1) and water were available at all times during housing and experimentation. All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.

2.1.3 Immune Challenge Protocols

2.1.3.1 Immune stimulation with LPS or zymosan

C57BL/6 mice were challenged intraperitoneally (i.p.) with lipopolysaccharide (LPS, derived from *Salmonella abortus equi*, L5885, Sigma, Poole, UK) at 100µg/kg or zymosan (Zymosan A from *Saccharomyces cerevisae*, Sigma, Poole, UK) at 5mg/kg. Control animals were challenged i.p. with 200µl sterile saline (0.9%, Fannin, UK)

2.1.3.2 Induction of allergic lung inflammation

A number of different models are currently used in pre-clinical studies to mimic human allergic asthma in the rodent[249]. Treatment with natural allergens, such as *Aspergillus fumigatus*[250], ragweed pollen[187] and HDM[251] have been all shown to mimic several features

of human asthma, including TH2 cytokine expression, eosinophilia and AHR. These models are undoubtedly physiologically relevant, as they involve real allergens. However, the mechanism by which inflammation develops in response to these allergens has not been well characterised[249]. In addition, repeated, long-term challenges with the allergens are often required to induce allergic lung inflammation, which involves repeated use of general anaesthesia, known to impact on the vagus nerve[252], induce pro-inflammatory cytokine expression[253] and increase the permeability of the BBB[254] (Appendix 9.6).

As a result, a well-established rodent model of allergic inflammation was used, based on the formation of OVA-immune complexes in the lung. Animals are immunised against OVA in conjunction with $Al(OH)_3$, which encourages the production of IgE, and skews to a TH2 type inflammatory response[249]. Following immunisation, a subsequent challenge with OVA in the airways (through intranasal, intratracheal or aerosol challenge) results in the formation of OVA immune complexes and promotes the development of a type III hypersensitivity reaction. The OVA-immune complex model is well-known to induce high circulating titers of IgE, TH2 cytokine expression, eosinophilia and AHR[249].

Acute Allergic Inflammation – Protocol A

To investigate the primary effect of allergic inflammation on the brain, an acute OVA-immune complex model was utilised, developed at GSK. At the University of Southampton, animals were challenged with OVA intranasally (i.n.), under isoflurane anaesthesia as the equipment necessary for OVA aerosolisation was not available. BALB/C mice were first immunised by an i.p. injection of 10µg OVA (Sigma, Poole, UK) and 2mg $Al(OH)_3$ (50µl Imject Alum, Pierce, Illinois, USA) in 0.2ml sterile saline on days 0 and 14. Control mice were challenged i.p. with sterile saline on days 0 and 14. All mice were then challenged i.n. with 50µg OVA in 50µl sterile saline on days 28, 29 and 30 under 3.5% isoflurane anaesthesia (IsofluraneVet, Merial Animal Health, Harlow, UK). (Figure 2.1)

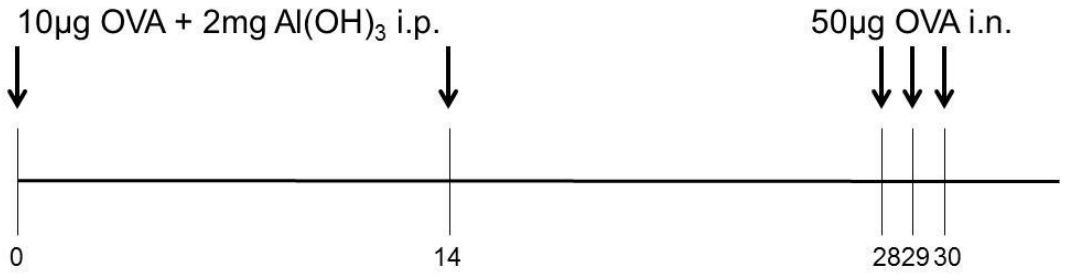


Figure 2.1 Challenge protocol A for the induction of acute allergic lung inflammation

Acute Allergic Inflammation – Protocol B

At GSK, animals were challenged with aerosolised OVA, to avoid the use of isoflurane, as per the following protocol. BALB/C mice were first immunised by an i.p. injection of 10µg OVA and 2mg $Al(OH)_3$ (Sigma, Poole, UK) in sterile Dulbecco's Phosphate Buffered Saline (DPBS, Invitrogen, UK) on days 0 and 14. Mice were subsequently challenged with aerosolised OVA (1% in DPBS) for 20 minutes a day, on days 21, 22 and 23, using a PulmoStar Nebuliser (DeVillbiss Healthcare, Pennsylvania, USA). Control mice were challenged with aerosolised sterile DPBS (Figure 2.2).

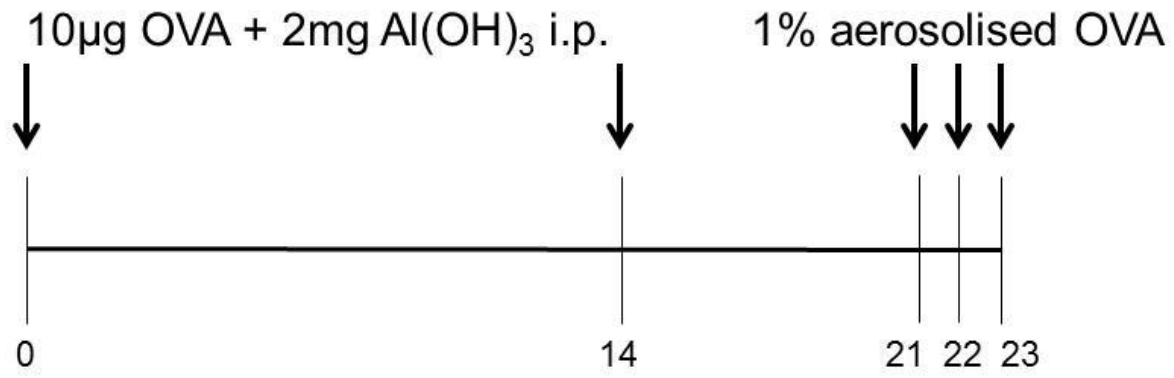


Figure 2.2 Challenge protocol B for the induction of acute allergic lung inflammation

Chronic Allergic Inflammation

To induce more chronic forms of allergic lung inflammation, OVA-immunised animals were challenged with further doses of aerosolised OVA, as per the following protocol. BALB/C mice were first immunised by an i.p. injection of 10µg OVA and 2mg $Al(OH)_3$ in sterile DPBS. Mice were subsequently challenged with aerosolised OVA (1% in DPBS) for 20 minutes a day on days 21-25, were not challenged for two days, and were

subsequently challenged on days 28-30 (sub-chronic, Figure 2.3A) using a PulmoStar Nebuliser. Alternatively, animals were challenged for 20 minutes a day on days 21-25, not challenged for two days, subsequently challenged on days 28-32, not challenged for two days and finally challenged on days 35-37 (chronic, Figure 2.3B) using a PulmoStar Nebuliser. Control mice were challenged with aerosolised sterile DPBS.

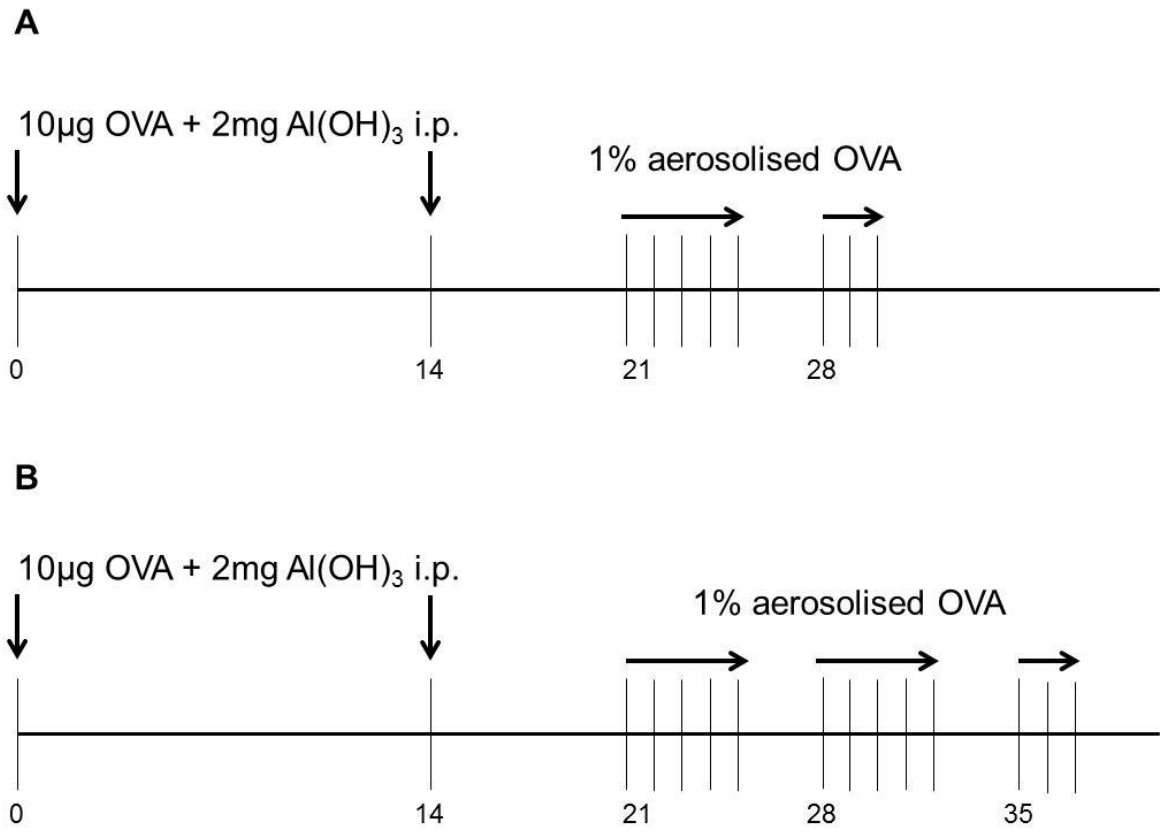


Figure 2.3 Challenge protocol for the induction of sub-chronic (A) and chronic (B) allergic lung inflammation

Severe allergic lung inflammation

To initially investigate the effect of allergic inflammation on gene expression in the brain, a pilot experiment was carried out, which involved the development of a severe model of allergic lung inflammation. BALB/C mice were first immunised by an i.p. injection of 50µg OVA in the presence of Alum (1:1 ratio, Imject Alum). Mice were subsequently boosted 3 times with 100µg OVA i.p. (weeks 5, 7 and 26) and once with 200µg OVA i.p. (week 30). In week 31, mice were challenged with 50µg OVA i.n. in 50µl sterile saline under 3.5% isoflurane anaesthesia on two consecutive days.

2.1.3.3 Immune stimulation with resiquimod

C57BL/6 or BALB/C mice were challenged i.p. or i.n. with resiquimod (R-848, Santa-Cruz Biotechnology, Texas, USA) at 5mg/kg in 0.2% Tween-80 (Sigma, Poole, UK) in sterile saline (0.9%) or DPBS under 3.5% isoflurane anaesthesia. The dose was chosen based on previous dose-response studies carried out in-house at GSK.

2.1.3.4 Immunomodulation of sub-chronic allergic lung inflammation with resiquimod

The protocol for immunomodulation was based on previous experiments carried out in-house at GSK. BALB/C mice were immunised by an i.p. injection of 50µg OVA and 10mg $Al(OH)_3$ in sterile DPBS. Mice were subsequently challenged with aerosolised OVA (1% in DPBS) for 20 minutes a day on days 21-25 and 28-29. On day 30, mice were challenged i.p. or i.n. with resiquimod at 5mg/kg in 0.2% Tween-80 in DPBS under 3.5% isoflurane anaesthesia. 30 minutes after administration of resiquimod, animals were challenged with aerosolised OVA (1% in DPBS) for 20 minutes. Behavioural analyses or terminal sample collection was carried out 2.5 hours after dosing of resiquimod as prior time-course experiments (Appendix 9.3) had shown this time point to be the peak of behavioural changes and circulating pro-inflammatory cytokine production.

2.1.3.5 Group size

Group size for individual studies was determined based on previous experiments and power analysis. At the University of Southampton, studies for measurement of circulating or central inflammatory mediators used group sizes of 3-5 animals, as per previous published literature from this lab[61,70], whereas group sizes of 5-10 animals were necessary for behavioural studies, due to high variability in animal behaviour. At GlaxoSmithKline, previous power calculations determined that studies for measurement of circulating or central inflammatory mediators required a group size of at least 6 animals per group, whereas behavioural studies needed group sizes of at least 8 animals per group. Group size for individual studies can be found in figure legends in respective results chapters.

2.1.4 Behaviours

2.1.4.1 Burrowing

Burrowing is a species-specific behaviour dependent on the integrity of the hippocampus and is thought to be a measure of both cognitive decline and anhedonia[255]. Previous studies have shown that mice injected with LPS i.p. fail to burrow over a space of two hours[61]. To perform the assay, a tube (20cm long, 6.8cm in diameter and sealed at one end) was filled with 200g of food pellets and placed in individual plastic cages with sawdust bedding. The tube was supported off the floor with a wooden block or metallic screws to prevent spillage of food pellets (Figure 2.4). Mice were individually placed in the cages for a period of two hours or overnight (16 hours), with access to water. The amount of pellets left in the tube (in grams) was measured after the specified time period and the amount displaced calculated. As the outcome of burrowing behaviour was determined through objective calculation of displaced pellets, without a subjective component, tests were not blinded. Mice were trained prior to experimentation, by one session of group overnight burrowing, one session of individual overnight burrowing and three to four sessions of individual 2-hour burrowing. The mice were acclimatised to behavioural testing room 30 minutes prior to testing. A baseline reading was performed prior to experimentation.

Figure 2.4– Burrowing behaviour. A mouse is placed in a plastic cage with sawdust bedding, containing a raised plastic tube filled with 200g of food pellets. The mouse is left in this cage for two hours or overnight and the amount of pellets left in the tube is measured after the specific time point.

2.1.4.2 Open Field

The open field assay is the most standardized measure of spontaneous activity in the rodent, giving an indication of general motor function, lethargy and anxiety behaviours[256]. At Southampton University, open field activity was analysed using a Med Associates Activity Monitor (Med Associates Inc., Vermont, USA). Mice were placed in a square aluminium container (27cm by 27cm) enclosed on four sides with 0.7 cm thick acrylic sheet surrounded by an opaque screen. Their activity, which includes total distance travelled and rears was recorded for 3 minutes each, using an automated laser-based system. As a consequence, these studies were not blinded. Mice were trained prior to experimentation, until a stable behavioural baseline reading was acquired. Mice were additionally acclimatised to the behavioural testing room at least 30 minutes prior to testing.

At GSK, open field activity was analysed using an open-field box fashioned from a rectangular, opaque, flat-bottomed plastic 30cm x 36cm box. Lines were drawn within the

box, to form rectangles of 9cm by 10cm (Figure 2.5). A mouse was placed in the centre of the box and "box entries" and rears were recorded over a period of 3 minutes. A "box entry" is a movement of the mouse into a rectangle, where both front paws and the head have entered the rectangle. A rear is a movement by which the mouse stands on its hind legs and has both front paws off the floor. Mice were trained on one occasion prior to experimentation, a baseline reading was taken the day before experimentation and the mouse was acclimatised to the behavioural testing room 30 minutes prior to testing. Due to the subjective nature of this test, the investigator was blinded.

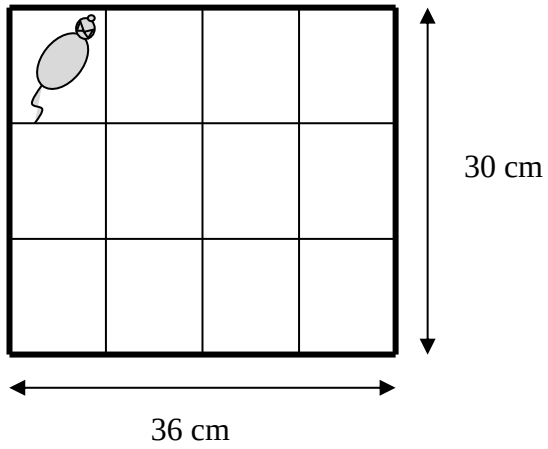


Figure 2.5 – Open field box. An open field box was fashioned from a rectangular, opaque, flat-bottomed plastic 30cm x 36cm box, where lines were drawn within the box to form rectangles of 9cm by 10cm. A mouse was placed in the centre of the box and entries into individual rectangles ("box entries") as well as rears were recorded over a period of 3 minutes.

At GSK, open field activity was also analysed using the Linton AM524 Single Layer X Y IR Activity Monitor (Linton Instrumentation, Norfolk, UK), in association with the Laboratory Animal Science (LAS) team. Mice were placed in a 30cm by 20cm container and their activity, including total movement and rears, was recorded for 5 minutes each, using an automated laser-based system. As a consequence, these studies were not blinded. Mice were trained on one occasion prior to experimentation.

2.1.4.3 Elevated Plus Maze

The elevated plus maze (EPM) is a well-accepted behavioural test for anxiety in rodents[256]. The EPM was built with Perspex (Poly(methyl methacrylate)) at dimensions of 50cm

length x 10cm depth by 40cm height for arms on an elevated MDF (medium density fibreboard) platform 50cm above the ground, as described previously[256]. Mice were placed in the centre of the EPM and the amount of time spent in the open arms over a 5-minute period was measured using a timer (Figure 2.6). Initial experiments involved training of the mice on two occasions prior to experimentation. Due to the rapid adaptation of the mice to the maze, later experiments were performed without training. Mice were acclimatised to behavioural testing room at least 30 min prior to testing. Due to the subjectivity of this test, the investigator was blinded.

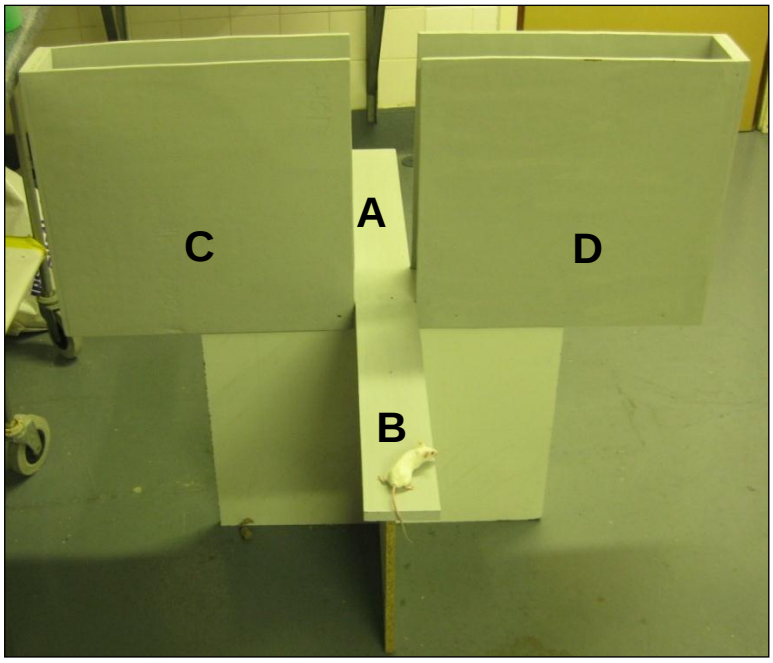


Figure 2.6 – Elevated Plus Maze. The elevated plus maze is a well-accepted behavioural test for anxiety in rodents. The mouse is placed in the centre of the maze and the time spent in the open arms (A and B) is measured over a 5 minute period. A control mouse spends equal amounts of time in the open and closed (C and D) arms, while an anxious mouse spends less time in the open arms.

2.1.4.4 Glucose Consumption

Glucose consumption is a behavioural assay believed to be a measure of anhedonia. To perform this assay, mice were housed overnight in individual cages provided with food and a pre-weighed bottle of 7.5% glucose (AnalaR Normapur, VWR International Ltd, Leicestershire, UK) solution. The glucose solution is weighed the next morning to determine the amount consumed overnight. A baseline reading is taken prior to experimentation. As the experiment was based on an objective weight calculation, the investigator was not blinded.

2.1.5 Tissue Harvesting

2.1.5.1 Tissue harvesting for immunohistochemistry

Mice were terminally anaesthetised with an i.p. injection of 150µl sodium pentobarbital (Pentoject, Animal Care, York, UK) and transcardially perfused with heparinised saline (0.1% heparin sodium (5000 units/ml, CP Pharmaceuticals, Wrexham, UK) in 0.9% saline). Brains and spleens were collected and embedded in Optimum Cutting Temperature (OCT, Sakura Finetek, Thatcham, UK), frozen in isopentane on dry ice and stored at -20°C. Lungs were inflated in a 1:1 solution of OCT and 0.9% saline, carefully excised and embedded in OCT, frozen in isopentane on dry ice and stored at -20°C. 10µm sections were cut, collected on 3-aminopropyltriethoxysilane (APES)-coated slides and stored at -20°C.

For free-floating immunohistochemistry, animals were perfused with heparinised saline followed by 4% paraformaldehyde (PFA, TAAB Laboratories, Berkshire, UK). Brains were collected and placed in 4% PFA overnight and subsequently transferred to a 30% sucrose (Fisher Scientific, Loughborough, UK) solution. Once brains had sunk, they were embedded in OCT, frozen in isopentane on dry ice and stored at -20°C. 40µm sections were cut on a cryostat, collected in cryoprotectant (see Appendix 9.1.1) and stored at -20°C.

2.1.5.2 Tissue harvesting for protein and qPCR analysis

Mice were terminally anaesthetised with an i.p. injection of 150µl of sodium pentobarbital and transcardially perfused with heparinised saline. Tissues (brain, spleen, and lung) were extracted and sectioned. Brains were cut in half and a thick coronal section (2mm, approximate coordinates: bregma-1mm – bregma-3 mm) enriching for the hippocampus was taken, as was the brainstem. Tissues were snap frozen in liquid nitrogen and stored at -80°C until further use.

2.1.5.3 Serum collection for analysis of inflammatory mediators

Mice were terminally anaesthetised with an i.p. injection of 150µl of sodium pentobarbital. At Southampton University, mice were exsanguinated by a puncture to the right atrium and

blood was collected in an eppendorf. Samples were centrifuged at 3000 rpm for 5 minutes. Serum supernatant was collected and stored at -20°C until further use. At GSK, mice were exsanguinated by an incision to the jugular vein and blood was collected in BD Microtainer® SST™ Tubes (BD, Oxford, UK), centrifuged at 8000g for 90s and stored at -20°C. These tubes contain a gel which separates serum from red blood cells, when centrifuged.

2.1.5.4 Bronchoalveolar lavage

Mice were terminally anaesthetised with an i.p. injection of 150µl sodium pentobarbital and exsanguinated via an incision to the jugular vein. An incision was made in the skin of the neck to insert a 22G Angiocath (BD, Maryland, USA) into the trachea. Using a syringe, 1ml of lavage buffer (see Appendix 9.1.2) was inserted into the lungs and then carefully extracted. Lavage fluid was kept on ice, until further use.

2.1.5.2 Peritoneal lavage

Mice were terminally anaesthetised with an i.p. injection of 150µl sodium pentobarbital and exsanguinated via an incision in the jugular vein. An incision was made in the skin of the lower right side of the abdomen and a 22G Angiocath was inserted into the peritoneal cavity. Using a syringe, 4ml of lavage buffer (see Appendix 9.1.2) was inserted into the peritoneal cavity, then carefully extracted. Lavage fluid was kept on ice, until further use.

2.2 Ex-vivo methods

2.2.1 Immunohistochemistry

2.2.1.1 Haematoxylin and eosin stain

Slides were dried for 30 min at 37°C and fixed in absolute alcohol (Fisher Scientific, Loughborough, UK) for 10 minutes. Slides were placed in haematoxylin for 10 seconds, rinsed with tap water, placed in acid alcohol for 12 seconds, rinsed with tap water, placed in eosin for 30 seconds, rinsed with tap water, dehydrated and coverslipped.

2.2.1.2 Free-floating immunohistochemistry for c-fos

C-fos is an intermediate early gene (IEG), which is acutely upregulated upon increased metabolic activity of a cell. C-fos immunohistochemistry is often used as a marker of neuronal activity.

To stain for c-fos immunoreactivity, cyroprotected sections were defrosted and washed in phosphate-buffered saline (PBS) followed by immunobuffer (see Appendix 9.1.3). They were initially quenched for endogenous peroxidase activity in 1% H_2O_2 (Sigma, Poole, UK) for 10 minutes. To block aspecific binding, sections were incubated for 1 hour in Normal Goat Serum (3%, Vector Labs, Peterborough, UK), followed by a 36 hour incubation in rabbit anti-mouse c-fos polyclonal antibody (Ab-5, Calbiochem, Merck, Middlesex, UK) at 1:40,000. Sections were then incubated in goat anti-rabbit secondary antibody (BA-4001, Vector Labs, Peterborough, UK) for 2 hours followed by a 2 hour incubation in Avidin-Biotin Complex (Vector Labs, Peterborough,UK) C-fos staining was detected using diaminobenzidine (DAB, Sigma, Poole, UK) as the chromogen, catalysed by 0.015% H_2O_2 and enhanced with nickel (0.005% di-ammonium nickel sulphate 6-hydrate, BDH GPR, VWR International Ltd, Leicestershire, UK). The sections were mounted onto gelatinised glass slides (see Appendix 9.2.1 for gelatinising protocol), dehydrated and coverslipped.

2.2.1.3 Immunohistochemistry for inflammatory markers

Immunohistochemistry was also carried out to detect inflammatory markers in the brain, spleen and lung tissue. CD68 is expressed on all macrophages and was therefore used as a generic inflammatory marker to detect all macrophage populations. Activated macrophages were detected by upregulation of CD11b and MHC Class II. Upregulation of FcγR1 was used to detect polarisation of M1 macrophages, whereas YM1 (expressed specifically on M2 macrophages) and Dectin-1 (expressed on M2 macrophages, but also other cell types) were used to specifically detect M2 macrophages[257].

Sections were dried and post fixed in absolute alcohol, and quenched for endogenous peroxidise activity in 1% H_2O_2. Sections were incubated in 2% Bovine Serum Albumin

(BSA, Fisher Scientific, Loughborough, UK) and 1% serum for one hour to block aspecific binding and consequently incubated in primary antibody (see Table 2.1) overnight. Sections were then incubated with secondary antibody (see Table 2.2) for 1 hour. For DAB immunohistochemistry, sections were incubated for 1 hour in Avidin Biotin Complex and staining was detected using DAB as the chromogen, catalysed by 0.015% H_20_2. Sections were counterstained with haematoxylin, dehydrated and coverslipped. For fluorescent immunohistochemistry, sections were incubated for 10 minutes in 1μg/ml (1:1000) Hoescht 33258 (Sigma, Poole, UK), which stains nuclei, and subsequently coverslipped.

Table 2.1

Primary antibodies used in immunohistochemical staining

Primary Antibody	Antibody Details	Catalogue Number	Distributor	Optimal concentration
CD11b (5c6)	Rat mAb	MCA711	AbD Serotec, UK	2µg/ml (1:500)
CD68	Rat mAb	MCA1957	AbD Serotec, UK	2µg/ml (1:500)
MHC Class II	Rat mAb	14-5321	eBioscience, UK	1µg/ml (1:500)
Dectin-1	Rat mAb	MAB17561	R&D Systems, UK	1µg/ml (1:500)
YM-1	Rat mAb	MAB2446	R&D Systems, UK	2µg/ml (1:500)
FcγR1 (AT152-9)	Rat mAb	-	Made in house	2µg/ml (1:500)
NMDAR1	Rabbit pAb	ab17345	Abcam, UK	1µg/ml (1:1000)
Mouse IgG	Sheep $F(ab')_2$ fragment-FITC	F2266	Sigma-Aldrich, UK	2µg/ml (1:500)
Collagen IV	Rabbit pAb	Ab6585	Abcam, UK	2µg/ml (1:500)
OVA	Rabbit pAb	W59413R	Biodesign International, USA	1µg/ml (1:1000)

Table 2.2

Secondary antibodies used in DAB and fluorescent immunohistochemical staining.

Secondary Antibody	Antibody Details	Catalogue Number	Distributor
Rabbit anti-rat IgG	Biotinylated	BA-4001	Vector Laboratories, UK
Goat anti-rabbit IgG	Biotinylated	BA-1000	Vector Laboratories, UK
Goat anti-rat IgG	AlexaFluor®546	A11081	Invitrogen, UK
Donkey anti-rat IgG	AlexaFluor®488	A21208	Invitrogen, UK
Donkey anti-rabbit IgG	AlexaFluor®488	A21206	Invitrogen, UK
Goat anti-rabbit IgG	AlexFluor®568	A11011	Invitrogen, UK

2.2.1.4 Immunohistochemical Analysis

Immunohistochemical analysis was performed using ImageJ (Image Processing and Analysis in Java) software. A picture was captured using a Leica DM5000 Microscope, at a specified magnification and light setting. Using ImageJ, images of c-fos immunhistochemical stains were initially thresholded to a value which was constant for pictures of the same area in all treatment groups. The area of interest was selected and a particle count was performed, based on size and circularity of the particles. The specific size and circularity assigned to c-fos stained nuclei was preliminarily adjusted by finding an average size and circularity of nuclei for all stains. Images of immunhistochemical stains of microglial phenotype were initially deconvoluted to separate the DAB and haemotoxylin channels. The DAB channel was thresholded, to a constant value for all pictures, and the total area of staining measured.

2.2.2 Serum, BAL and Tissue Immune Mediator Measurements

2.2.2.1 Tissue homogenisation

Brains and spleens were homogenised in lysis buffer (see Appendix 9.1.4) to form a 10% w/v solution. Lungs were homogenised in Hank's Based Salt Solution (HBSS, Invitrogen, Paisley, UK) to form a final concentration of 50mg/ml. All homogenisation was performed on ice.

2.2.2.2 Protein measurement

To analyse for protein content in tissue homogenates, the homogenates were first centrifuged (14,000 rpm at 4°C for 30 minutes). The supernatant was assayed using a BCA^{TM} Protein assay (Pierce, Illinois, USA) as per the manufacturer's instructions.

2.2.2.3 Cytokine measurements

Cytokines in tissue, serum or lavage fluid were measured using an MSD TH1/TH2 multiplex kit (IFN-γ, IL-1β, IL-2, IL-4, IL-5, KC/GRO/CINC, IL-10, IL-12 total, TNF-α),

a TNF-α singleplex kit or a Cytokine Panel 8 4-plex kit (IL-6, IFN-γ, KC, GM-CSF) for mouse cytokines (Mesoscale Discovery (MSD), Maryland, USA), according to the manufacturer's instructions. Cytokine measurements from tissue samples were compared against total protein content to provide a cytokine value per mg protein.

IFN-α in serum and bronchoalveolar lavage fluid (BALF) was measured using an eBioscience Mouse IFN-α Platinum enzyme-linked immunosorbent assay (ELISA) (eBioscience, Hatfield, United Kingdom), according to the manufacturer's instructions. Briefly, 50µl of Assay Buffer, 50µl of standard or sample and 50µl of Biotin Conjugate was added to each well of a 96-well plate, and incubated for 2 hours at room temperature (RT) on a plate shaker (600rpm). The plate was washed and incubated with 100µl Streptavidin-HRP for 1 hour at RT on a plate shaker (600rpm). The plate was washed and incubated with 100µl of 3,3',5,5'-Tetramethylbenzidine (TMB) Substrate Solution at RT in the dark for 30 minutes. 100µl of Stop Solution was added and the plate was read at 450nm on a plate reader. Sample concentration of IFN-α was determined by comparing against a 5-parameter standard curve.

IL-6 in serum was measured using a DuoSet® ELISA Development System (R&D Systems, Abingdon, UK), according to the manufacturer's instructions, with minor modifications. Briefly, a 96-well MaxiSorp™ (Nunc, Fisher Scientific, Loughborough, UK) plate was coated with capture antibody (2µg/ml) overnight at room temperature. Plates were subsequently blocked for aspecific binding with 1% BSA for 1 hour and incubated with supplied standard and serially diluted sample for 2 hours. Thereafter, plates were incubated with the provided detection antibody (400ng/ml) for 2 hours, followed by Poly-HRP (100pg/ml, Sanquin, Amsterdam, The Netherlands) for 20 minutes and substrate solution (see Appendix 9.1.5) for 5 minutes. The reaction was stopped with 0.1M H_2SO_4. To determine the optical density of the wells, the plate was read at 450nm. Concentrations were determined by interpolating optical density measurements from a 5-parameter standard curve, using GraphPad Prism Software.

2.2.2.4 Prostaglandin E metabolite measurement

Serum levels of prostaglandin E metabolites were measured according to the manufacturer's instructions (Cayman, Cambridge, UK). Briefly, serum samples (and

standards) were initially derivatized by addition of carbonate buffer followed by overnight incubation at 37°C. Samples were diluted (1:10) and incubated with PGEM AChE tracer and PGEM AChE antiserum for 20 hours at room temperature. Development of samples was achieved using Ellman's reagent. Samples were analysed as per the manufacturer's instructions.

2.2.3 qPCR Analysis

2.2.3.1 RNA isolation

RNA isolation was carried out using two different protocols and the yield and quality of RNA was compared, to determine the optimum protocol for subsequent quantitative PCR analysis.

Protocol A:

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Briefly, tissue was homogenised in 700µl β-mercaptoethanol (Sigma, Poole, UK) diluted 1:100 in RLT Buffer. The homogenate was transferred to a QIAshredder column (Qiagen, Crawley, UK) and spun at 13,000 rpm for 6 minutes. The flow-through was diluted 1:1 in 70% RNAse-free ethanol, transferred to an RNeasy column and spun at 13,000 rpm for 15s. The column was washed by adding 350µl RW1 Buffer and centrifuged at 13,000 rpm for 15s. DNA was digested using an on-column RNase-free DNase kit (Qiagen, Crawley, UK), according to the manufacturer's instructions. The column was subsequently washed with 350µl RW1 Buffer, followed by 500µl RPE Buffer. This was followed by the addition of 500µl RPE Buffer, which was centrifuged through the column at 13,000 rpm for 2 minutes. RNA was eluted by the addition of 30µl RNAse-free dH_2O, which was centrifuged through the column 13,000 rpm for 1 minute. Eluent was collected and stored at -80°C.

Protocol B:

Total RNA was extracted using the RNeasy Microarray Tissue Mini Kit (Qiagen, Crawley, UK), according to the manufacturer's instructions. Briefly, tissue was homogenised in 1ml QIAzol™ Lysis Reagent and incubated at room temperature for 5 minutes. 200µl chloroform (Fisher Scientific, Loughborough, UK) was added, vigorously mixed and

incubated at room temperature for 3 minutes. Homogenate was subsequently centrifuged at 12,000g for 15 minutes at 4°C. The top aqueous layer was extracted and diluted 1:1 in 70% RNase-free ethanol. This was transferred to an RNeasy column and centrifuged at 13,000 rpm for 15s. The column was washed adding 700µl RW1 Buffer and centrifuged at 13,000 rpm for 15s, followed by the addition of 500µl RPE Buffer and centrifuged at 13,000 rpm for 15s. This was followed by the addition of 500µl RPE Buffer, which was centrifuged through the column at 13,000 rpm for 2 minutes. RNA was eluted by the addition of 30µl RNAse-free dH_2O, which was centrifuged through the column 13,000 rpm for 1 minute. Eluent was collected and stored at -80°C.

2.2.3.2 Analysis of RNA quality

RNA quality was determined by analysing 1µl of the RNA sample on a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA). The NanoDrop® ND-1000 Spectrophotometer measures UV-visible absorbance (220nm-750nm) to determine the concentration, as well as the purity of RNA, by displaying the ratio of readings at 260nm and 280nm (A_{260}/A_{280}) and 260 and 230nm (A_{260}/A_{230}). Pure RNA has an A_{260}/A_{280} and A_{260}/A_{230} ratio between 1.8 and 2.1. An A_{260}/A_{280}value below 1.8 suggests protein contamination in the sample, whereas an A_{260}/A_{230} value below 1.8 suggests chaotropic salt or phenol contamination.

2.2.3.3 cDNA synthesis

Four hundred nanograms of RNA were converted to cDNA as per the standard protocol, using reagents from Applied Biosystems (Warrington, U.K.), unless otherwise stated. The appropriate amount of RNA was added to molecular biology grade water (Sigma, Poole, UK) to make up a volume of 7.7µl. This was added to 2µl of 10x RT Buffer, 4.4µl of 25mM $MgCl_2$, 4µl dNTPs Mix, 1µl random hexamers, 0.4µl RNAse inhibitor and 0.5µl Multiscribe RT. The mixture were incubated at 25ºC for 10 minutes, 48ºC for 30 minutes, 95°C for 5 minutes and cooled to 4°C. The resulting cDNA was stored at -20ºC until further use.

2.2.3.4 cDNA synthesis for use in RT² Profiler™ PCR Array and RT² qPCR Primer Assay

400ng of RNA was converted into cDNA for RT^2 Profiler™ PCR and qPCR Primer Array using the RT^2 First Strand Kit (SABiosciences, Qiagen, Crawley, UK) according to the manufacturer's instructions. Briefly, genomic DNA was eliminated by combining the 2µl Buffer GE, 400 nanograms of RNA and the appropriate amount of RNase-free dH_2O to a total volume of 10µl and heating to 42°C for 5 minutes. 10µl of RT cocktail (4µl BC3, 1µl P2, 2µl RE3, 3µl RNase-free dH_2O) was added to each sample and heated to 42°C for 15 minutes, followed by 95°C for 5 minutes to stop the reaction. 91µl of RNase-free dH_2O was added to each sample to make a total volume of 111µl. The cDNA was stored at -20°C until further use.

2.2.3.5 Primer design

Primers (Table 2.4) were designed using Primer3 software[258], using the parameters shown in Table 2.3. If possible, primers that crossed an exon-exon boundary were chosen, to avoid amplification of DNA. Returns were blasted for specificity using NCBI Primer Blast[259] and specific primers were synthesised and ordered from Sigma-Aldrich Custom DNA Oligos (Poole, UK). Specificity of primers was assessed through analysis of melting curves of a qPCR run with positive control cDNA (see Table 2.4), as well as by running the qPCR product on a standard DNA gel. To run the gel, a 1.8% agarose (Fisher Scientific, Loughborough, UK) gel containing 0.003% ethidium bromide (Fisher Scientific, UK) was made. A 2:5 mixture of loading buffer (0.25% bromophenol blue, 30% glycerol) and PCR product was added to the gel and run alongside a 100bp DNA ladder (GeneRule, Fermentas, Fisher Scientific, Loughborough, UK) at a voltage of 85mV. A primer was deemed specific if a single melting curve on the qPCR and a single band of the right size on the DNA gel was observed.

Table 2.3

Parameters of qPCR primer characteristics used when designing primers

	Minimum	Optimum	Maximum
Primer Size	10	20	30
Primer Tm	55	60	65
Primer GC%	45	50	55
Product Size	100	200	300
Self-Complementarity	1.00	2.00	3.00
3' Self - Complementarity	1.00	2.00	3.00

2.2.3.6 Quantitative PCR

Five microliters of the diluted cDNA (1:5 in molecular-grade dH_2O) generated was used for the quantitative PCR (qPCR) reaction. Per reaction, 0.06µl of each primer (100µM stock concentration) was added to 10µl of iTaq™ Universal SYBR Green Supermix (Bio-Rad Laboratories, Hertfordshire, UK), 5µl of diluted cDNA and 4.88 µl of molecular grade dH_2O (Invitrogen, Paisley, UK). The housekeeping genes glyceraldehyde-3-phosphate deyhydrogenase (GAPDH) or hypoxanthine-guanine phosphoribosyltransferase (HPRT) were measured in each sample. Analysis of C(t) values was carried out using comparative C(t) or $2^{\wedge-\Delta\Delta C(t)}$ method. This method of relative gene expression compares expression of a particular gene of interest to the expression of housekeeping gene across treated and control samples. A standard curve was constructed from cDNA from positive control samples (see Table 2.4), as a measurement of efficiency of the qPCR reaction. Serial one in five dilutions of the cDNA synthesised were made and a curve was plotted of the C(t) value versus the log of the concentration. Efficiency was calculated using the following formula:

$$\% \, Efficiency = (10^{-1}_{slope} - 1) * 100$$

Table 2.4

Mouse house-keeping gene and gene of interest primer sequences used for qPCR

Gene	Oligonucleotide	Sequence	Size (bp)	Positive Control
GAPDH	Forward Primer	5'TCCACCACCCTGTTGCTGTA-3'	**307**	Unstimulated Raw264.7 Cells
	Reverse Primer	5'TGAACGGGAAGCTCACTGG-3'		
HPRT	Forward Primer	5'TGGGCTTAACTGCTTT-3'	124	Unstimulated Raw264.7 Cells
	Reverse Primer	5'CTAATCACGACGCTGGGACT-3'		
IL-1β	Forward Primer	5'TGTGTTTTCCTCCTTGCCTC-3'	103	LPS-stimulated (4h) Raw264.7 Cells
	Reverse Primer	5'CTGCCTAATGTCCCCTTGAA-3'		
TNF	Forward Primer	5'CGAGGACAGCAAGGGACTAG-3'	275	LPS-stimulated (4h) Raw264.7 Cells
	Reverse Primer	5'GCCACAAGCAGGAATGAGAA-3'		
IL-6	Forward Primer	5′-TCCAGAAACCGCTATGAAGTTC-3′	72	LPS-stimulated (4h) Raw264.7 Cells
	Reverse Primer	5′-CACCAGCATCAGTCCCAAGA-3		
COX-2	Forward Primer	5'GGGTGTCCCTTCACTTCTTTCA-3'	77	LPS-stimulated (4h) Raw264.7 Cells
	Reverse Primer	5'TGGGAGGCACTTGCATTGA-3'		
TGFβ	Forward Primer	5'ATTCCTGGCGTTACCTTGG-3'	120	Allergic inflammation

				lung
	Reverse Primer	5'AGCCCTGTATTCCGTCTCCT-3'		
IL-4	Forward Primer	5'CCTCACAGCAACGAAGAACA-3'	153	Allergic inflammation lung
	Reverse Primer	5'CGAAAAGCCCGAAAGAGTC-3'		
GABARB	Forward Primer	5'TCAACACTGGATGACCTGGA-3'	137	Naive mouse hippocampus
	Reverse Primer	5'AAAAGTCCCACGATGATTCG-3'		
BDNF	Forward Primer	5'TTCACAGGAGACATCAGCAA-3'	200	Naive mouse hippocampus
	Reverse Primer	5'CCAACAAGAGACCACAGCAA-3'		

Primers were designed as described above and acquired from Sigma (Poole, UK). A positive control sample for testing of the primer is also shown.

2.2.3.7 RT² Profiler™ PCR Array

For simultaneous analysis of a multitude of genes, catalogued RT^2 Profiler™ PCR Arrays (SABiosciences, Qiagen, Crawley, UK) were used. Catalogued PCR Arrays profiled 84 target genes and 5 house-keeping genes (gusb, hprt, hsp90ab1, gapdh, actb) on a 384-well plate, allowing space for analysis of four samples (Figure 2.7). The protocol provided by the manufacturer was used. Briefly, 102µl of the cDNA synthesis reaction was added to 550µl of 2xRT^2 SYBR Green Mastermix (SABiosciences, Qiagen, Crawley, UK) and 448µl RNase-free water. Each sample was dispensed into appropriate wells, and the plate was centrifuged for 1 minute at 1000g at room temperature. Real-time PCR was carried out using an Applied Biosystems 7900HT real-time cycler. Analysis of C(t) values was carried out using comparative C(t) or $2^{\wedge -\Delta\Delta C(t)}$ method.

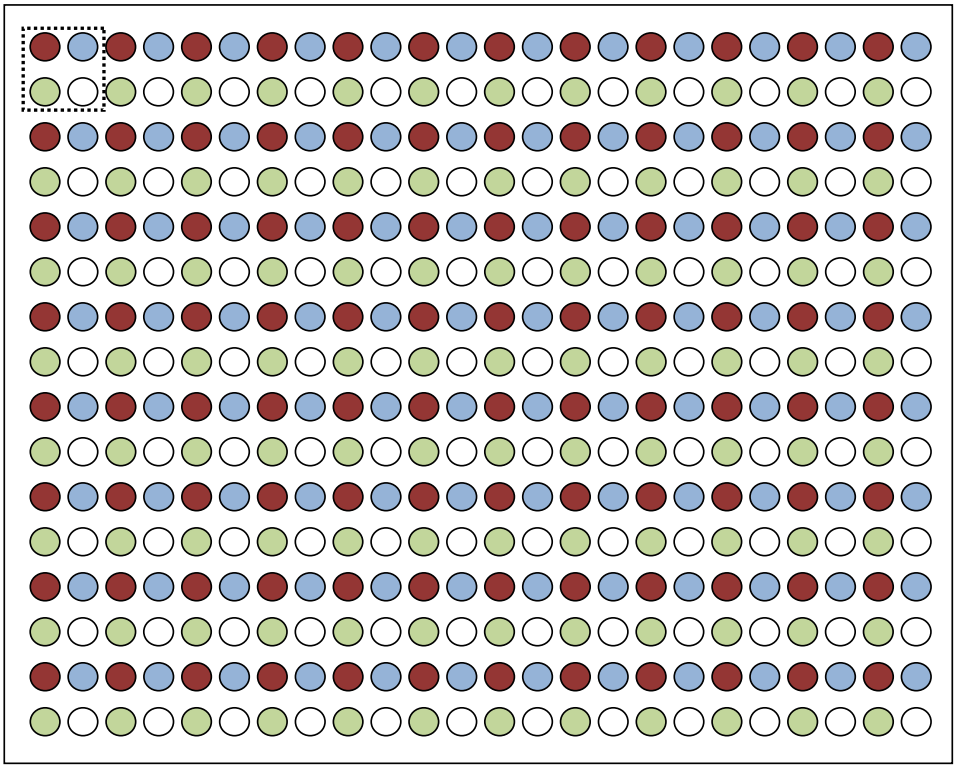


Figure 2.7 – Schematic of 384-well plate used for catalogued PCR Array. Each coloured well represents a different sample. The dashed-line box represents an area of analysis for one gene.

2.2.3.8 RT² qPCR Primer Assay

QPCR was carried out using reagents purchased from SABiosciences, unless otherwise specified. A mastermix was created using 12.5µl RT^2 SYBR Green Mastermix, 1µl RT^2 qPCR primer assay for the specific primer (Table 2.5) and 10.5µl RNase-free water for each sample. 1µl of cDNA synthesis reaction of each sample was added to the mastermix to make up a total volume of 25µl. A standard curve was created for each primer using cDNA created from XpressRef^{TM} Universal Total RNA (SABisociences, Crawley, UK). Two house-keeping genes (HPRT1 and HSP90AB1) were used as an internal comparison. Analysis of C(t) values was carried out using comparative C(t) or $2^{\wedge -\Delta\Delta C(t)}$ method.

Table 2.5

Mouse house-keeping gene and gene of interest primer sequences used for RT^2 qPCR Primer Assay

Gene	Band Size	Refseq Accession #	Reference Position
HPRT	83	NM_013556	902
HSP90AB1	105	NM_008302	2362
IL-1β	156	NM_008361	1080
TNF	93	NM_013693	879
IDO	173	NM_008324	986
GRIN1	91	NM_008169	2791
GRIN2D	163	NM_008172	2691
TAC1	102	NM_009311	900
TACR1	155	NM_009313	4846

Primers were designed by SABiosciences (Qiagen, Crawley, UK) where they have been experimentally validated for specificity and uniform efficiency[260]

2.2.4 Cells

2.2.4.1. Bronchoalveolar and peritoneal lavage fluid total cell counts

Peritoneal lavage fluid (PLF) and bronchoalveolar lavage fluid (BALF) was centrifuged (1200 rcf at RT for 5 minutes) and the pellet re-suspended in 1ml lavage buffer. PLF and BALF were mixed 1:1 with Trypan Blue (Sigma, Poole, UK) and the mixture was placed in a haemocytometer (Neubauer Chamber). The mean amount of live cells in five 4x4 square areas were counted. This value was multiplied by 20000 to represent the number of cells in 1ml.

2.2.4.2. Cytocentrifuge, Rapid Romanowsky stain and differential cell counts

175ul of PLF or BALF was applied to SuperFrost® glass slides (Menzel-Gläser, Thermo Scientific, Leicestershire, UK) by centrifugation at 200rpm for 5 minutes. The slides were then air-dried overnight and stained with a Rapid Romanowsky Stain (RA Lamb, Thermo Scientific, Leicestershire, UK). This involves a 30 second incubation in methanol, to fix the cells, a 30 second incubation in eosin and a final 1 minute incubation in methylene blue.

Percentage of eosinophils, neutrophils, macrophages and lymphocytes was determined by counting cells in 6 randomly selected fields at 40x magnification.

2.2.4.3 Flow cytometry

Flow cytometry is an intricate and informative technique used to count and analyse the presence of cell-types and other microparticles in a particular suspension. It involves the marking of cells with specific fluorescent antibodies, the movement of cell types in a hydronamically-focused stream of liquid, the shining of light at specific wavelengths at these cells, and the recording of the scattered and emitted fluorescent light. The pattern of scattered and emitted light gives an indication of the different cell types in the suspension and their expression of cell-surface markers.

Flow cytometry was carried out to identify the different cell populations present in BALF. The BALF was vortexed and 50µl was strained using Falcon tubes with Cell Strainer Caps (BD Biosciences, Oxford, UK), spun at 1500rpm for 15s at RT. To block aspecific binding, 10µl of 50µg/µl Mouse BD Fc Block™ (purified rat anti-mouse CD16/CD32, BD Pharmigen, BD Biosciences, Oxford, UK) was incubated with each sample for 15 minutes at RT. 1µl of each antibody (Table 2.6) was incubated with each sample for 45 minutes at 4°C in the dark. 200µl of IO Test (1:10 dilution) was incubated with each sample for 5 minutes. The plate was spun two times at 1500rpm for 5 minutes, the final resuspension containing 1µg/ml DAPI and 50µl flowcount beads in 200µl FACS buffer (see Appendix 9.1.6) and subsequently analysed on a FACSCanto II machine.

A differential cell count was performed by gating three cell populations (granulocytes, lymphocytes, monocytes) according to a forward/side scatter (FSC/SSC) plot. The gated lymphocyte population was further analysed according to expression of CD4, CD8 and CD19 (T vs. B lymphocyte) and expression of TIM3 and T1/ST2 (TH1 vs. TH2 cells, respectively).

Total cells were calculated according to the following formula:

$$Number\ of\ specific\ events * \left(\frac{1000}{\frac{NumberofFlowCountEvents}{982}} \right) * BAL\ Recovery$$

Table 2.6

Anti-mouse antibodies and reagents used in flow cytometry

Marker	Isotype	Dye	Laser	PMT	Catalogue Number	Supplier
CD45	Rat IgG2a κ	Pe-Cy7	Blue (488)	780/60	557659	BD Biosciences
CD4	Rat IgG2a κ	PerCP-Cy5.5	Blue (488)	670LP	550954	BD Biosciences
CD8	Rat IgG2a κ	Pe-Cy7	Blue (488)	780/60	552877	BD Biosciences
CD19	Rat IgG2a κ	APC	Red (633)	660/20	550992	BD Biosciences
TIM3	Rat IgG1 κ	PE	Blue (488)	585/42	12-5871-81	eBioscience
T1/ST2	Rat IgG1	FITC	Blue (488)	530/30	101001F	MD Bioproducts
Dapi	-	Pacific Blue	Violet (405)	450/50	D9542	Sigma
Flow-Count	Flow	AmCyan	Violet	510/50	7508092D	Beckman

2.2.4.4 Sysmex Cell Counting

For instant analysis of BALF, a Sysmex XT-2000iV was used to perform a differential cell count. The sysmex XT-2000iV analyses 80µl of BALF by analysis of forward scatter (FSC) and side scatter (SSC). BALF samples were vortexed and read by the Sysmex XT-2000iV. Analysis was performed as per manufacturer's instructions.

2.3 Statistical Analysis

All data was analysed using GraphPad Prism software (LaJolla, California, USA). All data was initially tested for normal distribution. Normally distributed data was analysed using parametric statistical tests, including the Student's t-test, one way ANOVA and two way

ANOVA. Significant results were further analysed using the Tukey post-hoc test for one way ANOVA and the Bonferroni or Sidak's post-hoc test for two way ANOVA. Data that was not normally distributed was log transformed using the formula Y=log(Y). The transformed data was tested for normal distribution and analysed as above. Non-normalised data was analysed using non-parametric statistical tests including the Mann-Whitney test and the Kruskal-Wallis test and followed by, if significant, the Dunns multiple-comparison post-test. The test used for each set of data is specified in the results section of each chapter.

Chapter 3: A comparison between the central effects of systemic or localised innate inflammatory responses

3.1 Introduction

Decades of study have shown that circulating mediators of the peripheral inflammatory response, including IL-1β and PGE_2 impact on the brain by acting on endothelial cells of the BBB[48,56,62,65,261], crossing into CVOs[38,90,91,262] or activating the vagus nerve[95,99,106]. This immune to brain communication results in changes in neurochemistry[263], increased central cytokine production[54,61], specific patterns of neuronal activity[264,265] and alterations in behaviour[266,267]. Collectively, these adaptations are important physiological changes following peripheral infection, key to the maintenance of homeostasis[3].

The majority of these studies have used bacterial or viral mimetics, such as LPS or poly I:C, which interact with TLRs to stimulate an immune response. This has undoubtedly provided a vast amount of information in the field of immune to brain communication, detailing the intricate pathways of neuroimmune cross-talk and pinpointing the foundations of sickness behaviour. However, the use of LPS only covers a fraction of the substantial immune response, focusing only on TLR4 and how systemic immune responses dominated by circulating pro-inflammatory cytokines impact on the brain.

The impact of localised inflammatory events on the brain has been widely ignored. Having particular prominence are allergic inflammatory events, which tend to be localised to a particular organ (e.g. lung or gut) and are dominated by the TH2 cytokines IL-4 and IL-5. Allergic inflammation affects 30-40% of people at some stage in their lives[268], but only a handful of studies have investigated how it impacts on the CNS[246,247,269] .

In this chapter, a rudimentary model of localised TH2-like inflammation was developed using the yeast cell-wall component zymosan. This acute model has comparable kinetics to peripheral inflammation following LPS challenge[270], where the effects on the CNS have been widely characterised. This therefore allowed a direct comparison of the impact of peripheral pro-inflammation versus localised TH2-like inflammation on the brain, in terms behavioural changes, central cytokine expression and neuronal activity.

3. 2 Methods

To compare the impact of innate TH1-like and TH2-like immune responses on the brain, two micro-organism components were used. C57BL/6 mice were challenged with LPS or zymosan, as described in section 2.1.3.1. Behavioural changes were analysed between 1 and 3 hours after challenge, as described in section 2.14. The open field and elevated plus maze assays were performed 1 hour after challenge; the burrowing assay was performed between 1 and 3 hours after challenge.

Tissue samples, including serum, peritoneal lavage, spleen and brain were collected 3 hours post-challenge, as detailed in section 2.1.5. Brain samples were analysed using immunohistochemistry for c-fos expression as detailed in section 2.2.1.2 and analysed for alterations in cytokine expression using qPCR, as detailed in sections 2.2.3.1 (protocol A), 2.2.3.4 and 2.2.3.6. Serum, spleen, brain and peritoneal lavage was analysed for cytokine expression using MSD technology, as detailed in section 2.2.2.3.

3.3 Results

3.3.1 Induction of Innate TH1-like and TH2-like Immune Responses

3.3.1.1. Systemic cytokine production

To confirm the induction of differential inflammatory profiles by LPS and zymosan, a collection of pro-inflammatory (TH1) and anti-inflammatory (TH2) cytokines were measured in the serum 3 hours after immune stimulation. The 3 hour time point was chosen, as this is the peak of pro-inflammatory cytokines production in the periphery following LPS[61] and zymosan administration[271]. Intraperitoneal administration of LPS resulted in a significant increase in serum levels of IL-1β ($F_{2,12}$=7.947, p=0.0072), TNF-α (H_2=10.29, p=0.0006), IL-6 (H_2=7.665, p=0.0029), IL-12 ($F_{2,9}$=20.53, p=0.0011) and IFN-γ (H_2=11.36, p=0.0001) as compared to the saline-treated control. In contrast, levels of these cytokines were not significantly elevated 3 hours post-zymosan administration, though there was a significant increase in IL-5 ($F_{2,12}$=8.240, p=0.0042) and KC ($F_{2,9}$=62.99, p<0.0001) as compared to the control (Figure 3.1).

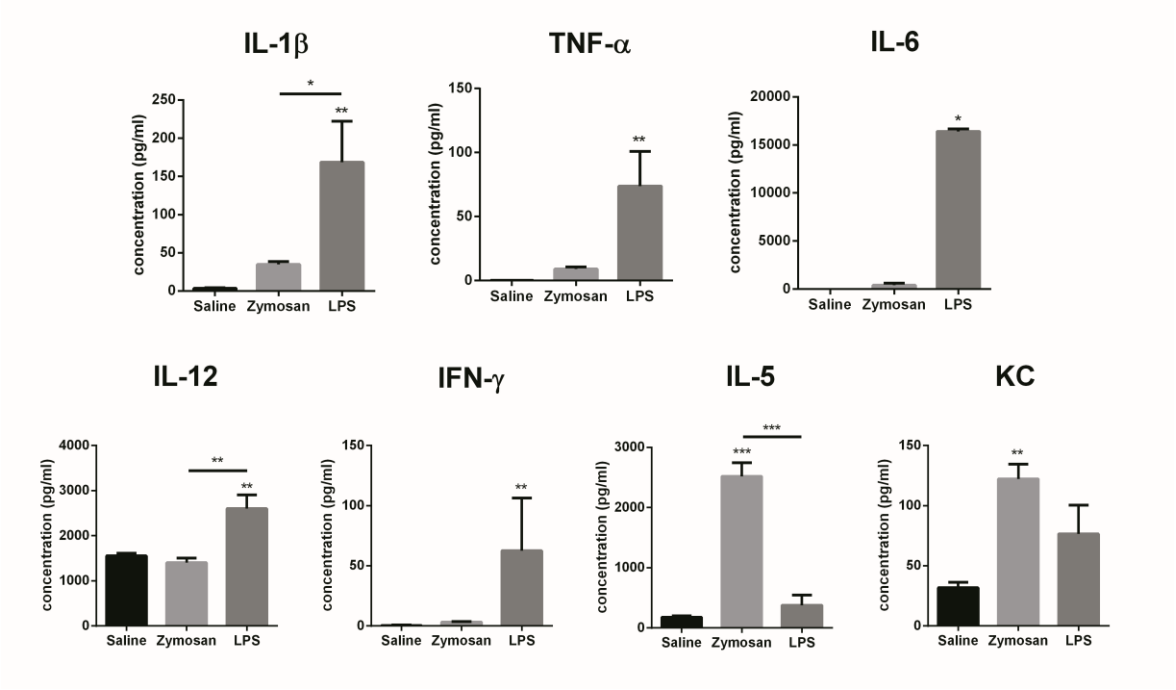


Figure 3.1 - Effect of i.p. LPS (100µg/kg) or zymosan (5mg/kg) administration on serum cytokine levels. A blood sample was collected 3 hours after immune stimulation; cytokine levels were measured using multiplex (IL-1β, TNF-α, IL-12, IFN-γ, IL-5, KC) or singleplex (IL-6) ELISA. Values are expressed as mean pg per ml serum ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. saline control, unless otherwise shown, using one way ANOVA followed by, if significant, a Tukey post-hoc test (IL-1β, IL-12, IL-5, KC) or Kruskal-Wallis test followed by a Dunn's post-hoc test (TNF-α, IL-6, IFN-γ). n=4-5.

3.3.1.2. Systemic prostaglandin production

It was been well-described that, in addition to the prototypical pro-inflammatory cytokines IL-1β, TNF-α and IL-6, peripheral PGE_2 plays a key role in the communication between the immune system and the brain[272]. Levels of serum PGE_2 were therefore measured following i.p. zymosan and LPS administration. Levels of serum PGE_2 were significantly elevated following LPS administration as compared to zymosan administration ($F_{2,15}$=14.07, p=0.0010) and saline control ($F_{2,15}$=14.07, p=0.0009). In contrast, there was no change in serum PGE_2 levels following zymosan administration as compared to saline control ($F_{2,15}$=14.07, p=0.9981) (Figure 3.2).

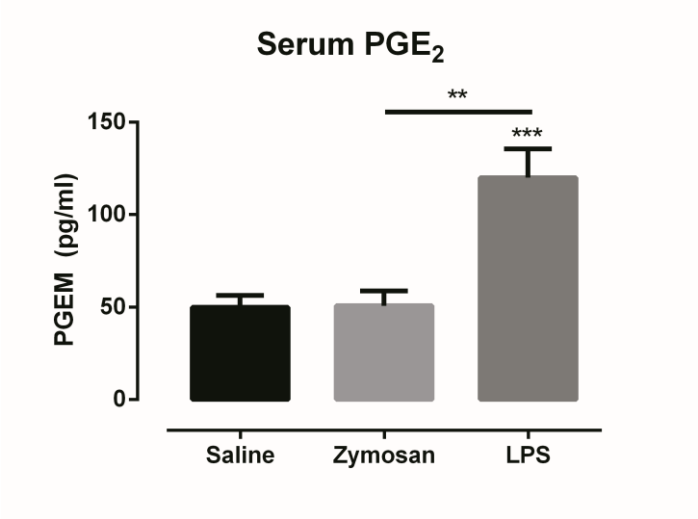


Figure 3.2 – Effect of i.p. LPS (100µg/kg) or zymosan (5mg/kg) administration on serum prostaglandin levels. A blood sample was collected 3 hours after immune stimulation; prostaglandin levels were measured using the prostaglandin metabolite EIA kit (Cayman, USA). Values are expressed as mean pg per ml serum ± SEM. **p<0.01, ***p<0.001 vs. saline control, unless otherwise shown, using one way ANOVA followed by a Tukey post-hoc test. n=5.

3.3.1.3 Peritoneal cells

Initial circulating cytokine measurements suggested that zymosan did not induce a systemic pro-inflammatory response. Zymosan has previously been shown to induce a localised inflammatory response, with increased leukocyte infiltration into the peritoneum following peritoneal challenge[273] and is in consequence often used as a model of peritonitis[274]. The numbers and type of peritoneal cells were therefore analysed following zymosan and LPS administration. Zymosan challenge was associated with a significant increase in total cells in the peritoneum (Figure 3.3A), as compared to LPS challenge ($F_{2,9}$=5.929, p=0.0478) and saline control ($F_{2,9}$=5.929, p=0.0299). A differential cell count (Figure 3.3B) revealed that zymosan challenge was additionally associated with a significant rise in peritoneal neutrophils compared to LPS challenge (p=0.0047) and the saline control (p=0.0015), as well as a substantial, but non-significant rise in peritoneal eosinophils as compared to LPS challenge (p=0.1266) and saline control (p=0.0666).

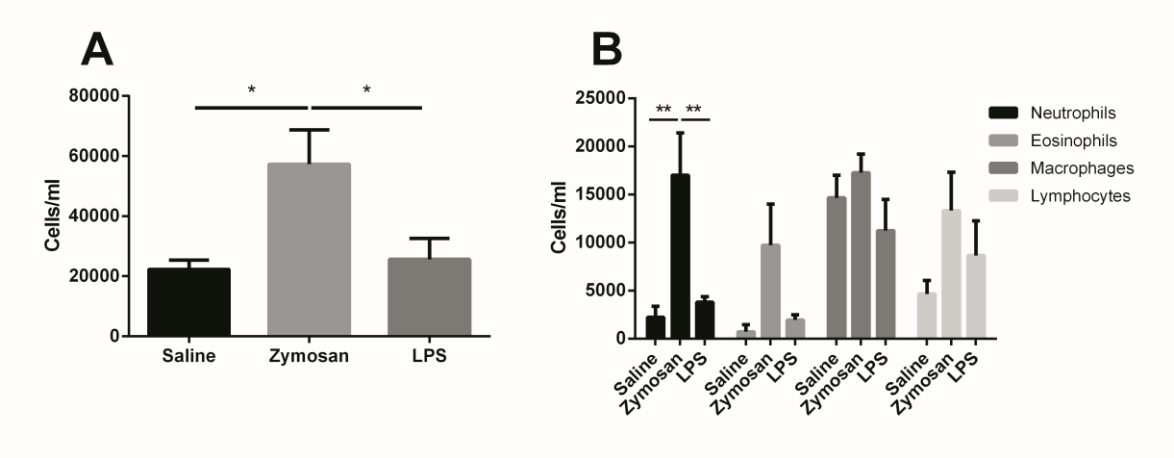


Figure 3.3 - Effect of i.p. LPS (100µg/kg) or zymosan (5mg/kg) administration on total cells (A) and individual neutrophil, eosinophil, macrophage and lymphocyte (B) counts in the peritoneum. Peritoneal lavage was performed 3 hours following immune stimulation; total cells were counted using a haemocytometer; differential cells were quantified using a Rapid Romanowsky stain; values are expressed as mean cells per ml recovered lavage ± SEM. *p<0.05, **p<0.01 using a one way ANOVA, followed by a Tukey post-hoc test (A) or a two way ANOVA, followed by a Tukey post-hoc test (B). n=4

3.3.1.4 Peritoneal cytokines

Peritoneal lavage was also assessed for presence of TH1 and TH2 cytokines (Figure 3.4). Zymosan administration was associated with a significant 4-fold increase in IL-1β ($F_{2,9}$=158.6, p<0.0001), a 1.2 fold increase in TNF-α ($F_{2,9}$=22.72, p=0.00060), an almost 6-fold increase in IL-5 ($F_{2,9}$=39.45, p<0.0001) and a 4-fold increase in IL-4 ($F_{2,9}$=172.4, p<0.0001), as compared to the saline-treated control. In contrast, LPS administration was associated with a significant 9-fold increase in IL-12 (p=0.0134) and an almost 55-fold increase KC (p=0.0071), as compared to saline administration.

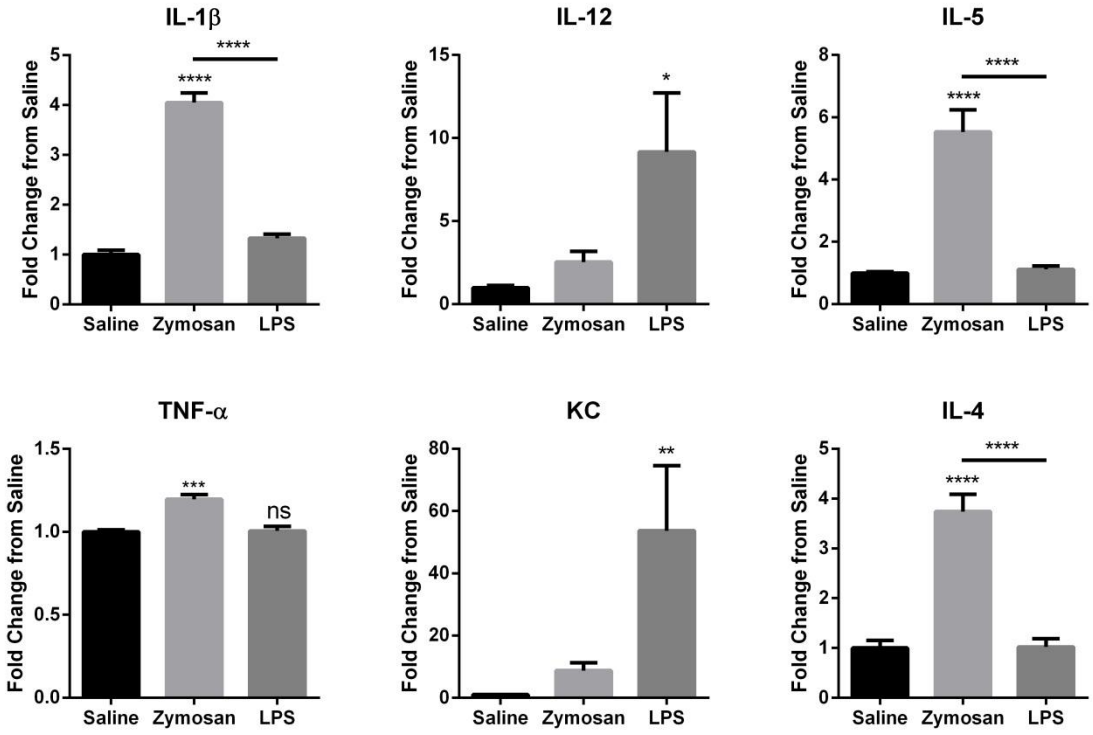


Figure 3.4 – Effect of i.p. LPS (100µg/kg) or zymosan (5mg/kg) administration on peritoneal cytokines. Peritoneal lavage was performed 3 hours following immune stimulation, cytokines were measured using a multiplex ELISA. Values expressed as mean fold change over saline ± SEM. *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001 vs. saline control unless otherwise shown, using a one way ANOVA followed by a Tukey post-hoc test (IL-1β, TNF-α, IL-5 and IL-4) or Kruskal-Wallis test, followed by a Dunn's post-hoc test (IL-12, KC). n=4

3.3.2 The Differential Effect of LPS and Zymosan on Behaviour

Upon confirmation of the induction of distinct systemic inflammatory profiles, the effects of LPS and zymosan on behaviour were investigated, as a primary indicator of immune to brain communication. Behaviours were assayed using three different behavioural tasks, each exploring a different component of sickness behaviour. The open field task examines general movement and gives an indication of lethargy and anxiety. The elevated plus maze (EPM) is a well-established test for anxiety. Burrowing behaviour is a species-typical behaviour dependent on an intact hippocampus and investigates learning and memory, as well as anhedonia. It has previously been shown that LPS induces a reduction in all three of these behavioural tasks[61,275].

LPS administration was associated with clear changes in behaviour in the open field and burrowing assays. There was a significant decline in activity ($F_{2,23}$=5.049, p=0.0199) (Figure 3.5A) and rears ($F_{2,22}$=9.304, p=0.0012) (Figure 3.5B) in the open field assay, as well as a significant decline burrowing behaviour ($F_{2,19}$=102.4, p<0.0001) (Figure 3.5C) following LPS administration, as compared to saline control. No changes in the EPM were observed (Figure 3.5D). In contrast, following zymosan administration, there was no change in activity (p=0.6778) or rears (p=0.5451) in the open field. Zymosan induced a significant reduction in burrowing behaviour (p<0.0001), as compared to saline control, albeit to a much lesser degree when compared to LPS. Finally, zymosan induced a reduced activity in the open arms of the EPM, although this did not reach significance when compared to control.

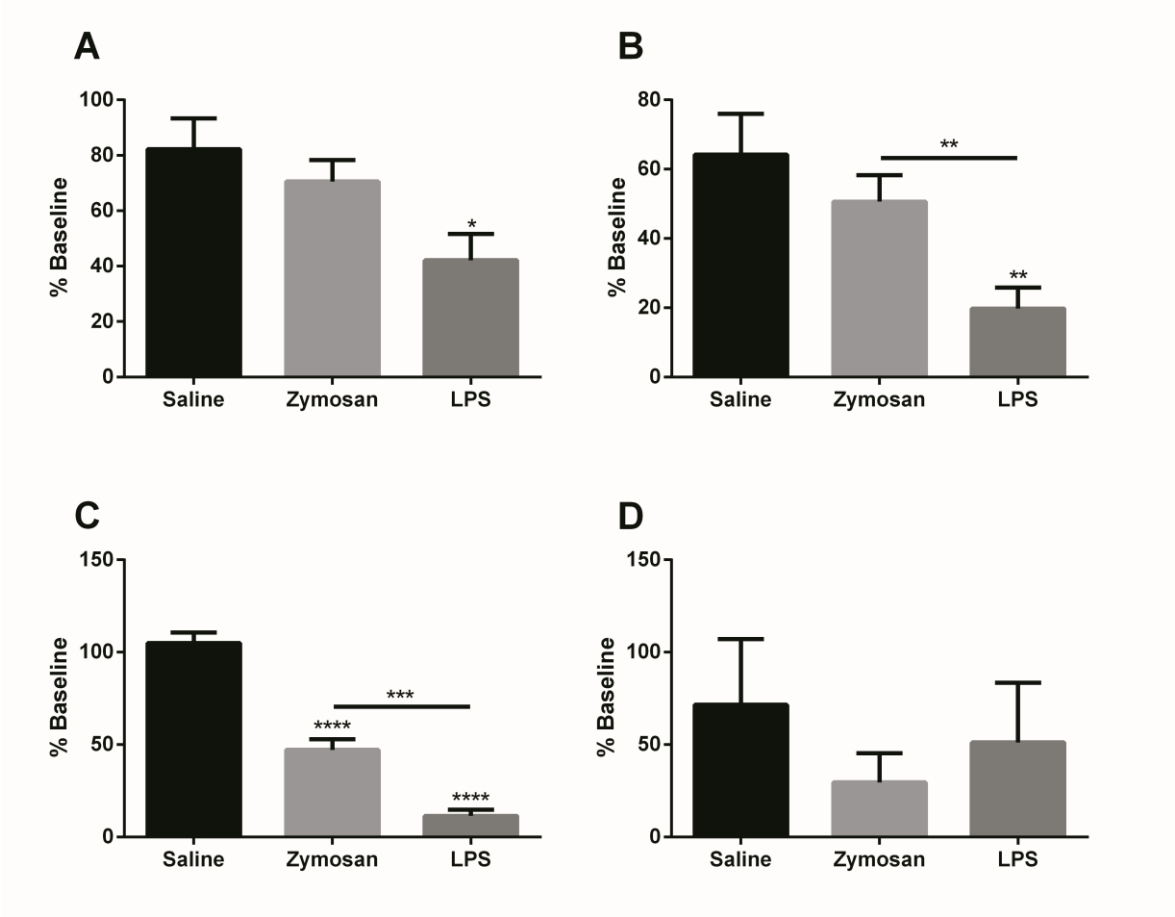


Figure 3.5 – Effect of i.p. LPS (100µg/kg) or zymosan (5mg/kg) administraiton on total activity (A) and rears (B) in the open field, burrowing behaviour (C) and behaviour in the elevated plus maze (D). Burrowing behaviour was assayed between 1 and 3 hours after immune stimulation. Behaviour in the open field apparatus and elevated plus maze was assayed between 2 and 3 hours post immune stimulation over a period of 3 or 5 minutes, respectively. A baseline measurement was taken one day prior to immune stimulation. Values are represented as mean percent of baseline measurement ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs saline control unless otherwise shown using one way ANOVA followed by, if significant, a Tukey post-hoc test. n=5-10.

3.3.3 The Effect of Zymosan and LPS Administration on Cytokine Protein and mRNA Expression in the Brain

To further assess whether zymosan administration impacted on the brain, protein and mRNA levels of cytokines were measured in the hippocampus and brainstem. Protein levels of IL-1β, TNF-α, IL-12, KC, IL-4 and IL-5 were measured, as were mRNA levels of TNF-α and IL-6. There were no significant changes in protein levels of IL-1β, TNF-α, IL-4 or IL-5 in the hippocampus (Figure 3.6) or brainstem (Figure 3.7) following LPS or zymosan administration, though a trend towards an increase following LPS administration was evident. In contrast, LPS administration was associated with significant elevations in

protein levels of IL-12 (H_3=7.426, p=0.0196) in the brainstem, and KC in the brainstem (H_2=9.103, p=0.0227) and hippocampus (H_2=11.08, p=0.0030), as compared to saline control. No changes in levels of IL-12 or KC were evident following zymosan administration.

Although no significant change in protein levels were detected, LPS administration was associated with a significant increase in mRNA levels of TNF-α in the hippocampus (H_2=9.380 p=0.0267) and brainstem (H_2=8.540, p=0.0141), but not IL-6. No changes in mRNA levels of TNF-α or IL-6 were seen following zymosan administration (Figure 3.8).

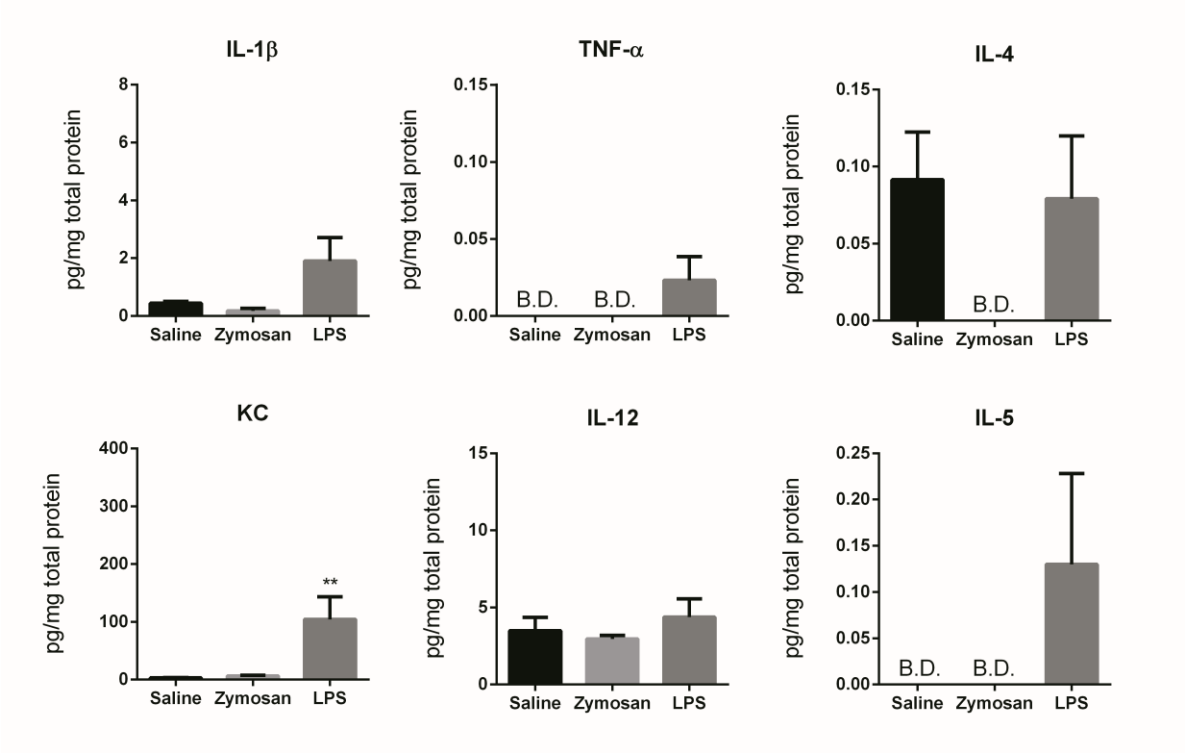


Figure 3.6 - Effect of i.p. LPS (100µg/kg) or zymosan (5mg/kg) administration on cytokine protein expression in the hippocampus. Brain tissue was collected 3 hours after immune stimulation; cytokines were measured using a multiplex ELISA. Values are expressed as mean pg per mg of total protein ± SEM. **p<0.01 vs. saline control using Kruskal-Wallis Test followed by a Dunn's post-hoc test. n=4-5. B.D. = below detection limit of the assay.

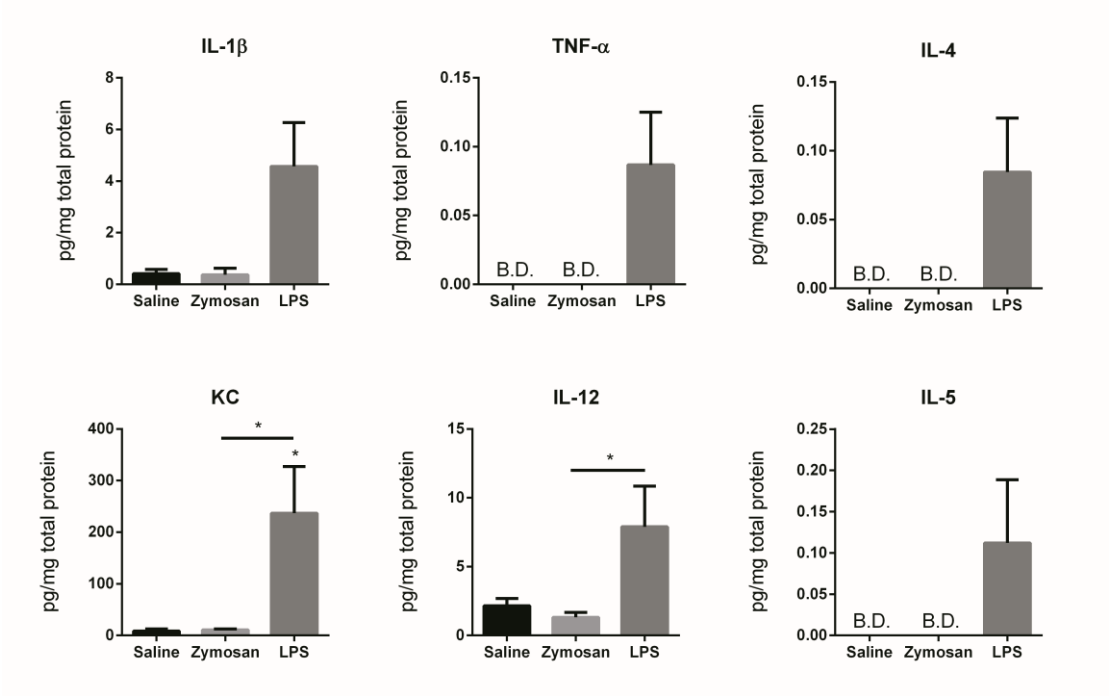


Figure 3.7 - Effect of i.p. LPS (100µg/kg) or zymosan (5mg/kg) administration on cytokine protein expression in the brainstem. Brain tissue was collected 3 hours after immune stimulation; cytokines were measured using a multiplex ELISA. Values are expressed as mean pg per mg of total protein ± SEM. *p<0.05 vs. saline control using Kruskal-Wallis Test followed by a Dunn's post-hoc test. n=4-5 B.D. = below detection limit of the assay.

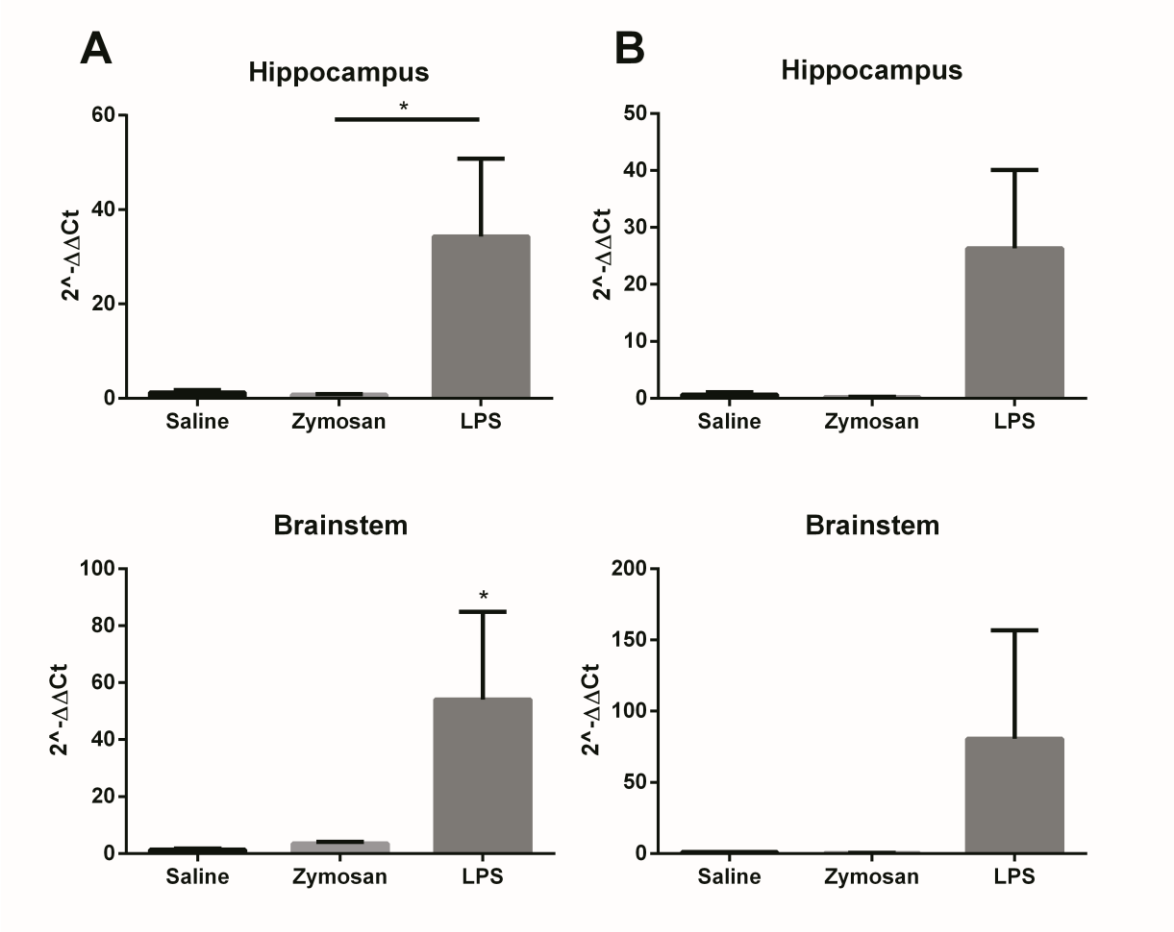


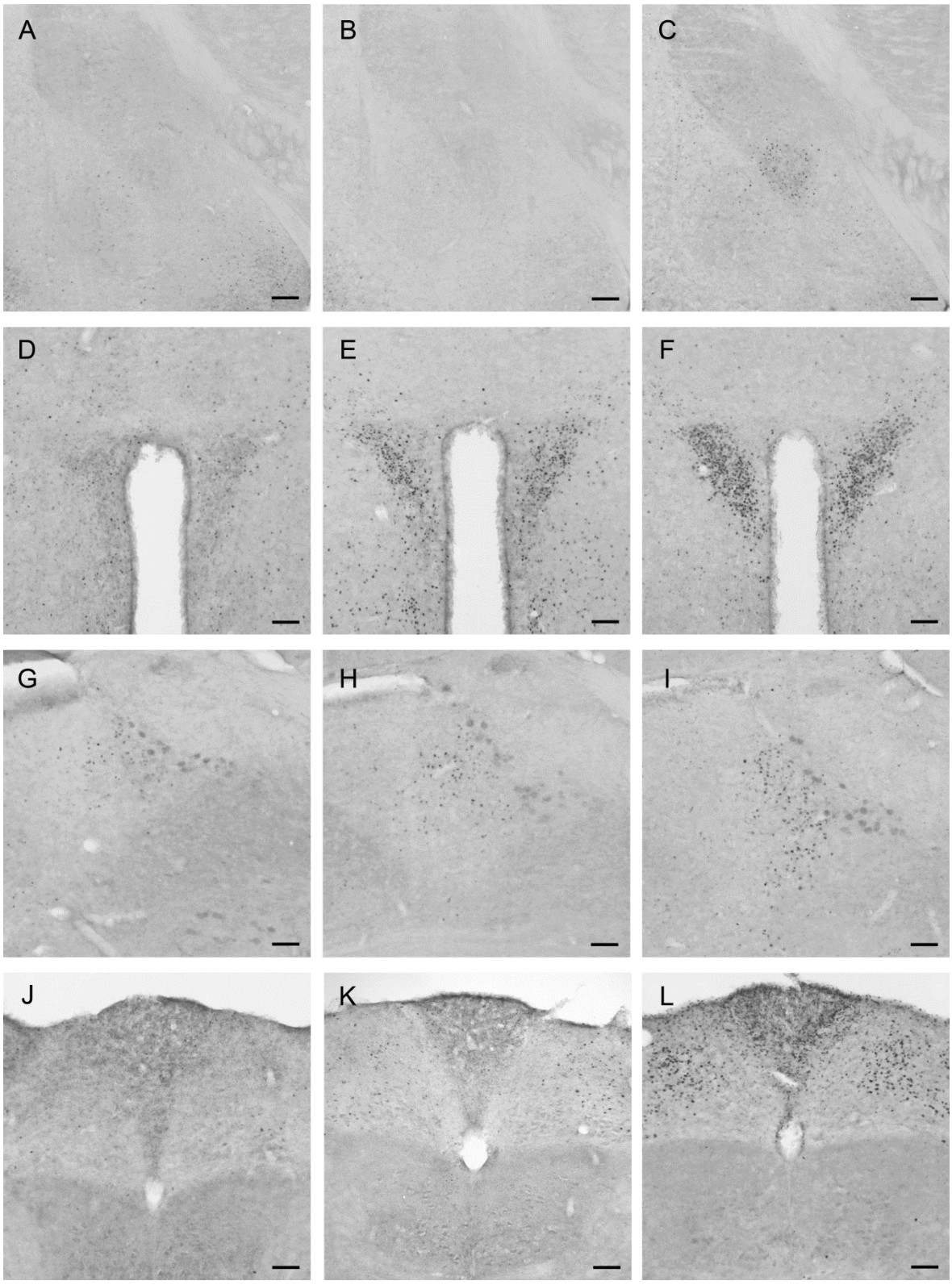
Figure 3.8 - Effect of i.p. LPS (100µg/kg) or zymosan (5mg/kg) administration on TNF-α (A) and IL-6 (B) mRNA expression in the hippocampus and brainstem. Brain tissue was collected 3 hours after immune stimulation; cytokines were measured using SYBR Green qPCR. Values are expressed as mean fold change from saline, relative to GAPDH expression ± SEM. *$p<0.05$ vs. saline control, unless otherwise shown, using Kruskal-Wallis Test followed by a Dunn's post-hoc test. n=5

3.3.4 The Effect of Zymosan and LPS Administration on c-fos Expression

As a final assessment as to whether zymosan administration impacted on the brain, levels of neuronal activity were measured by immunohistochemically staining for c-fos. C-fos is an intermediate early gene (IEG) that is transiently induced upon transcriptional activation of a cell. The expression of c-fos is commonly used as an indicator of the activation status of a cell, often in neurons. C-fos expression was initially investigated at 30 minutes, 3 hours and 6 hours following LPS and zymosan administration, and it was found that expression peaked at 3 hours (*data not shown*). This time point was consequently chosen for further investigation. C-fos expression was analysed in various areas of the brain 3 hours post peripheral administration of zymosan or LPS, particularly focusing on areas that

have previously shown increased c-fos expression in response to immune stimulation with LPS[49], including the paraventricular nucleus of the thalamus (PVA), paraventricular nucleus of the hypothalamus (PVN), the median preoptic nucleus (MnPO) central nucleus of the amygdala (CeA), the hippocampus (Hp), the lateral parabrachial nucleus (LBPN) the locus coeruleus (LC), the nucleus of the solitary tract (NTS) and the area postrema (AP).

LPS and zymosan induced different patterns of c-fos expression throughout the brain (Figure 3.9). LPS administration was associated with a widespread expression of c-fos over the entire neuraxis of the brain, including in the PVN, MnPO, CeA, LC, LPBN and NTS, as well as circumventricular organs (CVOs), including the SFO and AP. The number of nuclei stained for c-fos following LPS administration were significantly increased in the PVN (p=0.0451), CeA (p=0.0005) and NTS (p=0.0020) as compared to saline control. Similar to LPS, zymosan was associated with substantial increases in c-fos expression in the PVN, LC, NTS and AP, though at levels not significantly above saline control. No differences in c-fos expression between LPS and zymosan treated animals and controls were apparent in the PVA or dentate gyrus of the hippocampus (Figure 3.10).


A
B
C
D
E
F
G
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L

Figure 3.9 – Representative images of c-fos expression in the CeA (A-C), PVN (D-F), LC (G-I), NTS and AP (J-L) after i.p. administration of saline (A, D, G, J), zymosan (B, E, H, K) and LPS (C, F, I, L). Tissue was collected 3 hours after immune stimulation and immunohistochemically stained for c-fos expression. Representative of n=5. Scale bar: 100μm

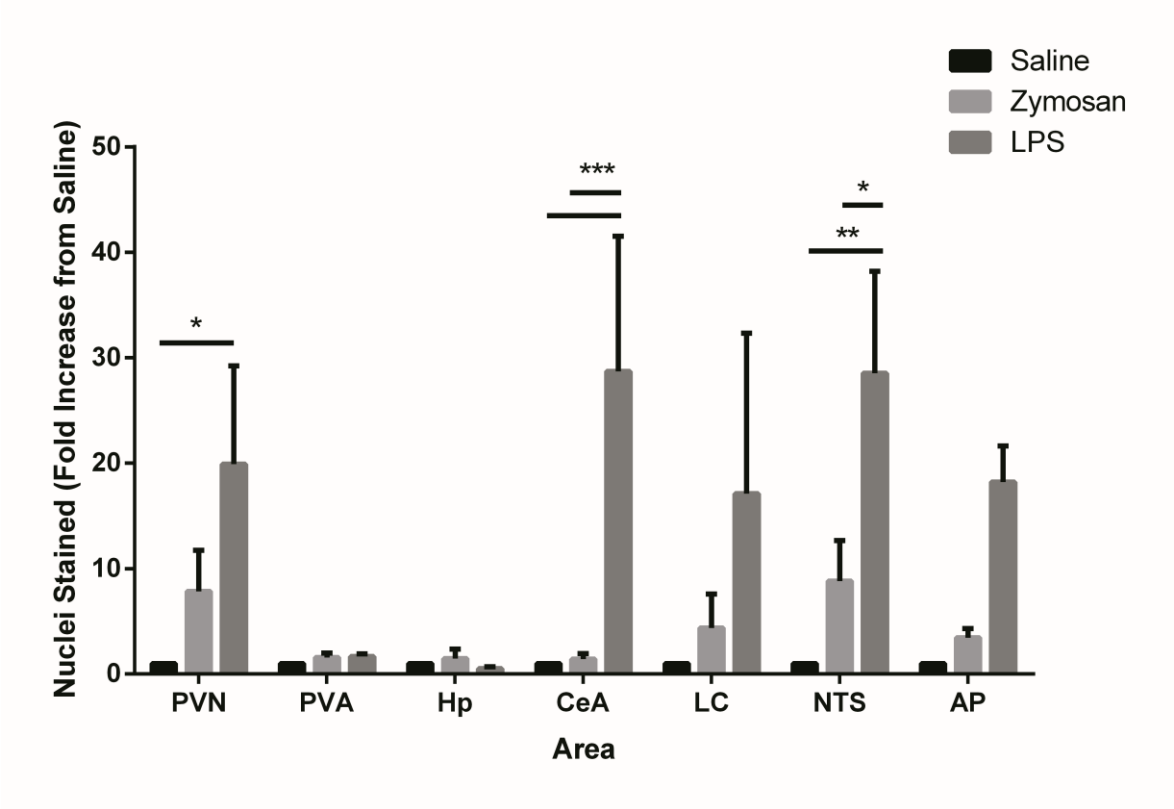

Figure 3.10 – Quantification of c-fos immunohistochemistry in PVN, PVA, hippocampus (Hp), CeA, LC, NTS and AP following i.p. administration of LPS (100μg/kg) or zymosan (5mg/kg). Brain tissue was collected 3 hours after immune stimulation; c-fos expression was assessed using immunohistochemistry and analysed using ImageJ software. Values are expressed as the fold change of nuclei stained in each respective area. *p<0.05, **p<0.01, ***p<0.001 using two way ANOVA followed by a Tukey's post-hoc test. n=5.

3.4 Discussion

It is well established that systemic immune responses induced by bacterial or viral mimetics, such as LPS or poly I:C, impact on the brain, largely due to the presence of circulating pro-inflammatory cytokines and prostaglandins[34,272]. However the effect of other inflammatory events on the CNS, such as responses dominated by TH2 cytokines, or localised inflammation, key features of allergic lung inflammation, has been widely ignored. Here, a localised innate immune response dominated by TH2 cytokines was found to communicate with the brain, inducing small behavioural changes and a discrete

pattern of neuronal activity, despite the lack of circulating pro-inflammatory cytokines and prostaglandins.

3.4.1 Zymosan Induces Acute Granulocytic Inflammation

In order to determine the effect of a localised TH2-like inflammatory response on the brain, an inflammatory stimulus was chosen with similar characteristics to LPS, to which the effects on the brain have been widely characterised[37,44,49,61,64,65]. Like LPS, zymosan is a micro-organism cell-wall component that interacts with PRRs and induces an acute innate inflammatory response.

As previously reported in the literature[61,132], systemic LPS administration resulted in significantly high levels of circulating pro-inflammatory cytokines, IL-1β, TNF-α and IL-6, as well as the TH1 cytokines IL-12 and IFN-γ. In contrast, these pro-inflammatory cytokines were not detected following zymosan administration, at the dose used (5mg/kg). Instead, high levels of the circulating TH2 cytokine and eosinophil chemoattractant IL-5 and neutrophil chemoattractant KC (the mouse homologue of IL-8) were detected. This polarisation towards a TH2 type immune response has previously been reported *in vitro*[276,277] in response to zymosan.

Accompanying the rise in circulating TH2 cytokines and granulocytic chemoattractants, zymosan induced a localised granulocytic response, characterised by infiltration of eosinophils and neutrophils into the peritoneum, a response that was absent following LPS administration. This was associated with localised production of pro-inflammatory cytokines IL-1β and TNF-α, as well as increased levels of localised and circulating TH2 cytokines IL-4 and IL-5. This rise in circulating IL-5 and KC, both of which are granulocyte chemoattractants, further explains the mobilisation and infiltration of granulocytes into the peritoneum. The development of localised granulocytic inflammation is corroborated by previous studies, where peritoneal administration of zymosan, when compared to LPS, results in significantly higher leukocyte infiltration into the peritoneum[273], underpinning the wide use of zymosan as a peritonitis model[274].

Zymosan is a β-glucan that interacts with TLR2 and the C-type lectin Dectin-1[278], the latter of which is known to be expressed on a variety of innate immune cells, including mast

cells[279]. Mast cell activation triggers the transcription of the pro-inflammatory cytokines IL-1β and TNF-α, as well as IL-4 and IL-5[1], cytokines produced locally in response to zymosan, suggesting a role for mast cells in zymosan-induced peritoneal inflammation. Indeed, previous studies have shown attenuated peritonitis in response to zymosan, in cromolyn-treated or mast-cell deficient WBB6F1 mice[280]. In contrast, mast cells express TLR4, but not the co-receptor CD14, and are therefore unresponsiveness to LPS if soluble CD14 is not present[281]. This, alongside the knowledge the LPS freely enters the circulation following peritoneal challenge[132], provides an explanation for the discrepancy in localised versus systemic pro-inflammatory responses following zymosan or LPS administration, respectively.

3.4.2 Attenuated Classical Sickness Behaviours and Lack of Central Cytokine Production following Zymosan Administration

Upon establishing differential innate immune responses, the effect of these immune responses on behavioural changes and central cytokine production was compared. As previously reported, LPS administration was associated with a significant downregulation of activity and rears in the open field, as well as a significant abrogation of burrowing behaviour, suggestive of the classical sickness behaviours lethargy and anhedonia. This occurred alongside an increase in pro-inflammatory cytokine expression in the hippocampus and brainstem. In contrast, zymosan administration did not affect behaviour in the open field, nor induce central cytokine expression, only resulting in a moderate, though significant, decline in burrowing behaviour.

Classical sickness behaviours, such as visualised by a decline in locomotor activity in the open field and decreased burrowing behaviour, have repeatedly been shown to be associated with circulating and central prostaglandins and circulating and central pro-inflammatory cytokines[61,70,275,282,283] . Peripheral administration of IL-1β has been shown to reduce open field activity[275], although the role of peripheral IL-1β has been disputed[61], and central IL-1β has been shown to have a more prominent role[284]. Additionally, indomethacin, an inhibitor of COX-1/2 activity, significantly attenuates LPS-induced decline in locomotor activity and burrowing behaviour[70,282], suggesting a role for prostaglandins. Similarly in this study, a significant decline in open field activity was observed following LPS administration, associated with a rise in circulating prostaglandins

and central IL-1β. These immune mediators are absent following zymosan administration, consequently not resulting in a change in locomotor activity. In contrast, however, despite the absence in these inflammatory mediators, zymosan is associated with a significant decline in burrowing behaviour.

Alterations in behaviours such as open field or burrowing and associated rises in central inflammatory mediators can be attributed to binding of the LPS to TLR4 on the cerebral endothelium. Indeed, at the dose given, LPS has previously shown to induce central PGE_2 production[61] and has been shown here, and previously, to elevate central cytokine expression, irrespective of peripheral cytokine expression[61]. Cerebral endothelial cells are additionally known to express TLR2[57], raising the possibility that circulating zymosan, if present, can trigger the central prostaglandin production, influencing burrowing behaviour. Indeed, higher doses of zymosan (50mg/kg) have been shown to induce COX-2 expression in the brain, as well as increase levels of central PGE_2[285], suggesting a means by which zymosan could alter burrowing behaviour. In this study, and after this dose of zymosan (5mg/kg), however, prostaglandins were not measured, and this postulate can therefore not currently be verified.

It should be noted that changes in burrowing behaviour are highly sensitive to even low levels of inflammation, with almost complete abrogation of behaviour following very low doses (0.5μg/kg) of LPS[61]. Contrastingly, higher doses of LPS are necessary to observe changes in open field activity. It is possible that low levels of LPS were present in the zymosan used, possibly accounting for the small, but non-significant elevation in circulating IL-1β and the reduction in burrowing, but not open field behaviour.

3.4.3 A Role for the Vagus Nerve in Zymosan-induced Changes in Neuronal Activity

In this study, it was found that zymosan induced specific patterns of neuronal activity, as measured by c-fos expression, despite the lack of significant elevation in systemic pro-inflammatory cytokines and prostaglandins. As previously shown[49], LPS induces widespread c-fos activation in the brain, including in the VLM, NTS, LPBN, LC, PVN, MnPO, BNST and the CeA. In contrast, zymosan-induced patterns of c-fos expression

were limited, expression only found in the NTS, LC and PVN, and were less robust, in terms of number of nuclei stained in each region.

Enhanced c-fos expression following peripheral immune stimulation is dependent on several pathways of immune to brain communication, including via activation of the endothelium, through interaction with cells in the CVOs and via the vagus nerve[2]. The specific pattern of neuronal activity following zymosan administration, particularly in the NTS and PVN mirrors patterns previously demonstrated following localised inflammation, specifically in the gut[112,248]. Both aforementioned studies demonstrate a role for the vagus nerve in transferring signals from the periphery to the brain. Indeed, it is well described that the NTS, which receives inputs from vagal afferent fibres, projects directly to the PVN, forming an ascending immunosensitive pathway directly from the periphery. Subdiaphragmatic vagotomy has been shown to inhibit activation of the PVN, and block ACTH secretion (a direct consequence of PVN activation) in response to peripheral LPS[106,286], suggesting a critical role for the neuronal pathway of immune to brain communication in activation of these brain regions. The discrete pattern of c-fos expression and the localised nature of the inflammatory event following zymosan administration would suggest that inflammatory events in the peritoneum induce neuronal changes via the vagus nerve.

Indeed, zymosan was found to induce localised production of IL-1β, which has previously been shown to bind vagal afferent fibres and transmit a signal from the immune system to the brain[94]. Zymosan is also known to trigger localised production of prostaglandins[285] and is a proficient inducer of complement[287], specifically C5a, both of which have been shown to impact on the brain via interaction with the vagus nerve[95,288]. As discussed above, zymosan has the ability to activate mast cells, resulting in lipid mediator metabolism and pro-inflammatory cytokine production. Activation of mast cells has previously been shown to be a critical in neuronal immune to brain communication in localised allergic gut inflammation[245], indicating a mechanism by which zymosan administration may result in increased neuronal activity in the CNS.

Previous studies investigating the impact of localised inflammatory responses on the brain found clear upregulation of c-fos expression in the CeA, a limbic structure associated with fear conditioning. Contrastingly, despite the localised nature of the inflammatory event,

zymosan did not induce any significant or noticeable upregulation of c-fos expression in this area. The CeA has previously been shown to receive projections from the external LPBN, which in turn receives projections from the NTS[289], together forming an ascending immunosensitive pathway that is upregulated following gut inflammation[112]. Though this study did show upregulation of c-fos in the NTS following zymosan administration, no changes were seen in the LPBN. This may, in part, due to the localisation of the inflammatory response (gut vs. peritoneum), as well as the strength of the inflammatory response.

3.4.4 Alterations in Anxiety Behaviour – Due to Localised Nature of Inflammation?

Localised inflammatory events, which result in these discrete patterns of neuronal activation, have previously been shown to induce anxiety like behaviours[108,290]. Though in this study no significant changes were found in anxiety behaviour following zymosan administration, there was a trend towards a reduction in the time spent in the arms of the EPM, indicative of increased anxiety behaviour. This may be associated with an increase in c-fos expression in the LC, an area that has previously been associated with stress[291]. The lack of a clear response in the assay may have partly been due to the variability of the assay and the low n-number, but also due to the repeated exposure of the mice to the EPM. Exposure of animals to familiar surroundings may reduce detectability of anxiety behaviours.

3.5 Conclusion

In this chapter it was found that localised inflammatory events, dominated by TH2 cytokines and granulocyte chemoattractants, impact on the brain, inducing attenuated changes in behaviour and discrete increases in neuronal activity. This occurs in a pathway independent of circulating pro-inflammatory cytokines or prostaglandins and is most likely due to immune to brain communication via the vagus nerve, as evidenced by the particular pattern of neuronal activity observed. Localised granulocytic inflammatory events are important features of allergic diseases, such as asthma; this study alludes to the likely possibility that asthmatic inflammation has the ability to communicate with, and impact on, the brain.

Chapter 4: The impact of acute allergic lung inflammation on the brain

4.1 Introduction

It has long been recognised that allergic inflammatory events, including asthma, affect behaviour. As early as the 19th century, scientists were aware of a phenomenon known as the "rose effect", in which a flower to which one was allergic could induce thoughts of anxiety and ultimately avoidance behaviour[204]. More recently, many studies have provided links between asthma and mental health[292]. Asthma patients, or patients with self-reported respiratory disease, are over 50% more likely to experience anxiety disorders, panic attacks, or depression as compared to non-asthmatic patients[293-298]. A review of the epidemiological literature calculated that 6.5-24% of asthmatics suffer from panic disorders, compared to 1-3% of the general population[299]. This impact of asthma on behaviour has recently been attributed to changes in neuronal circuitry in the brain[300].

It is additionally well established that the lung parenchyma is innervated by neuronal fibres of the vagus nerve, specifically the bronchopulmonary c-fibres, which are known to be activated by inflammatory events in the airways, such as allergic inflammation[199]. The vagus nerve, as detailed in Chapter 1, is a key pathway by which the immune system communicates with the brain, having particular prominence in localised inflammatory events[108,301].

In the previous chapter, it was found that unlike systemic pro-inflammation, localised inflammation, dominated by granulocyte recruitment, had little overt impact on sickness behaviour, although there was a trend towards anxiety behaviour. This was associated with discrete patterns of neuronal activity, which mirrored changes exhibited following vagus-dependent immune to brain communication following gut inflammation[112,248]. Correspondingly, localised inflammatory events have recurrently been shown to induce anxiety-like behaviours[244,290]. These results repeatedly suggest that localised inflammatory events impact on anxiety behaviour in rodent models via interaction with the vagus nerve.

In this chapter, we investigate how another form of localised granulocytic inflammation, namely acute allergic lung inflammation, impacts on the brain, by investigating alterations in classical sickness behaviours and anxiety, and exploring changes in c-fos expression throughout the neuraxis.

4.2 Methods

4.2.1 Challenge Protocol

To induce acute allergic lung inflammation, a protocol was developed, based on a well-established mouse model of allergic lung inflammation involving the formation of ovalbumin (OVA) immune complex in the lung (as reviewed by Nials and Uddin[249]). This is detailed in section 2.1.3.2.

4.2.2. Behavioural Analysis

Behavioural analysis was carried out on day 28 (the first day of intranasal OVA challenge, referred to hereafter as day 1), day 29 (referred to hereafter as day 2), day 30 (referred to hereafter as day 3) and day 32 (referred to hereafter as day 5), as detailed in section 2.1.4. Analysis of burrowing behaviour was carried out between 1 and 3 hours after OVA challenge and overnight on day 1, 2 and 3. Analysis of glucose consumption was carried out overnight on days 1, 2 and 3. Analysis in the open field apparatus was carried out 2 hours after OVA challenge on day 1 and 3 and a at a similar time point on day 5. Behavioural measurements in the elevated plus maze (EPM) were also initially carried 2 hours after OVA challenge on days 1 and 3 and at a similar time point on day 5, but was, in a subsequent study, only carried out on day 5.

4.2.3. Sample Collection

Bronchoalveolar lavage fluid (BALF) and serum samples were collected 3 hours after OVA challenge on days 1 and 3 or at a similar time point on day 5. BALF cell content was analysed as detailed in sections 2.2.4.1 and 2.2.4.2. Cytokine levels in BALF and serum were analysed using MSD technology as detailed in section 2.2.2.3. Brain samples for analysis of c-fos expression were collected on day 1 only and analysed as detailed in sections 2.2.1.2 and 2.2.1.4.

4.3. Results

4.3.1. Induction of Allergic Lung Inflammation

4.3.1.1 Bronchoalveolar lavage cells counts

To confirm if the challenge protocol employed resulted in allergic lung inflammation, a differential cell count was performed on the cells of the BALF. Following the first OVA challenge (day 1, Figure 4.1A), significantly higher levels of neutrophils (p=0.0234) were found in BALF of OVA immunised and challenged animals, as compared to controls. Overall, however, immunisation had no effect on cell numbers in BALF on day 1 ($F_{1,20}$=0.4673, p=0.5021). Similarly, immunisation did not have a significant effect on cell numbers on day 3 ($F_{1,32}$=0.1823, p=0.6723). However, there were significantly higher levels of eosinophils in OVA immunised animals as compared to controls on day 3 (p=0.0018). In contrast, non-immunised controls had significantly higher levels of neutrophils on day 3 (p=0.0112) than immunised animals (Figure 4.1B). On day 5 (Figure 4.1C), immunisation had no significant effect cell counts in BALF ($F_{1,28}$=3.989, p=0.0556) and non-immunised controls had significantly higher levels of BALF macrophages (p<0.0001) than immunised animals. Analysis of eosinophil numbers over time, however, (Figure 4.1D) revealed that immunisation had a significant effect on eosinophil number in BALF ($F_{1,20}$=113.8, p<0.0001), and eosinophil number was significantly dependent on day of sample collection ($F_{2,20}$ =13.02, p=0.0002). Eosinophil numbers were significantly elevated on day 1 (p=0.0326), peaking on day 3 (p<0.0001) and staying significantly elevated on day 5 (p<0.0001) in BALF of immunised animals, as compared to non-immunised controls.

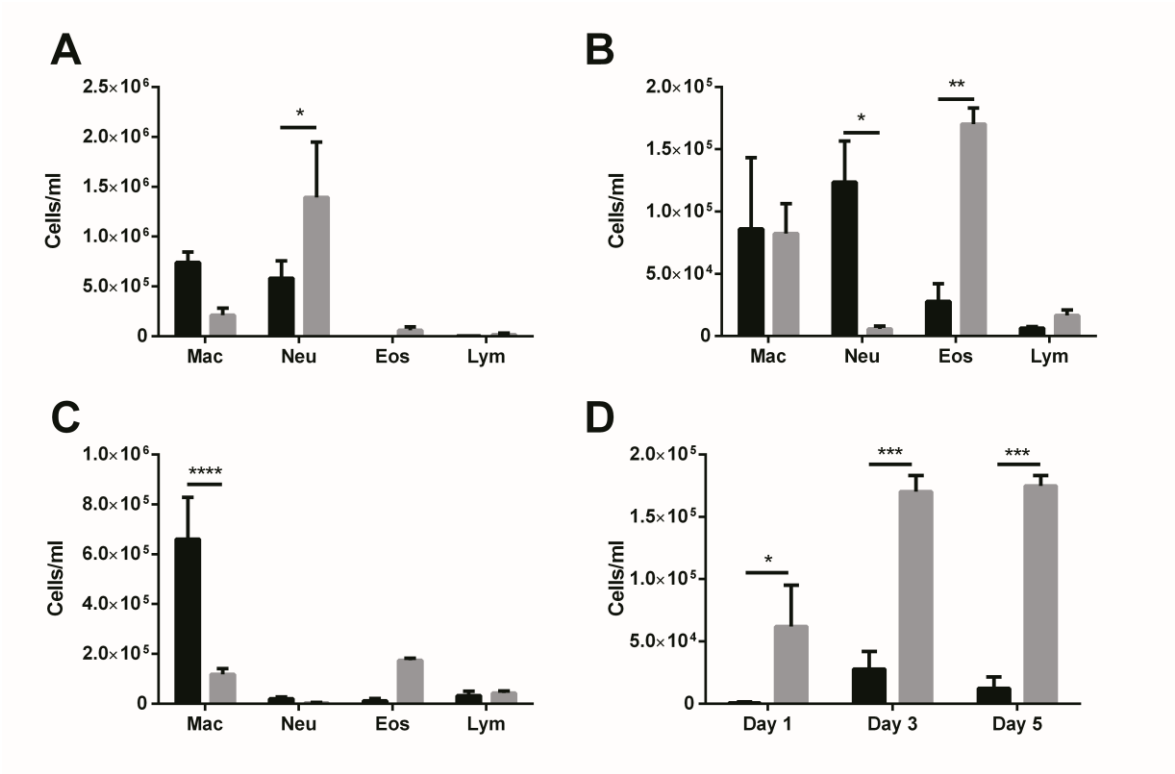

Figure 4.1 - Effect of OVA immunisation and i.n. challenge on cell numbers in the BALF on the first day (day 1, A) and third (day 3, B) day of OVA challenge and two days after the final OVA challenge (day 5, C). (D) Effect of OVA immunisation and i.n. challenge on eosinophil numbers in the bronchoalveolar lavage fluid on days 1, 3 and 5. Terminal lavage samples were collected 3 hours after OVA challenge on day 1 and day 3 or at an equivalent time on day 5; total cells were counted with a haemocytometer and a differential cell count was carried out through analysis of a Rapid Romanowsky stain. Values represented as mean total cells per ml lavage fluid ± SEM. Black bars are non-immunised and OVA challenged controls; grey bars are immunised and OVA challenged animals. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. control using a two way ANOVA followed by a Sidak's post-hoc test. n=5.

4.3.1.2 Bronchoalveolar lavage cytokines

To further determine the establishment of allergic lung inflammation, a panel of TH1 and TH2 cytokines and chemokines were measured in the BALF (Figure 4.2). OVA immunisation had no effect on levels of IFN-γ ($F_{1,18}$=0.2046, p=0.6564), IL-12 ($F_{1,18}$=4.190, p=0.0555) IL-1β ($F_{1,18}$=0.6536, p=0.4294), TNF-α ($F_{1,18}$=0.3034, p=0.5885) , IL-10 ($F_{1,18}$ = 0.6275, p=0.4836) or KC ($F_{1,18}$=1.930, p=0.1817) in BALF. In contrast, immunisation had a significant effect on levels of TH2-cytokines IL-4 ($F_{1,18}$=4.437, p=0.0495) and IL-5 ($F_{1,18}$=38.52, p<0.0001) in BALF. IL-4 was significantly elevated in BALF of OVA-immunised animals on day 3 (p=0.0031), as compared to non-immunised controls; IL-5 was significantly elevated in BALF of OVA-immunised animals on day 3

(p<0.0001) and day 5 (p=0.0294), as compared to non-immunised controls. The results also showed significantly elevated levels of IL-12 (p=0.0041) in BALF of non-immunised controls, as compared to OVA-immunised animals on day 3.

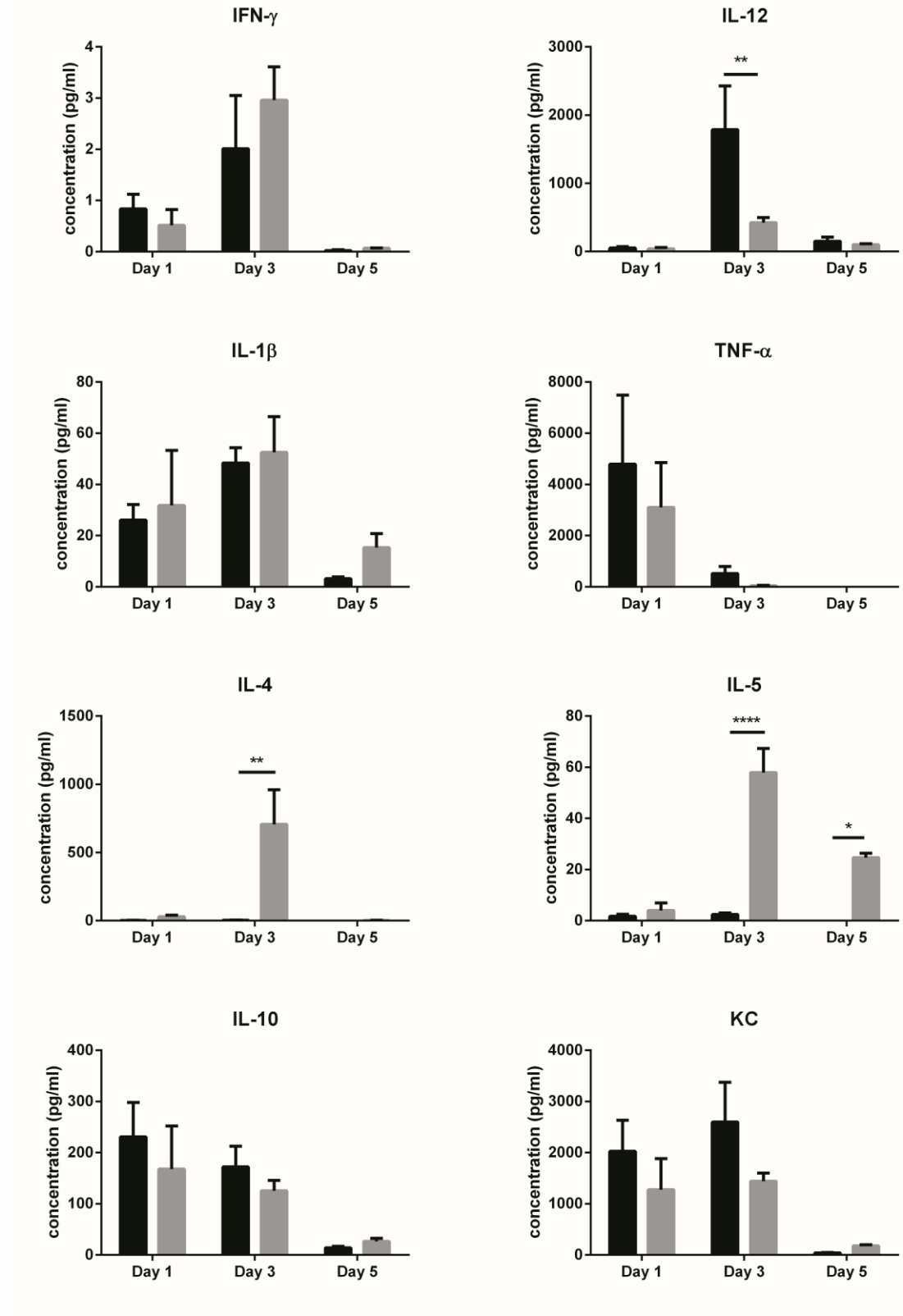
IFN-γ
concentration (pg/ml)
0
1
2
3
4
Day 1
Day 3
Day 5
IL-12
concentration (pg/ml)
0
1000
2000
3000
**
Day 1
Day 3
Day 5
IL-1β
concentration (pg/ml)
0
20
40
60
80
Day 1
Day 3
Day 5
TNF-α
concentration (pg/ml)
0
2000
4000
6000
8000
Day 1
Day 3
Day 5
IL-4
concentration (pg/ml)
0
500
1000
1500
**
Day 1
Day 3
Day 5
IL-5
concentration (pg/ml)
0
20
40
60
80
****
*
Day 1
Day 3
Day 5
IL-10
concentration (pg/ml)
0
100
200
300
400
Day 1
Day 3
Day 5
KC
concentration (pg/ml)
0
1000
2000
3000
4000
Day 1
Day 3
Day 5

Figure 4.2 – Effect of OVA immunisation and i.n. challenge on levels of cytokines in BALF on the first (day 1) and third (day 3) days of i.n. OVA challenge and two days after the final OVA challenge (day 5). Terminal BALF samples were collected 3 hours after OVA challenge on days 1 and 3 or at an equivalent time on day 5; BALF was analysed for cytokine content using a multiplex ELISA. Values expressed as mean pg cytokine per ml BALF ± SEM. Black bars represent non-immunised and OVA challenged controls; grey bars represent immunised and OVA challenged animals. *p<0.05, **p<0.01, ****p<0.0001 vs. control using a two way ANOVA followed by, if significant, a Sidak's post-hoc test. n=5

4.3.1.3 Serum cytokines

To determine the nature of inflammatory event, whether it was localised or systemic, a panel of TH1 and TH2 cytokines were measured in the serum on days 1, 3 and 5 (Figure 4.3). Immunisation had no effect on levels of IFN-γ ($F_{1,22}$=1.442, p=0.2426) in the serum. In contrast, intranasal challenge in immunised mice had a significant effect on serum levels of IL-12 ($F_{1,22}$=25.84, p<0.0001), IL-1β ($F_{1,22}$=13.96, p=0.0011), TNF-α ($F_{1,22}$=15.61, p=0.0007), IL-4 ($F_{1,22}$=14.15, p=0.0011), IL-5 ($F_{1,22}$=62.17, p<0.0001), IL-10 ($F_{1,22}$=8.283, p=0.0087) and KC ($F_{1,22}$=39.19, p<0.0001), with significant, but marginal elevation of serum levels of IL-12 on day 5 (p=0.0004), IL-1β on day 5 (p=0.0470) and TNF-α on day 5 (p=0.0018), as well as significant elevation of IL-4 on day 1 (p<0.0001), IL-5 on day 1 (p<0.0001) and day 3 (p<0.0001) and KC on day 1 (p<0.0001).

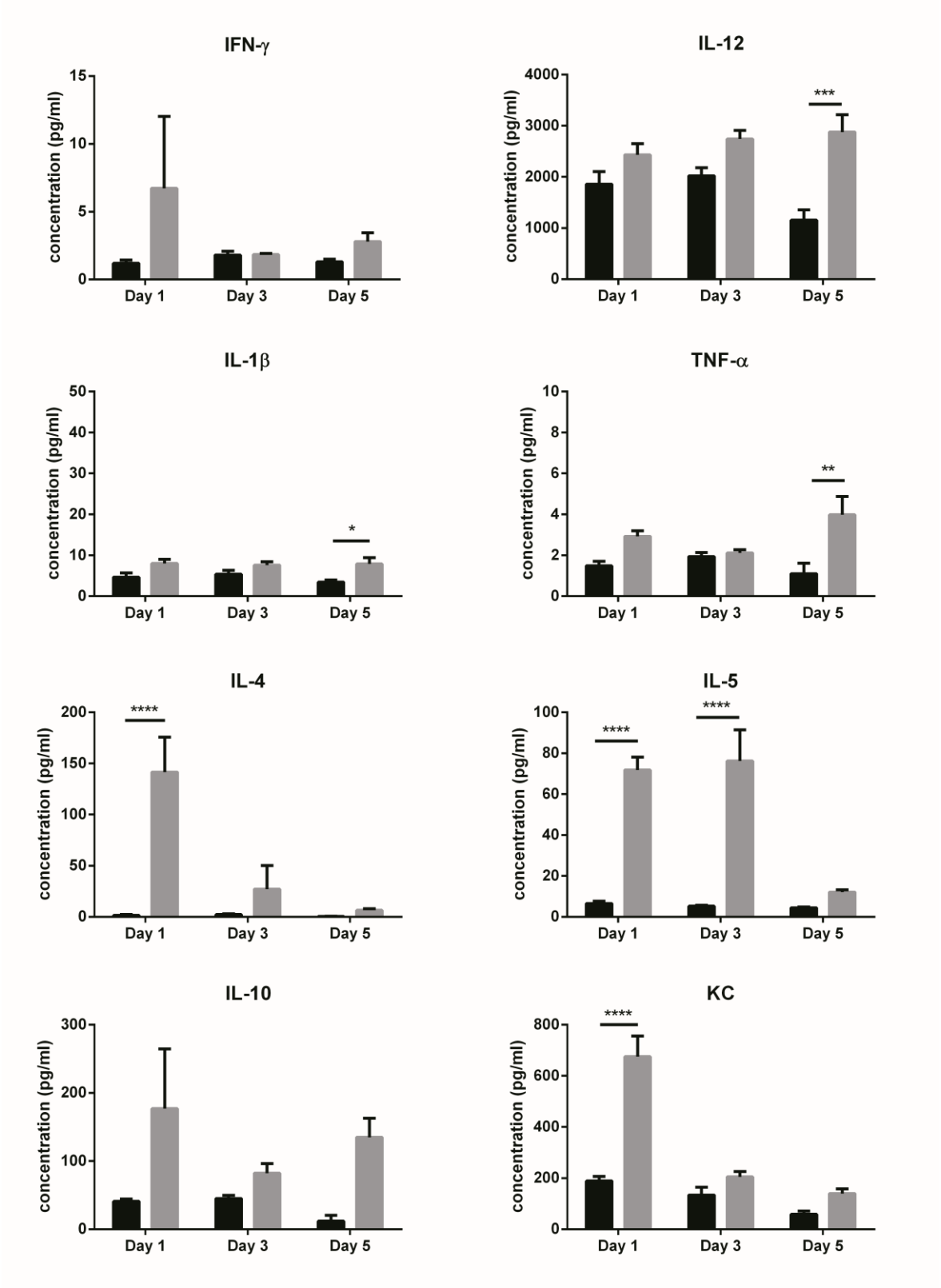
IFN-γ
concentration (pg/ml)
15
10
5
0
Day 1
Day 3
Day 5
IL-12
concentration (pg/ml)
4000
3000
2000
1000
0
***
Day 1
Day 3
Day 5
IL-1β
concentration (pg/ml)
50
40
30
20
10
0
*
Day 1
Day 3
Day 5
TNF-α
concentration (pg/ml)
10
8
6
4
2
0
**
Day 1
Day 3
Day 5
IL-4
concentration (pg/ml)
200
150
100
50
0
****
Day 1
Day 3
Day 5
IL-5
concentration (pg/ml)
100
80
60
40
20
0
****
****
Day 1
Day 3
Day 5
IL-10
concentration (pg/ml)
300
200
100
0
Day 1
Day 3
Day 5
KC
concentration (pg/ml)
800
600
400
200
0
****
Day 1
Day 3
Day 5

Figure 4.3 – Effect of OVA immunisation and i.n. challenge on levels of cytokines in serum on the first (day 1) and third (day 3) days of i.n. OVA challenge and two days after the final OVA challenge (day 5). Terminal serum samples were collected 3 hours after OVA challenge on day 1 and 3 or at an equivalent time on day 5; serum was analysed for cytokine concentration using a multiplex ELISA. Values expressed as mean pg cytokine per ml serum ± SEM. Black bars represent non-immunised and OVA challenged controls; grey bars represent immunised and OVA challenged animals. **p<0.01, ****p<0.0001 vs. control using a two way ANOVA followed by, if significant, a Sidak's post-hoc test. n=5

4.3.2. Behavioural Changes

Upon confirming the establishment of a TH2-type inflammatory event in the lung, behavioural changes were investigated as a functional readout of immune to brain communication. Various behaviours were assayed, including burrowing behaviour and glucose consumption, as measures of anhedonia, activity in the open field, as a measure of lethargy, as well as rears in the open field and activity in the elevated plus maze (EPM), as measures of anxiety. OVA immunisation and challenge had no significant effect on burrowing behaviour over 2 hours ($F_{1,6}$=0.3144, p=0.3744) or overnight ($F_{1,6}$=1.280, p=0.3011), nor did it have a significant effect on glucose consumption ($F_{1,6}$=0.2967, p=0.6056) (Figure 4.4). Similarly, OVA immunisation and challenge had no significant effect on activity ($F_{1,58}$=0.9989, p=0.3217) or rears ($F_{1,53}$=0.2337, p=0.6308) in the open field or activity in the EPM ($F_{1,59}$=2.421, p=0.1250), when measurements were taken on days 1, 3 and 5. Despite this, there was a significant reduction in the percent of time spent in the open arms of the EPM, as compared to the baseline measurement, in immunised animals compared to non-immunised controls on day 5 (p=0.0404). When the EPM assay was carried out on day 5 only, there was a trend towards a decrease in the time spent in the open arms by immunised animals, as compared to non-immunised controls (Figure 4.5), though this was not significant (t_{18}=1.5.19, p=0.1461).

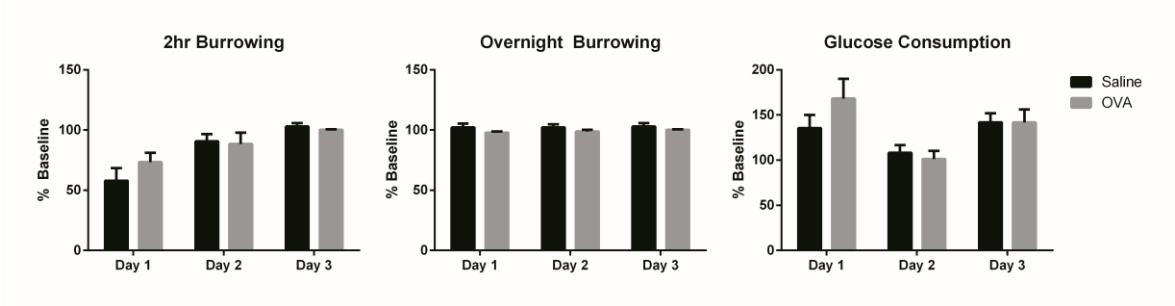

Figure 4.4 – Effect of OVA immunisation and i.n. challenge on burrowing behaviour (2hour and overnight) and glucose consumption on days 1, 2 and 3 of i.n. OVA challenge. 2hour burrowing was carried out between 1 and 3 hours after OVA challenge. Overnight burrowing and glucose consumption was carried out overnight after each challenge. A baseline measurement was taken at a similar time on the day before the first challenge. Values are expressed as mean % of the baseline measurement ± SEM. Statistical analysis was carried out using a two way ANOVA.

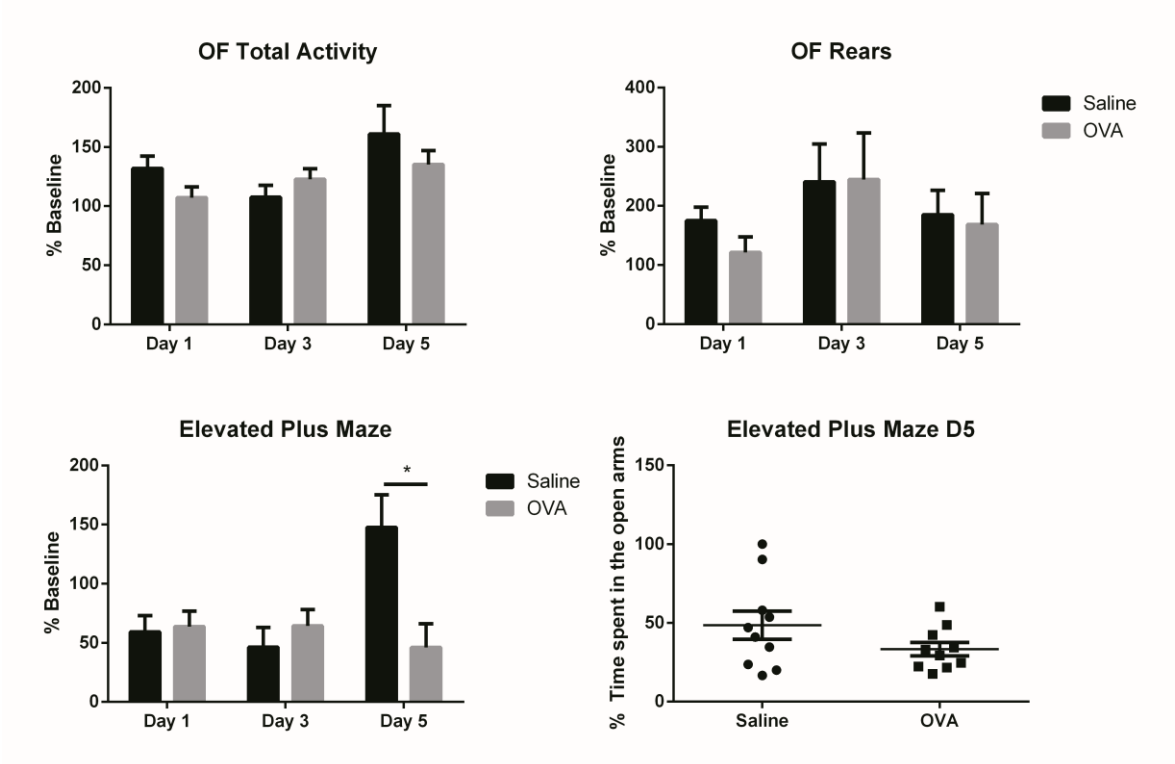

Figure 4.5 – Effect of OVA immunisation and i.n. challenge on activity and rears in the open field (OF) and EPM on days 1, 3 and 5, as well as EPM on day 5 only. Behavioural analysis in the OF and EPM was carried out between 2 and 3 hours after i.n. OVA challenge on days 1 and 3, or at a similar time on day 5. For OF and EPM time-course: a baseline measurement was carried out at a similar time the day before the first challenge; values are expressed as mean % of the baseline measurement ± SEM. *$p<0.05$ vs. saline control using a two way ANOVA followed by a Sidak's post-hoc test. For EPM on day 5 only: values are expressed as mean % of time spent in the open arms ± SEM. Statistical analysis was carried out using a Student's t-test.

4.3.3 Changes in Neuronal Activity

Despite the lack of overt changes in sickness behaviour following OVA immunisation and challenge, immune to brain communication following allergic lung inflammation was further investigated by examining neuronal activity in the brain, in the form of c-fos expression. As in the previous chapter, all regions in the brain from Bregma to Bregma -8.12 mm were analysed for c-fos immunoreactivity.

C-fos immunoreactivity was examined on all three days of OVA challenge. However, as c-fos is an indicator of acute changes to peripheral homeostasis and not chronic alterations [302], expression was only observed after the first day of OVA challenge. On this day, c-fos expression was evident only in the locus coeruleus (LC) in the hindbrain and in the nucleus of the solitary tract (NTS) in the brainstem in immunised animals (Figure 4.6). Quantification of expression indicates that c-fos expression in the NTS (t_4=6.033, p=0.0038) and LC (t_4=7.389, p=0.0018) was significantly higher in immunised animals, as compared to non-immunised controls (Figure 4.7).

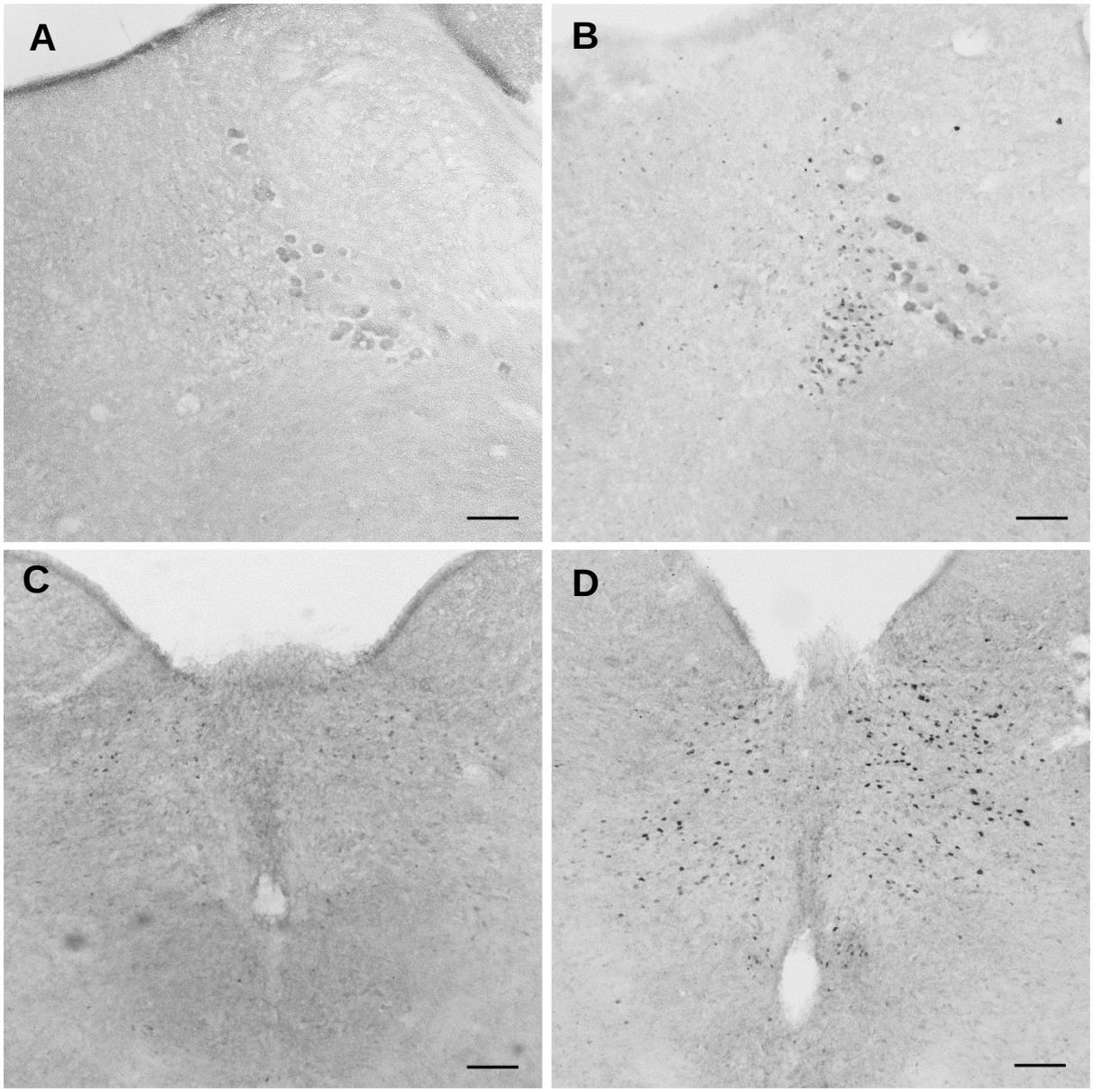

Figure 4.6 - Representative images of c-fos immunoreactivity in the LC (A and B) and NTS (C and D) in non-immunised controls (A and C) and immunised (B and D) animals on day 1 of i.n. OVA challenge. n=3; Scale bar = 100μm

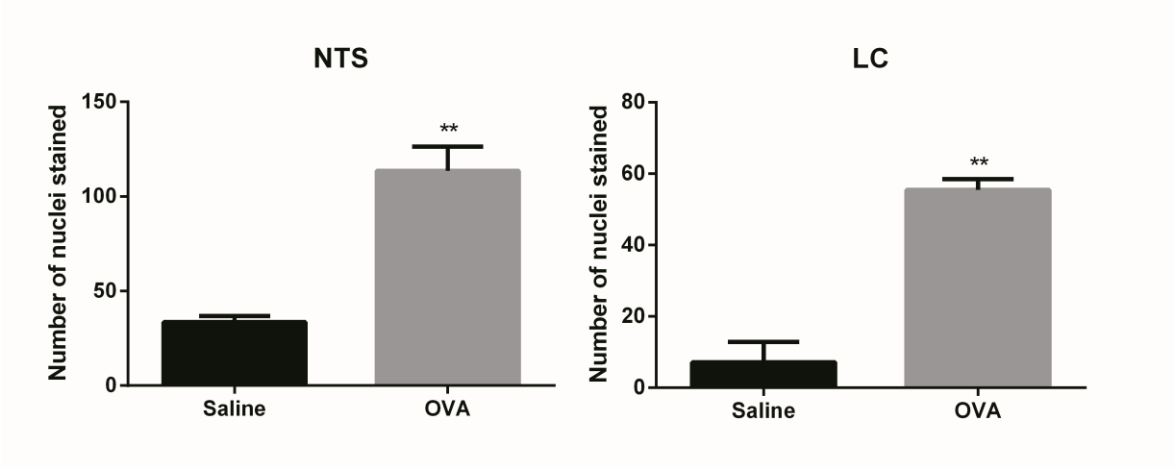

Figure 4.7 - Quantification of c-fos immunohistochemistry in NTS and LC following OVA immunisation and i.n. challenge. Brain tissue was collected 3 hours after OVA challenge on day 1; c-fos expression was assessed using immunohistochemistry and analysed using ImageJ software. Values expressed as mean number of nuclei stained in the respective area ± SEM. **$p<0.01$ vs. control using the Student's t-test.

4.4 Discussion

It is well established that systemic inflammatory events dominated by pro-inflammatory (IL-1β, TNF-α, IL-6) and TH1 (IFN-γ, IL-12) cytokines impact on the brain, inducing sickness behaviours and specific patterns or neuronal activation. In the previous chapter it was demonstrated that localised inflammatory events in the peritoneum, dominated by granulocytes, granulocyte chemoattractants and TH2 cytokines (IL-4, IL-5, KC) also induce changes in neuronal activity and evident, but significantly less marked, changes in behaviour. Here we provide evidence to suggest that localised granulocytic inflammation in the lung, dominated by TH2 cytokines, similarly impacts on the brain, inducing highly discrete patterns of neuronal activity, and altering specific behaviours.

4.4.1 Establishing TH2 Lung Inflammation

To establish an acute TH2 type allergic inflammatory event in the lung, a protocol was developed based on a well-established mouse model of allergic lung inflammation. This OVA immune-complex model involves the immunisation of an animal against OVA, together with the adjuvant aluminium hydroxide ($Al(OH)_3$), which is known to promote the development of a TH2 type immune response and encourage the development of

immunoglobulin IgE[303]. Upon subsequent challenge with OVA, immune complex is formed and a type III hypersensitivity reaction is established, resulting in the cross-linking of IgE on the surface of mast cells, leading to the production of TH2 cytokines and the infiltration of lymphocytes and eosinophils. The OVA immune complex model has previously been shown to mimic asthma by triggering airway hyperresponsiveness, airway remodelling, recruitment of eosinophils and TH2 cytokine production[249].

The results clearly demonstrate that a TH2 type allergic inflammatory event is established in the lung using the model developed. There is a progressive and significant increase in eosinophil numbers in the BALF, alongside significant elevations in TH2 cytokines IL-4 and IL-5 in the BALF and serum, both characteristic features of allergic lung inflammation in humans[22]. Increases in TH2 cytokines occur earlier in the serum, peaking on the first day of OVA challenge, as compared to the BALF, where TH2 cytokines peak at day 3. The early rise in systemic TH2 cytokines reflects an initial recruitment of eosinophils and lymphocytes from bone marrow and regional lymph nodes to the lungs. This is a key bridging event between the early and late phase of allergic lung inflammation, occurring at 3 to 6 hours after allergen challenge, in which cytokines and other immune mediators produced by lung-resident mast cells enter the circulation and recruit further immune cells to the lung. This subsequently results in the localised development of TH2-type inflammation in the lung, dominated by eosinophils and TH2 lymphocytes, evident by the peak in eosinophil number and TH2 cytokines in the BALF on day 3.

A mild inflammatory response was also found to develop in control animals, which were not immunised, but challenged intranasally with OVA. Analysis of BALF cell counts demonstrates an initial rise in macrophage numbers on day 1, neutrophilia on day 3 and a second rise in macrophage numbers on day 5. This is associated with an elevation of BALF IL-12 on day 3, a cytokine that has previously been shown to be critical for neutrophil activation[304]. Overall, this would suggest that an inflammatory response may have been mounted against OVA alone. It is worth noting that OVA was not tested for LPS content and may thus have been contaminated with endotoxin, which is a known driver of classical macrophage activation, IL-12 production and neutrophilia[305]. The subsequent rise in macrophage numbers on day 5, two days after OVA challenge, may reflect resolution of inflammation, as it is associated with an evident decrease in pro-inflammatory and TH1 cytokines in both BALF and serum. However, further analysis of

macrophage phenotype would be necessary to confirm this postulate. The experiment has been repeated, in which control animals were immunised, but challenged with saline (Appendix 9.4). With this control, an inflammatory response in the lungs was absent.

4.4.2 Impact of TH2 Lung Inflammation on Behaviour

Upon confirming that a TH2 type inflammatory event developed in the airways, behavioural changes were measured as an indicator of immune to brain communication. TH2 lung inflammation had no impact on burrowing behaviour or glucose consumption, measured on all three days of OVA challenge. Burrowing behaviour is a species-typical behaviour dependent on an intact hippocampus[255]. Although not completely defined, reductions in burrowing behaviour are believed to be markers of reduced cognition, as animals are trained to learn the behaviour prior to experimentation, as well as anhedonia, as it is believed that rodents take pleasure out of this behaviour. Hence, TH2 lung inflammation has no evident impact on cognition or anhedonia. This is further corroborated by the lack of changes in glucose consumption, also a measure of anhedonia, as a glucose solution is believed to be a palatable and pleasurable drink for rodents. To further determine the impact of TH2 lung inflammation on behaviour, activity and rears in the open field was measured. Reductions in these behaviours are known to occur in rodents following LPS treatment[61], are measures of lethargy; however, no changes in activity or rears were apparent following TH2 lung inflammation. The lack of typical sickness behaviours is not surprising, as clinical symptoms of illness are not typically seen in asthmatic patients.

Rears are additionally believed to reflect anxiety behaviour[306], where a reduction in rears suggests an increase in anxiety. This assay, alongside the EPM, was used as a measure of anxiety, but similarly, no changes in this behaviour were apparent following TH2 lung inflammation, when measured over time. However, these results may be an artefact of the repeated exposure to the assays, including training and baseline exposure. Animals quickly adapt to new environments and would, quite possibly, show few obvious signs of anxiety when placed in familiar surroundings. As a result, the EPM assay was repeated, where a single measurement was recorded two days after the final OVA challenge. No significant change in behaviour was evident, though there was a trend towards a decrease in time spent in the open arms of the EPM in animals with TH2 lung inflammation,

suggestive of increased anxiety. This trend is corroborated by both rodent models of allergic inflammation, which show increased anxiety[108,247], as well as epidemiological studies, which suggest that individuals with asthma have a two-fold higher risk of developing mood disorders such as anxiety[307]. The trend in anxiety may additionally be related to the localised nature of the inflammation, as localised allergic and pro-inflammatory events in the gut have previously been shown to induce anxiety-like behaviour[108,290].

4.4.3 Impact of TH2 Lung Inflammation on Neuronal Activity

Despite the lack of overt changes in behaviour following allergic lung inflammation, discrete increases in neuronal activity as measured by c-fos expression clearly verify the occurrence of immune to brain communication. Allergic lung inflammation resulted in a distinct pattern of neuronal activity in the brain, centred only in the LC (hindbrain region) and NTS (brainstem region), with no evidence of neuronal activity in the forebrain. This differs substantially from the patterns of neuronal activation following systemic inflammation induced by LPS, as well as localised peritoneal inflammation induced by zymosan, as evidenced in Chapter 3, suggesting that both composition and location of an inflammatory response is central to the impact on the CNS. This is undoubtedly related to the immune to brain communication pathways employed.

As explained in Chapter 3 and widely in the literature, immune stimulation with LPS, which induces pro-inflammatory cytokine and prostaglandin production, activates the endothelium, cells of the CVOs and the vagus nerve, resulting in widespread c-fos expression across the neuraxis. Contrastingly, zymosan, which induced a localised peritoneal response, and a small, but non-significant upregulation in circulating IL-1β, induced neuronal activation in discrete areas including the NTS, LC and PVN, postulated to dominantly be due to localised vagal stimulation and, to a lesser degree, activation over the endothelium or via CVOs. The precise pattern of neuronal activity following TH2 lung inflammation similarly gives an indication of the pathway of immune to brain communication employed. The particular subdivision of the NTS that becomes activated is the caudomedial NTS (cmNTS) (Figure 4.8), an area that has previously been shown to be the location of the terminal afferents of the cardiopulmonary c-fibres in cats[308] and rats[206]. Pulmonary c-fibres, as described in Chapter 1, are part of the vagus nerve and are

stimulated by endogenous chemical stimulation, such as by mediators of inflammation[220,222]. It has been well-described in the literature that allergic lung inflammation impacts on these fibres, resulting in both axonal and central reflexes[199]. This indicates that allergic inflammatory events in the lung communicate to the brain via the bronchopulmonary c-fibres of the vagus nerve, resulting in activation of the NTS.
In the field of immune to brain communication, it is generally believed that signalling via the vagus nerve is dependent on local production of IL-1β and PGE_2, due to the expression of functional IL-1 and EP3 receptors on vagal paraganglia[94,95]. No increase in IL-1β was found locally in the BALF; PGE_2, however, was not measured. It has been well described that early phase responses in allergic inflammation, which occur between 1 and 3 hours after allergen exposure, induce mast cell degranulation and the release of lipid metabolites, such as prostaglandins[142]. It has additionally been shown that signalling via the vagus nerve in allergic inflammation is dependent on mast cell degranulation[245], suggesting that immune to brain communication in this model of allergic lung inflammation may dependent on PGE_2 production in the lung parenchyma.

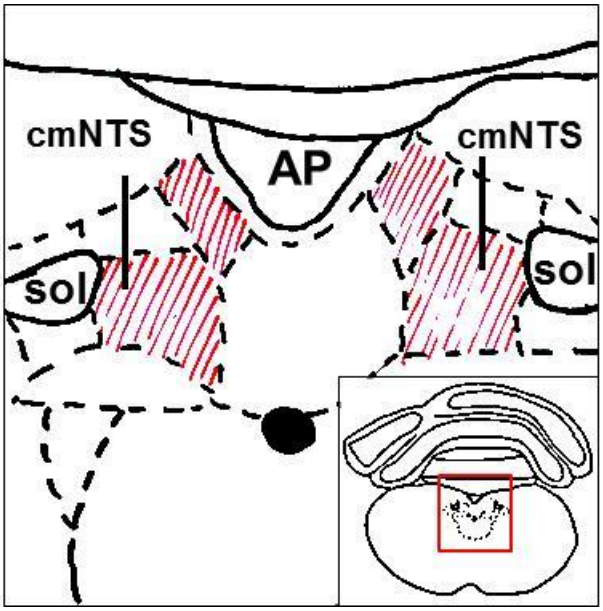

Figure 4.8 – Demonstration of the location of staining the caudomedial NTS (cmNTS), shaded red. Approximate coordinates: Bregma – 7.75

Similarly, activation of the LC has repeatedly been shown to be induced by vagus nerve stimulation[309], and is additionally known to receive projections from the NTS[310], suggesting that the increased activity in this area is directly due to inflammatory events in

the lung. Activation of the LC has additionally been shown to be related to stress behaviour (as reviewed by Sved et al[291]), demonstrating increases in c-fos expression following stress protocols[311]. Increased activity in the LC may be related to triggering later adaptive mechanisms, such as trends in anxiety behaviour seen on day 5, providing a preliminary biological explanation for the increased risk of anxiety and panic disorders evident in human asthmatics[307]. The lack of c-fos expression in the forebrain regions, including limbic structures, may similarly explain why behaviours such as burrowing behaviour and glucose consumption were unchanged following allergic lung inflammation.

It is worth noting that the NTS is often described as the "relay" station of immune to brain communication, receiving signals from the nearby area postrema, the endothelium and the vagus nerve, and projecting to a constellation of forebrain areas via the LPBN (see Figure 1.5 of Chapter 1). Retrograde labelling studies have demonstrated that external regions of the LPBN, which are activated upon systemic immune challenge[312], receive inputs from cmNTS, but also from the parvicellular, intermediate and commissural NTS, as well as the area postrema[313]. Despite this, no activation was seen in the LPBN following allergic lung inflammation. To date and to my knowledge, nobody has investigated if activation of multiple areas of the NTS is required for activation of the LPBN and consequent activation of forebrain structures. The data in this chapter suggests that activation of a single subnucleus in the NTS is not sufficient to activate the LPBN and forebrain regions. Indeed, in Chapter 3, increased c-fos expression in the medial NTS, as well as the AP was evident, as well as increases in c-fos expression in forebrain regions such as the PVN, suggesting that activation of multiple brainstem subnuclei is necessary for activation of forebrain regions. Indeed, following localised inflammatory events in the gut, a more widespread distribution of c-fos in the rostral lateral, rostral central, caudal dorsomedial, caudal medial and caudal central subnuclei of the NTS is apparent, together with c-fos expression in forebrain regions such as LPBN, CeA and PVN[112,248].

4.5 Conclusion

The results in this chapter demonstrate that an acute, localised and lung-based TH2 type inflammatory event results in activation of discrete hindbrain and brainstem nuclei, without overt induction of sickness behaviour. This differs greatly from the impact of systemic

inflammatory events on the CNS, such as induced by LPS, which lead to widespread neuronal activation and clear behaviour changes. The results highlight the importance of investigating the impact of localised inflammation on the brain using models for a common inflammatory disease in humans. Studying the effect of systemic inflammation on the CNS using LPS will help provide an overview of the multiple pathways by which the immune system communicates with the brain, but may not provide a physiological explanation for how real infections and inflammatory events affect the CNS.

In this chapter, it was additionally demonstrated that the effect of an allergic inflammatory event on the peripheral neuronal fibres, specifically the vagal c-fibres, is relayed to the terminal synapses in the brainstem. The next clear step is to investigate whether a chronic allergic inflammatory event leads to continuous activation of this area of the brainstem, and whether this has an impact on neurochemistry or neuroplasticity, consequently impacting on the innate immune system of the brain.

Chapter 5: The effect of chronic allergic lung inflammation on neuroplasticity and inflammation in the CNS

5.1 Introduction

Bronchopulmonary c-fibres are afferent fibres of the vagus nerve which project from the lung compartment to the caudomedial nucleus of the solitary tract (cmNTS) in the brainstem[203]. In response to inflammatory stimuli, these fibres elicit a central nervous system (CNS) reflex, a physiological phenomenon necessary for protection of lungs against inhaled irritants, through the induction of apnoea, shallow breathing, cough, bronchoconstriction and mucous secretion[314]. In asthma, however, this reflex is exaggerated, resulting in more frequent physiological effects, ultimately contributing to the symptoms of the disease[235].

It is well-documented that allergic lung inflammation is associated with a multitude of genotypical and phenotypical changes in bronchopulmonary c-fibres, the peripheral component of the CNS reflex. In response to inflammation, these fibres release neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), augmenting inflammation and resulting in a vicious cycle of continually increased inflammation, termed neurogenic inflammation[210]. In addition, inflammation has been shown to alter expression and sensitivity of numerous receptors on the surface of these fibres[226-228,231], leading to hypersensitivity and subsequent augmentation of the CNS reflex. These changes are believed to play a key role in establishing the symptoms of asthma.

Despite the breadth of work dedicated to understanding the role of peripheral fibres in asthmatic inflammation, few studies have considered the role of the central component of the CNS reflex in reflex exaggeration and asthma symptoms. The CNS reflex is believed to involve the release of glutamate and the neurokinin substance P at the first central synapse of afferent c-fibres in the cmNTS, both of which play a role in the CNS reflex-evoked cough response, as inhibition of N-methyl-D-aspartate (NMDA) glutamate receptors and neurokinin receptors (NK1) in the NTS prevent cough[315]. Increases in SP concentration in the NTS have been shown to sensitise the cough reflex[238], and airway inflammation has repeatedly shown to alter concentrations of SP in the NTS[236,237], implying a SP-dependent central sensitisation of the reflex in response to airway inflammation. In addition, extended tobacco smoke (ETS)-induced lung inflammation has shown to induce plasticity in the NTS[239], a glutamate-dependent phenomenon[316] that would

result in increased activity of efferent fibres, and subsequent exaggeration of the CNS reflex.

In the previous chapter, we found that acute allergic inflammation resulted in discrete neuronal activation, specifically in the cmNTS of the brainstem. In this chapter, we investigate whether continuous or chronic allergic lung inflammation leads to continual activation of this area and thus plasticity of neuronal fibres in the brainstem and whether this is associated with central inflammatory responses.

5.2 Methods

5.2.1 Pilot Experiment

To initially investigate the effect of chronic allergic lung inflammation on the brain, a pilot experiment was carried out using a severe model of allergic lung inflammation, as detailed in section 2.1.3.5. Two days after the final OVA challenge, lung tissue was analysed for inflammation macroscopically, histochemically (as detailed in section 2.2.1.1) and immunohistochemically for macrophage markers (as detailed in section 2.2.1.3); circulating TH2 cytokines were additionally measured using MSD technology, as detailed in section 2.2.2.3. Brainstem samples were subsequently analysed for expression of neuroplasticity and inflammation-associated genes, as detailed in sections 2.2.3.1 (protocol B), 2.2.3.4 and 2.2.3.7.

5.2.2 Challenge Protocol for Chronic Allergic Lung Inflammation

To induce chronic allergic lung inflammation, a series of protocols were developed based on a well-established mouse model of allergic lung inflammation involving the formation of immune complex in the lung, as detailed in sections 2.1.3.3 and 2.1.3.4. Each protocol involved an increasing number of OVA challenges, to induce acute, sub-chronic and chronic lung inflammation.

5.2.3 Sample Collection

Two days after the final OVA challenge, bronchoalveolar lavage fluid (BALF) was analysed for cellular composition as described in sections 2.2.4.3 and 2.2.4.4. Brainstem samples were analysed for expression of neuroplasticity and inflammation-associated

genes as detailed in sections 2.2.3.1 (protocol B), 2.2.3.4, 2.2.3.7 and 2.2.3.8. Hippocampus and brainstem sections were analysed immunohistochemically for expression of microglial markers, and for presence of IgG, as detailed in section 2.2.1.3.

5.3 Results

5.3.1 Confirmation of the Establishment of Severe TH2 Type Inflammation

To investigate whether TH2 lung inflammation had an impact on the expression of neuroplasticity- and inflammation-associated genes in the brainstem, a pilot experiment was conducted, in which animals were hyperimmunised against OVA. Repeated immunisation and boosting was carried out to induce a severe inflammatory response upon re-exposure to OVA.

5.3.1.1. Macroscopic examination of lung tissue

To confirm that hyperimmunised and challenged animals exhibited a severe form of TH2 type inflammation in the lungs, lung tissue was initially analysed macroscopically (Figure 5.1). Compared to lungs of non-immunised animals, lungs from hyperimmunsed animals appeared oedemous, consolidated, haemorrhagic and hyperaemic, suggestive of increased and severe inflammation.

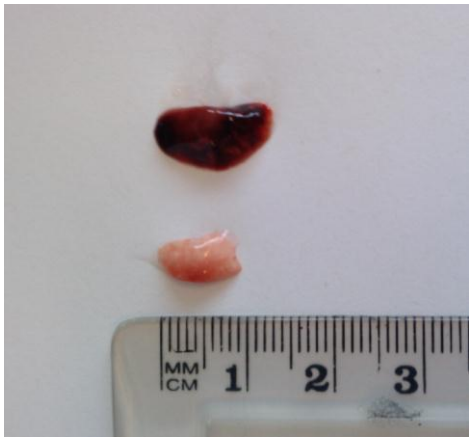

Figure 5.1 - Macroscopic examination of upper lobe of lung from OVA- (top) and saline- (bottom) challenged animals

5.3.1.2 Analysis of immune-complex formation in lung parenchyma

Lung tissue was further analysed by immunohistochemical staining of IgG and OVA, to determine the formation of immune complexes. Lungs from hyperimmunised animals show increased immunoreactivity for OVA and increased colocalisation of IgG and OVA than lung tissue from non-immunised animals (Figure 5.2), suggesting the formation of OVA immune complexes.

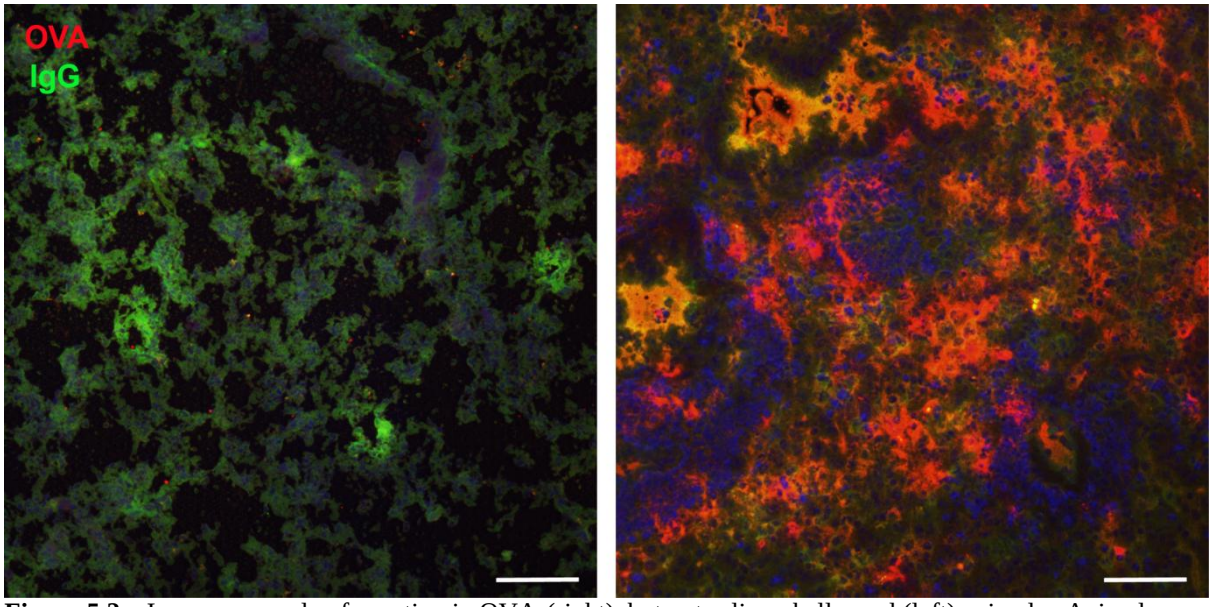

Figure 5.2 – Immune complex formation in OVA-(right), but not saline-challenged (left) animals. Animals were culled two days after the final OVA challenge; lungs were inflated with a 1:1 mixture of OCT and saline and frozen in OCT in isopentane on dry ice. Lung sections were immunohistochemically stained for OVA (red) and IgG (green). Representative of n=3. Scale bar: 75µm

5.3.1.3 Histochemical analysis of lung tissue

Haemotoxylin and eosin (H&E) stain of lung tissue revealed increased inflammation in the lung parenchyma of hyperimmunised, as compared to non-immunised, animals, with the formation of perivascular cuffs (Figure 5.3B) in hyperimmunised animals. In addition, the H&E stain shows evidence of thickening of submucosal layers in immunised animals (Figure 5.3D), but not non-immunised animals, which could contribute to airway narrowing.

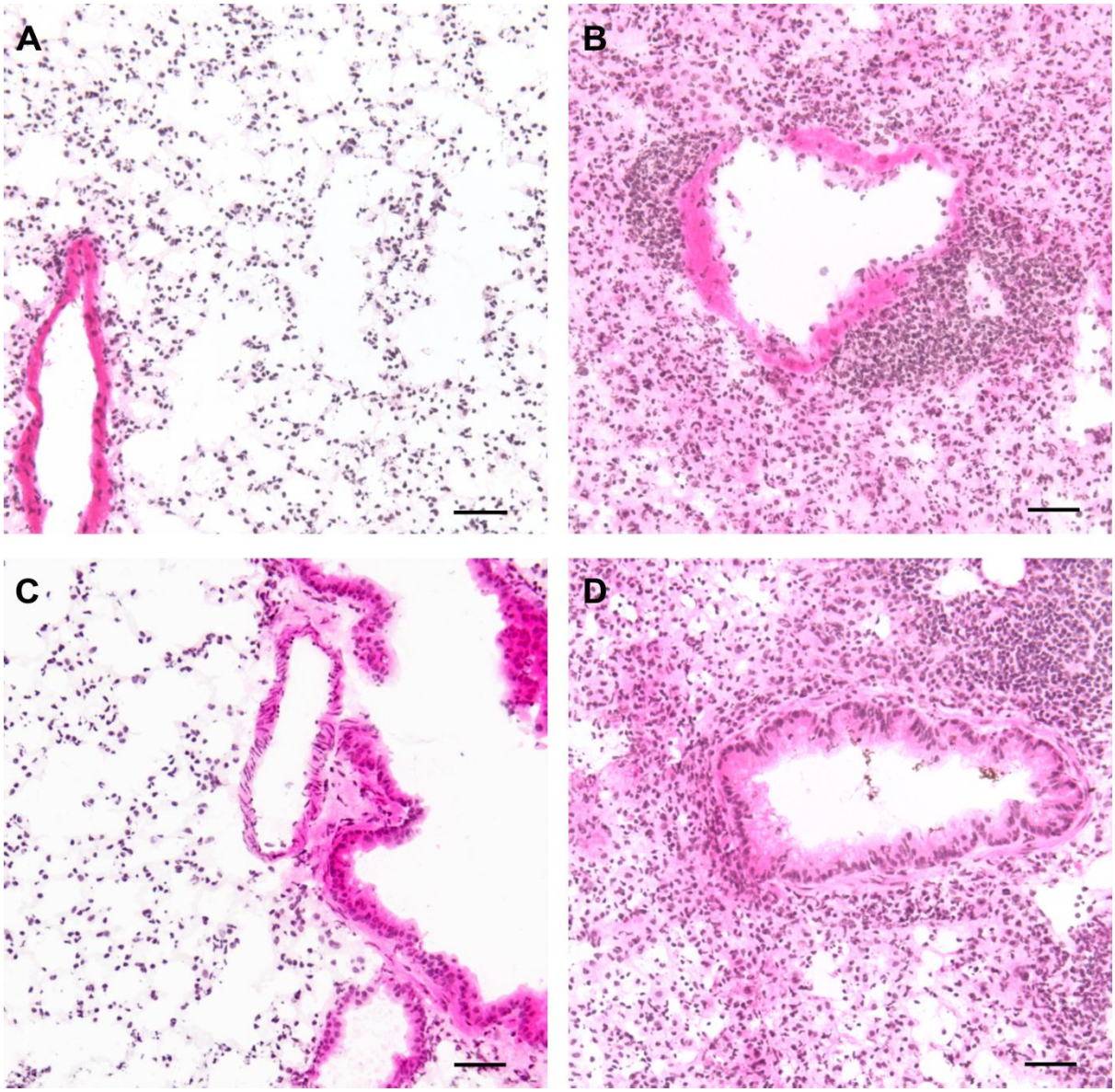

Figure 5.3 – Representative H&E stain of lung tissue in saline- (A, C) and OVA-challenged (B, D) animals. Animals were culled two days after final OVA challenge; lungs were inflated with a 1:1 mixture of OCT and saline and frozen in OCT in isopentane on dry ice. Representative of n=3. Scale bar: 100µm.

5.3.1.4 Immunohistochemical analysis of macrophage polarisation in lung tissue

To determine the nature of inflammation in the lungs of hyperimmunised animals, lungs were immunohistochemically stained for the presence of macrophages, through analysis of CD68 expression, as well as macrophage polarisation, through analysis of the expression of alternatively activated macrophage markers Dectin-1 and YM1 (Figure 5.4). Lungs from hyperimmunised animals show increased immunoreactivity for CD68, Dectin-1 and YM1, as compared to lungs from non-immunised animals.

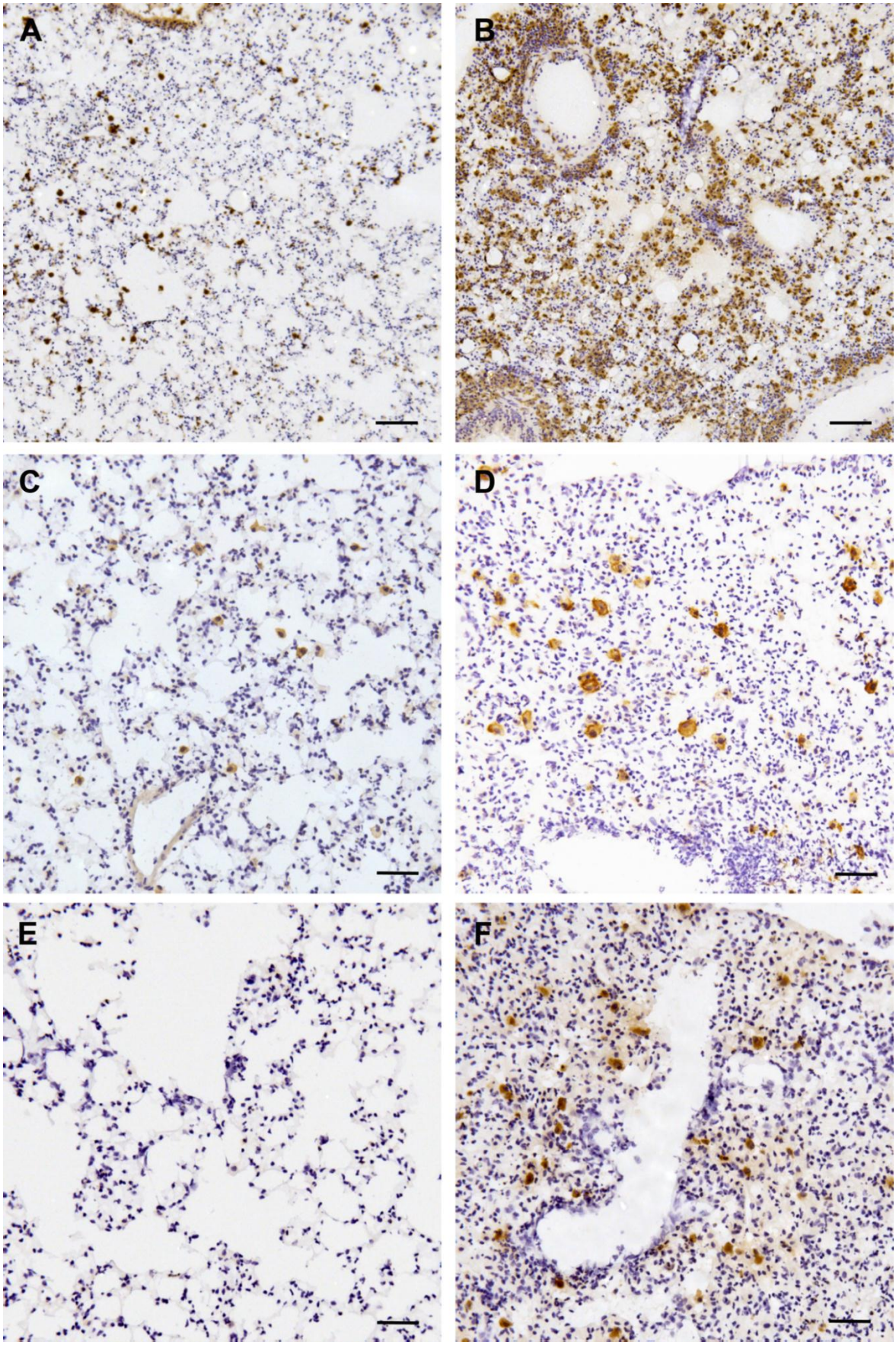
A
B
C
D
E
F

Figure 5.4 – Representative immunohistochemical stain of CD68 (A-B), Dectin-1 (C-D) and YM1 (E-F) in saline (A, C, E) and OVA (B, D, F) challenged animals. Animals were culled two days after final OVA challenge; lungs were inflated with a 1:1 mixture of OCT (optimum cutting temperature) and saline and frozen in OCT on dry ice. n=3. Scale bar A-F: 100µm.

5.3.1.5 Serum cytokines

To determine the nature of the inflammatory event, TH2 cytokines (IL-4, IL-5) were measured in the serum of hyperimmunised animals (Figure 5.5). Serum from hyperimmunised animals had substantially, but not significantly, elevated levels of IL-4 (t_4=1.410, p=0.2315) an IL-5 (t_4=1.782, p=0.1493), as compared to serum from non-immunised controls, suggesting the development of TH2-type inflammation.

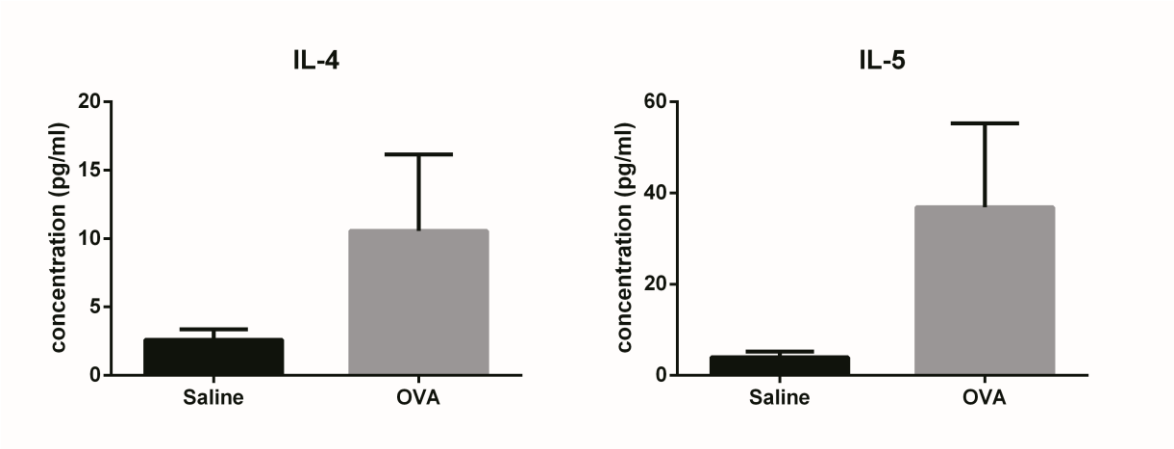

Figure 5.5 – The effect of OVA challenge on serum levels of IL-4 and IL-5. A terminal serum sample was collected two days after the final OVA challenge. Cytokine levels were measured using a multiplex ELISA. Values expressed as mean concentration in pg per ml serum ± SEM. Statistical analysis carried out using Student's t-test. n=3

5.3.2. The Effect of Severe TH2 Type Lung Inflammation on Gene Expression in the Brainstem

Upon confirming the establishment of severe TH2-type lung inflammation, the effect on the brain was investigated by analysing the expression of a collection of neuroplasticity- and inflammation-associated genes in the brainstem using multiplex qPCR array technology.

5.3.2.1 Genes associated with neuroplasticity

The expression of 84 genes associated with neuroplasticity was analysed. Only genes that were up- or downregulated above or below two-fold were considered physiologically relevant. These genes were subsequently grouped into categories, dependent on their specific roles within the cell (Table 5.1). Severe lung inflammation was associated with upregulated expression of 33 genes in the brainstem, eight of which were significantly upregulated, and downregulation of 2 genes, none of which were significant. Camk2g, Gnai1, Nr4a1, Synpo, Homer1, Plat, Dlg4, Grin1, Grin2d, Gria3 and Grm1 were significantly upregulated. A full list of genes and their regulation can be found in Appendix 9.5.

Table 5.1

Regulation of genes associated with neuroplasticity in the brainstem of mice with severe allergic lung inflammation

Category	Sub-category	Gene	Regulation	p-value
Cell Proliferation and Differentiation	Intracellular Signalling Molecules	Adcy1	5.35	00.110
		Akt1	2.02	0.495
		Camk2a	9.52	0.231
		Camk2g	4.44	0.008
		Gnai1	2.42	0.039
		Mapk1	2.11	0.110
		Nos1	3.725	0.252
		Pim1	4.64	0.197
		Ppp1ca	2.50	0.059
		Prkcc	3.04	0.138
		Prkg1	2.41	0.140
	Transcription Factors	Cepbd	23.00	0.188
		Creb1	3.01	0.836
		Jun	2.40	0.088
		Nr4a1	5.20	0.008
	Growth Factors/Neurotrophins	Ntf5	-2.05	0.283

Axonal Growth and Synapse Formation	Cell adhesion	Ncam1	-3.20	0.379
		Synpo	4.48	0.033
	Axonal Growth	Adam10	2.26	0.230
		Homer1	3.10	0.044
		Plat	3.03	0.045
	Synapse formation and organisation	Dlg4	2.55	0.047
		Nptx2	4.17	0.155
Glutamate Signalling	NMDA Receptor Signalling	Grin1	4.96	0.045
		Grin2a	3.78	0.064
		Grin2c	4.85	0.138
		Grin2d	4.82	0.028
		Kif17	2.22	0.162
	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) Receptor Signalling	Gria1	2.50	0.134
		Gria2	2.31	0.081
		Gria3	2.17	0.008
	Metabotropic Glutamate Receptor Signalling	Grm1	5.21	0.041
		Grm2	4.66	0.087
		Grm3	2.17	0.101
		Grm4	4.12	0.097

Regulation values in table represent an average regulation in OVA immunised and challenged animals as compared to OVA-immunised and saline-challenged controls. Data was analysed using the Student's t-test, using SABiosciences Data Analysis Software. n=3

5.3.2.2 Genes associated with inflammation

The expression of 84 genes associated with inflammation was analysed. Only genes that were up- or downregulated above or below two-fold were considered physiologically relevant (Table 5.2). Severe lung inflammation was associated with upregulated expression of six genes, none of which were significant, and downregulated expression of

3 genes, where IL17b was significantly downregulated. A full list of genes and their regulation can be found in Appendix 9.5.

Table 5.2
Regulation of genes associated with inflammation in the brainstem of mice with severe allergic lung inflammation

Category	Gene	Regulation	p-value
CCL Chemokines	Ccl17	-2.07	0.301
	Ccl24	-2.91	0.295
	Ccl17	3.88	0.369
CXCL Chemokines	Cxcl13	2.39	0.215
Interleukins	Il16	5.12	0.074
	Il17b	-3.06	0.034
Interleukin Receptors	IL2rb	3.10	0.081
Complement	C3	3.13	0.668
Lipid Metabolites	Lta	2.69	0.351

Regulation values in table represent an average regulation in OVA immunised and challenged animals as compared to OVA-immunised and saline-challenged controls. Data was analysed using the Student's t-test, using SABiosciences Data Analysis Software. n=3

5.3.3. The Effect of Acute, Sub-chronic and Chronic TH2 Type Lung Inflammation on Gene Expression in the Brainstem

Having established that severe acute lung inflammation was associated with neuroplasticity and inflammatory changes in the brainstem, we next investigated the effect of a more physiological and chronic form of lung inflammation on the brainstem.

5.3.3.1 Confirming the establishment of an acute, sub-chronic and chronic inflammatory events in the lung

To confirm the establishment of acute, sub-chronic and chronic TH2 lung inflammation, a differential cell count in the BALF was carried out. Three days of OVA challenge ("acute") had a significant effect on total cells in the BALF ($F_{1,16}$=15.90, p=0.0011), where BALF from OVA challenged animals had significantly higher levels of eosinophils (p=0.0054) than BALF from animals challenged with DPBS (Figure 5.6A). Eight days of OVA challenge ("sub-chronic") had a significant effect on total cells in the BALF ($F_{1,16}$=30.33, p<0.0001), where BALF from OVA challenged animals contained significantly higher levels of eosinophils (p<0.0001) and lymphocytes (p=0.0212) than BALF from DPBS challenged controls (Figure 5.6B). Similarly, 13 days of OVA challenge ("chronic") had a significant effect on total cells in the BALF ($F_{1,16}$=63.24, p<0.0001), where BALF from

OVA challenged animals contained significantly higher levels of eosinophils (p<0.0001) and lymphocytes (p=0.0029) than BALF from DPBS challenged controls (Figure 5.6C).

The number of days of OVA challenge had a significant effect on eosinophil count in the BALF ($F_{2,12}$=9.221, p=0.0038) where there was a substantial increase in eosinophil number from 3 days of challenge to 8 days of challenge, followed by a mild decrease at 13 days of challenge (Figure 5.6D). In addition, there was a significant interaction between number of days of OVA challenge and challenge treatment (OVA vs. DPBS) on eosinophil count in the BALF ($F_{2,12}$=9.196, p=0.0038).

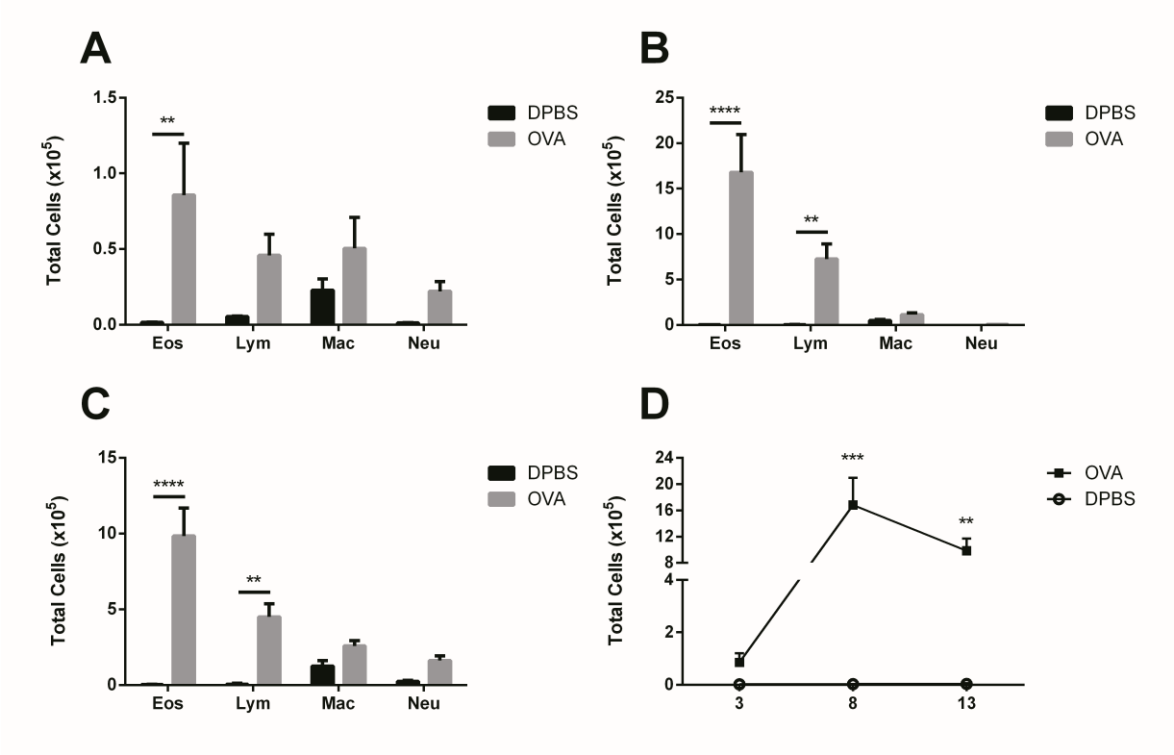

Figure 5.6 – The effect of OVA challenge on the inflammatory cell profile in the BALF. All animals were immunised with OVA and $Al(OH)_3$ on day 0 and day 14. From day 28 animals were challenged with aerosolised 1% OVA in DPBS for 20 minutes a day for 3 (A), 8 (B) or 13 (
C) days. Control animals were challenged with aerosolised DPBS. Animals were culled 2 days after the final OVA challenge. BAL was collected and differential cell analysis was carried out using flow cytometry or Sysmex analysis. (D) Change in eosinophil count over treatment days. **p<0.01; ***p<0.001; ****p<0.0001 vs. DPBS-treated control using two way ANOVA followed by Sidak's multiple-comparison post-test. n=3

5.3.3.2 The effect of acute, sub-chronic and chronic allergic lung inflammation on expression of neuroplasticity associated genes in the brainstem

Upon confirming the establishment of acute, sub-chronic and chronic forms of allergic lung inflammation, changes in neuronal activity were investigated. In previous chapters, c-fos was used as a marker of neuronal activity. However, as previously noted c-fos is an indicator of acute changes in neuronal activity and would therefore be undetectable following 3, 8 or 13 days of lung inflammation. As a result, genes associated with synaptic plasticity, a consequence of long-term alterations in neuronal activity, were investigated.

As in the pilot experiment, 84 genes associated with synaptic plasticity were investigated. Genes that were up- or down-regulated above or below two-fold are shown in Table 5.3. The genes shown in the table were analysed using SABiosciences software. Acute allergic lung inflammation was associated with a trend towards an upregulation of Gria1, Ntf3 and Ywhaq and downregulation of Tnf. Sub-chronic allergic lung inflammation was associated with significant 2-fold upregulation of Junb (p=0.005) and 3-fold upregulation of Kif17 (p=0.032), as well as a trend towards a downregulation of Adcy8. Chronic allergic lung inflammation was associated with significant 2-fold downregulation of Egr1 (p=0.007) and 3-fold downregulation of Fos (p=0.050), a trend towards a downregulation of Dlg4, Egr4, Gnai1, Gria1, Nr4a1 and Ywhaq and significant 2-fold upregulation of Ntf5 (p=0.022). A full list of genes and their regulation can be found in Appendix 9.5.

Table 5.3
Regulation of genes associated with neuroplasticity in the brainstem of mice with acute, sub-chronic or chronic allergic lung inflammation

Acute			**Sub-chronic**			**Chronic**		
Gene	*Regulation*	*p-value*	*Gene*	*Regulation*	*p-value*	*Gene*	*Regulation*	*p-value*
Gria1	4.1997	0.745	**Adcy8**	-2.09	0.125	**Dlg4**	-2.69	0.095
Ntf3	2.201	0.313	**Junb**	2.282	0.005	**Egr1**	-2.42	0.007
Tnf	-47.95	0.373	**Kif17**	3.12	0.032	**Egr4**	-4.53	0.133
Ywhaq	7.915	0.605				**Fos**	-2.96	0.050
						Gnai1	-18.5	0.224
						Gria1	-15.5	0.256
						Nr4a1	-2.77	0.127
						Ntf5	2.03	0.022
						Ywhaq	-8.82	0.714

Regulation values in table represent an average regulation in OVA immunised and challenged animals as compared to OVA-immunised and saline-challenged controls. Data was analysed using the Student's t-test, using SABiosciences Data Analysis Software. n=3

To further investigate the impact of acute, sub-chronic and chronic allergic lung inflammation on the expression of genes associated with synaptic plasticity in the brainstem, alterations in gene expression were analysed manually and graphed, to uncover kinetics of gene expression (trend analysis). Manual analysis involved individually determining a 2^ΔΔC(t) values for each sample, rather than determining an average for treated or control groups, as carried out by SABiosciences Data Analysis software.

As depicted in Figures 5.7 to 5.9, a trend is apparent in change in expression of a subset of genes over time. No change in gene expression in the brainstem is evident after 3 or 13 days of OVA challenge, as compared to control brainstem. In contrast, there is a rise in gene expression after 8 days of OVA challenge, as compared to control brainstem. This trend is apparent in a subset of glutamate receptors (Figure 5.7), where there is a significant interaction between the number of days of challenge and the challenge treatment (OVA vs. DPBS) on the expression of Grin1 ($F_{2,12}$=4.926, p=0.0274), Grin2a ($F_{2,12}$=5.020, p=0.0260), Grin2d ($F_{2,12}$=4.465, p=0.0355), Gria2 ($F_{2,12}$=4.547, p=0.0339) and Gria3 ($F_{2,12}$=5.162, p=0.0241). In addition, animals challenged with 8 days of OVA have significantly higher levels Grin1 (p=0.0482) and Grin2d (p=0.0487) in the brainstem, as compared to the brainstem of control animals. A similar pattern of expression was apparent for Grm1, Grm3 and Grm5, though there was no significant interaction between the number of days of challenge and the challenge treatment.

The trend is also apparent in a number of genes associated with dendrite outgrowth and synaptogenesis (Figure 5.8), where there is a significant interaction between the number of days of challenge and the challenge treatment on expression of Adam10 ($F_{2,12}$=4.052, p=0.0452), Homer1 ($F_{2,12}$=5.616, p=0.0190), Kif17 ($F_{2,12}$=7.352, p=0.0082), Ncam1 ($F_{2,12}$=6.846, p=0.0104) and Nptx2 ($F_{2,12}$=4.142, p=0.0429). In addition, animals challenged with 8 days of OVA have significantly higher levels of Grip1 (p=0.0472), Kif17 (p=0.0039) and Ncam1 (p=0.0198) in the brainstem, as compared to the brainstem of control animals. A similar pattern of expression was apparent for Dlg4, Grip1 and Synpo, though there was no significant interaction between the number of days of challenge and the challenge treatment.

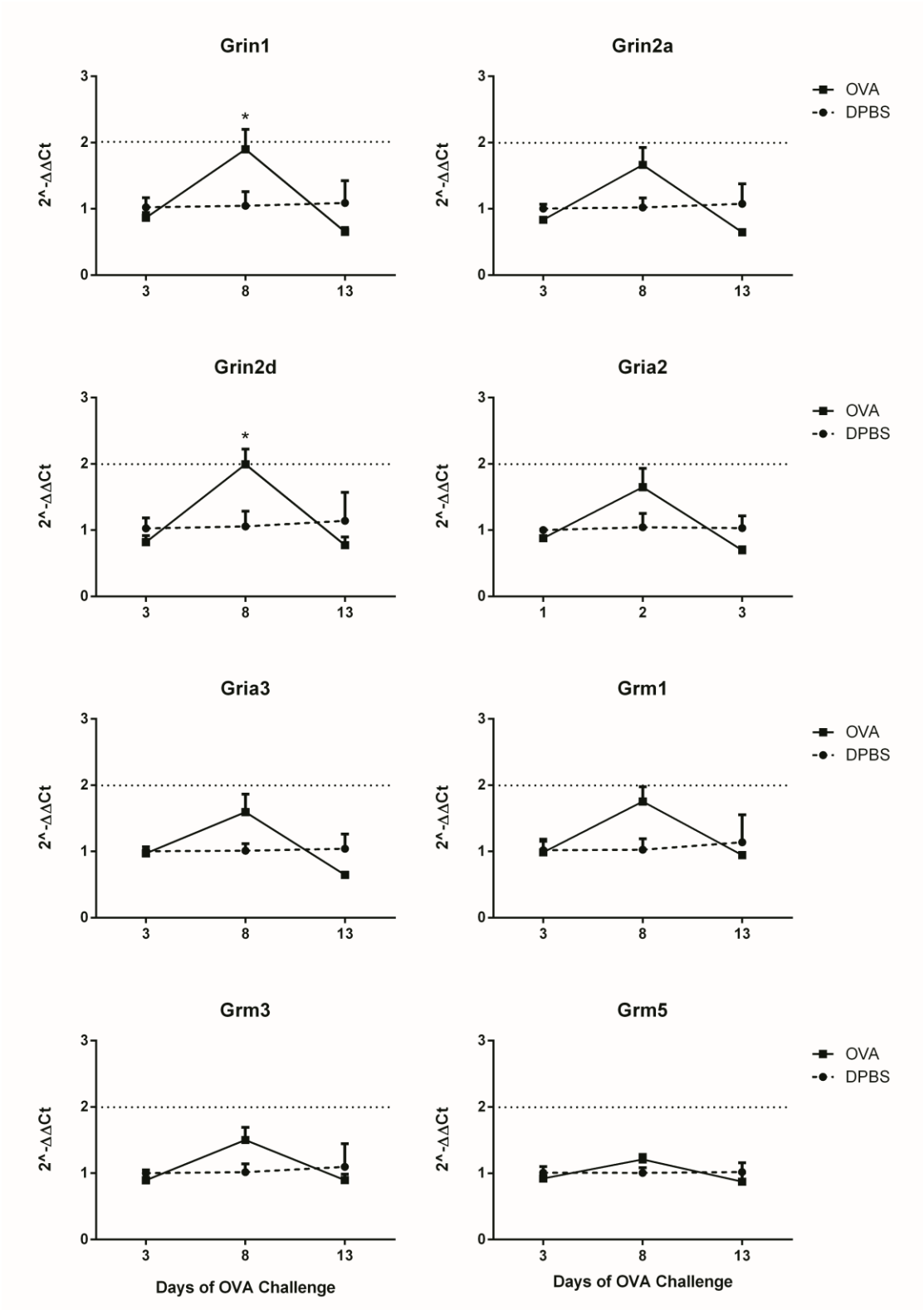

Figure 5.7- The effect of OVA challenge on expression of glutamate receptors in the brainstem. A terminal brainstem sample was collected two days after the final OVA/DPBS challenge and snap-frozen. Gene expression was measured using the RT^2 PCR Profiler™ Array for synaptic plasticity genes. Values are represented as mean fold change from control (2^ΔΔCt) ± SEM using HPRT and HSP90AB1 as reference genes. *$p<0.05$ vs. control using two way ANOVA followed by the Sidak's post-hoc test. n=3

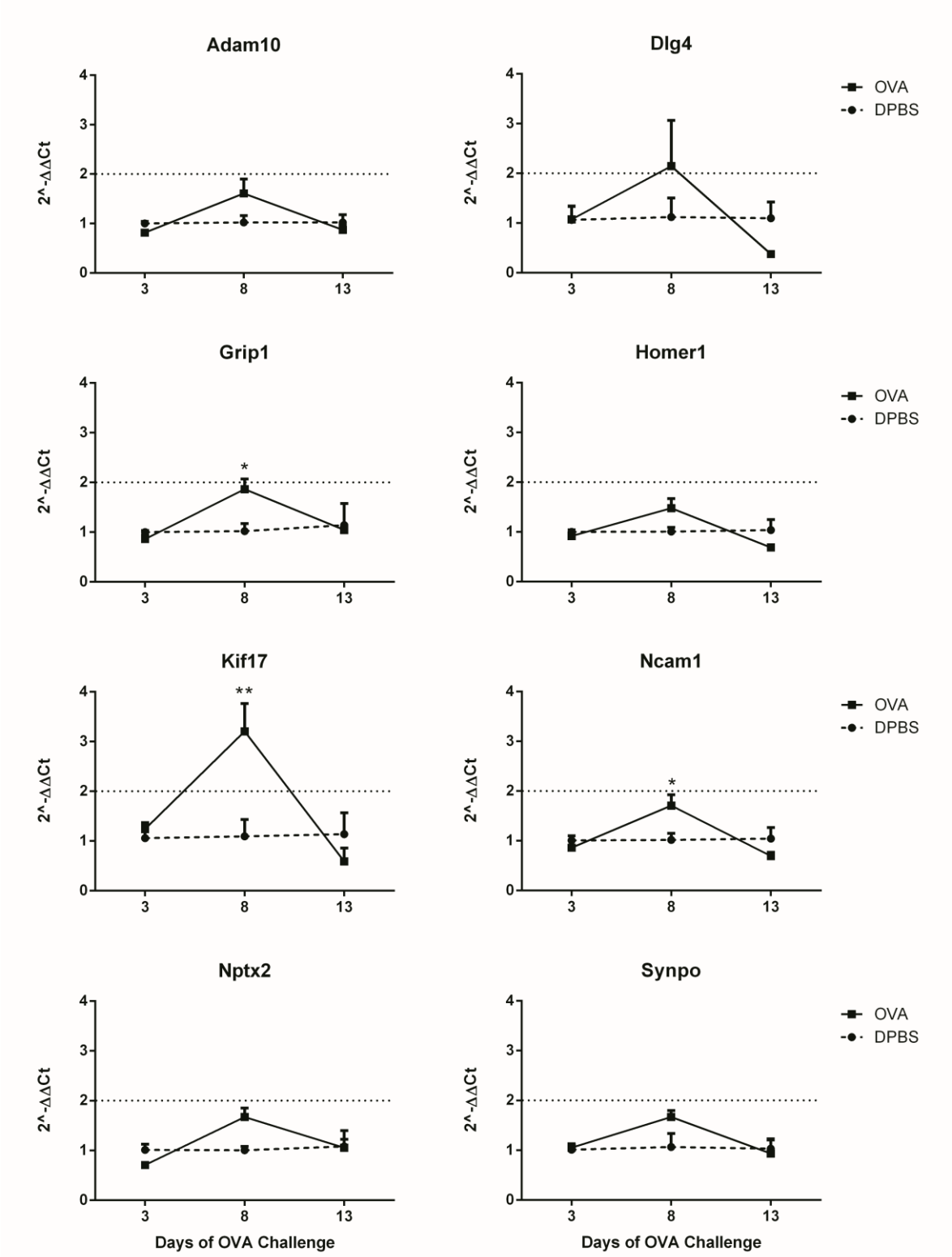

Figure 5.8- The effect of OVA challenge on expression of genes associated with axonal growth and synapse formation in the brainstem. A terminal brainstem sample was collected two days after the final OVA/DPBS challenge and snap-frozen. Gene expression was measured using the RT^2 PCR Profiler™ Array for synaptic plasticity genes. Values are represented as mean fold change from control (2^ΔΔCt) ± SEM using HPRT and HSP90AB1 as reference genes. *p<0.05, **p<0.01 vs. control using two way ANOVA followed by the Sidak's post-hoc test. n=3

A similar trend was apparent in a subset of genes encoding intracellular signalling molecules and transcription factors (Figure 5.9). There was a significant interaction between the number of days of challenge and the challenge treatment on expression of Akt1 ($F_{2,12}$=6.374, p=0.0130) and Junb ($F_{2,12}$=6.238, p=0.0139). Animals challenged with 8 days of OVA had significantly higher levels of Akt1 (p=0.0246) and Junb (p=0.0325) in the brainstem, as compared to the brainstem of control animals. A similar pattern of expression was apparent for Camk2g, though there was no significant interaction between the number of days of challenge and the challenge treatment.

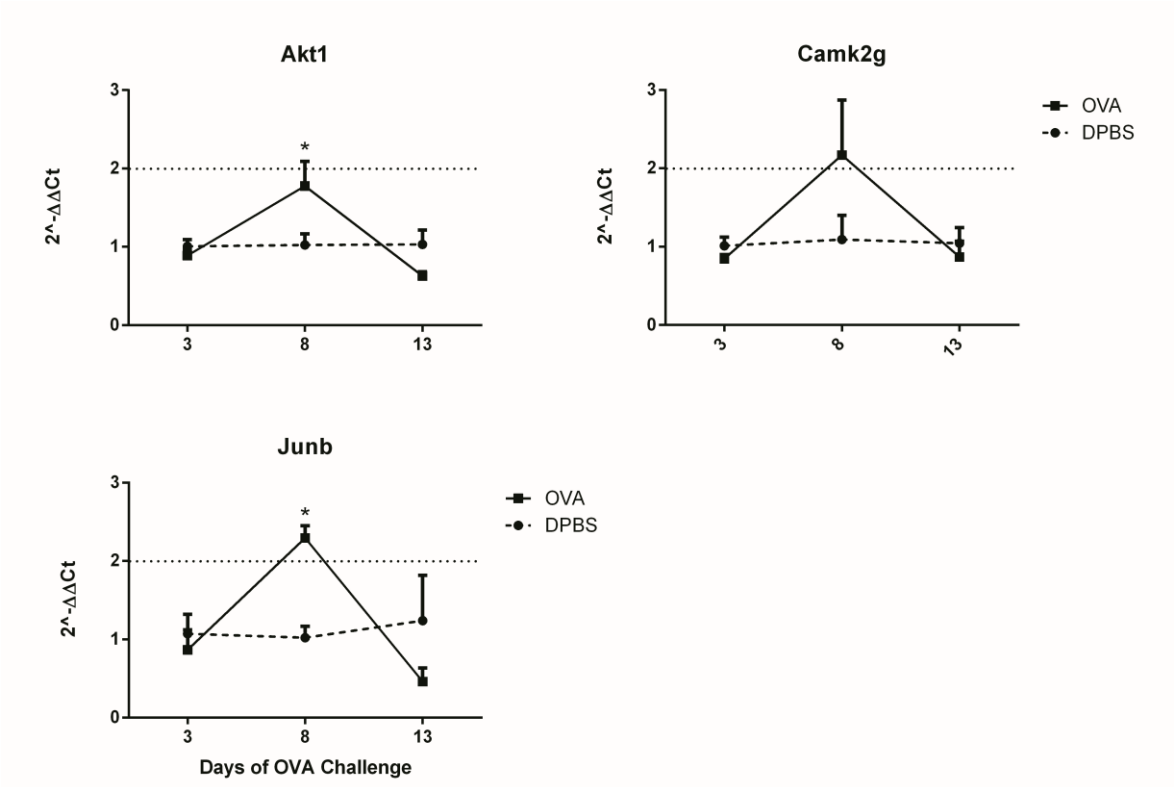

Figure 5.9- The effect of OVA challenge on expression of genes encoding intracellular signalling molecules and transcription factors in the brainstem. A terminal brainstem sample was collected two days after the final OVA/DPBS challenge and snap-frozen. Gene expression was measured using the RT^2 PCR Profiler^{TM} Array for synaptic plasticity genes. Values are represented as mean fold change from control (2^ΔΔCt) ± SEM using HPRT and HSP90AB1 as reference genes. *p<0.05 vs. control using two way ANOVA followed by the Sidak's post-hoc test. n=3

5.3.3.3 The effect of acute, sub-chronic and chronic allergic lung inflammation on expression of Tac1 and Tacr1 in the brainstem

Previous studies have shown that central SP and its receptor NK1 play a key role in the cough reflex[315], where increases in central SP have been associated with symptoms of asthma, such as dyspnoea and breathlessness[237]. It has also been demonstrated that tachykinins such as SP are upregulated in vagal ganglia in an OVA-immune complex model of asthma[317]. As a result, expression of Tac1 (which encodes the protachykinin, the precursor protein for SP) and Tacr1 (which encodes NK1) were measured (Figure 5.10).

OVA challenge had no significant effect on the expression of NK1 ($F_{1,12}$=1.921, p=0.1909). However, a trend was apparent in the expression of Tac1 in the brainstem of OVA challenged animals, as compared to control. There was no difference in expression of Tac1 in the brainstem of animals challenged with 3 or 13 days of OVA, but there was a significant increase in expression of Tac1 in the brainstem of animals challenged with 8 days of OVA, as compared to control (p=0.0292). This was associated with a significant interaction between days of OVA challenge and challenge treatment ($F_{2,12}$=6.632, p=0.0115).

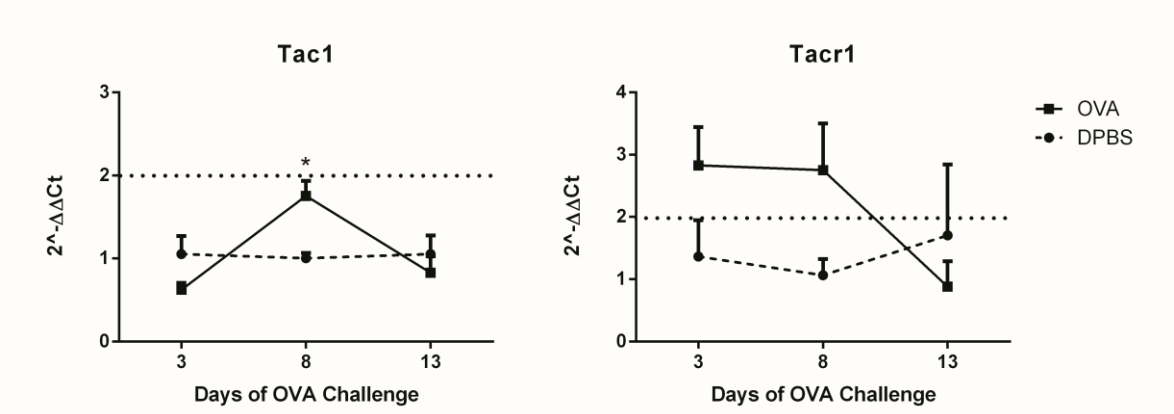

Figure 5.10- The effect of OVA challenge on expression of Tac1 and Tacr1 in the brainstem. A terminal brainstem sample was collected two days after the final OVA/DPBS challenge and snap-frozen. Gene expression was measured using the RT^2 qPCR primer assays for Tac1 and Tacr1. Values are represented as mean fold change from control (2^ΔΔCt) ± SEM using HPRT and HSP90AB1 as reference genes. Statistical analysis carried out using two way ANOVA. n=3

5.3.3.4 The effect of acute, sub-chronic and chronic allergic lung inflammation on expression of inflammation associated genes in the brainstem

To further investigate the effect of acute, sub-chronic and chronic allergic lung inflammation on the brain, changes in the expression of inflammation-associated genes were analysed. As in the pilot experiment, 84 genes associated with inflammation were investigated. Genes that were up- or downregulated above or below two-fold are shown in Table 5.4. The genes shown in the table were analysed using SABiosciences software. Acute allergic lung inflammation was associated with a trend towards an upregulation of five genes (Ccl11, Ccl17, Ccl22, Ccr2 and Il4), as well as a trend towards a downregulation of four genes (Ccl20, Ccl8, Il3, Tnf). Sub-chronic allergic lung inflammation was associated with a trend towards an upregulation of four genes (Ccl3, Ccr7, Il2rb, Xcr1), downregulation of Cxcl9 and a significant 4-fold upregulation of Ccl4 (p=0.007). Chronic allergic lung inflammation was associated with a trend towards an upregulation of four genes (Cxcl13, Il10rb, Il1a, Il1b), a trend towards a downregulation of five genes (Ccl19, Ccl2, Ccl3, Ccl7 and Ccr1) and a significant 3-fold upregulation of Il1f8 (p=0.032).

Table 5.4
Regulation of genes associated with inflammation in the brainstem of mice with acute, sub-chronic or chronic allergic lung inflammation

Acute			**Sub-chronic**			**Chronic**		
Gene	*Regulation*	*p-value*	*Gene*	*Regulation*	*p-value*	*Gene*	*Regulation*	*p-value*
Ccl11	10.2	0.374	**Ccl3**	2.04	0.298	**Ccl19**	-3.38	0.649
Ccl17	553	0.374	**Ccl4**	3.61	0.007	**Ccl2**	-2.71	0.677
Ccl20	-2.09	0.179	**Ccr7**	2.49	0.307	**Ccl3**	-2.11	0.185
Ccl22	3.27	0.515	**Cxcl9**	-2.81	0.065	**Ccl7**	-2.10	0.184
Ccl8	-2.36	0.533	**Il2rb**	2.54	0.174	**Ccr1**	-2.47	0.479
Ccr2	4.94	0.219	**Xcr1**	2.71	0.052	**Cxcl13**	2.38	0.314
Il3	-2.95	0.076				**IL10rb**	2.02	0.224
Il4	3.01	0.106				**IL1a**	2.24	0.783
Tnf	-2.537	0.0881				**IL1b**	3.11	0.150
						IL1f8	2.90	0.032

Regulation values in table represent an average regulation in OVA immunised and challenged animals as compared to OVA-immunised and saline-challenged controls. Data was analysed using the Student's t-test, using SABiosciences Data Analysis Software. n=3

As previously, change in gene expression following acute, sub-chronic and chronic allergic lung inflammation was further assessed by trend analysis, by manually analysing and

graphing change in gene expression with increasing OVA challenges in order to uncover patterns associated with chronicity and severity of lung inflammation.

Overall, three trends were apparent in change in expression of inflammatory genes with increasing challenges of OVA. As previously evident with genes associated with neuroplasticity, the expression of subset of genes associated with inflammation was increased following 8 days of OVA challenge, as compared to control, with no difference in expression following 3 or 13 days of OVA challenge, as compared to control (Figure 5.11). Xcr1 (Xcl1 receptor) expression was significantly upregulated following 8 days of OVA challenge (p=0.0067), associated with a significant interaction between number of days of challenge and challenge treatment ($F_{2,12}$=5.901, p=0.0164). A similar trend was evident in the expression of Ccl4, though it were not associated with a significant interaction between number of days of challenge and challenge treatment ($F_{2,12}$=2.598, p=0.1155).

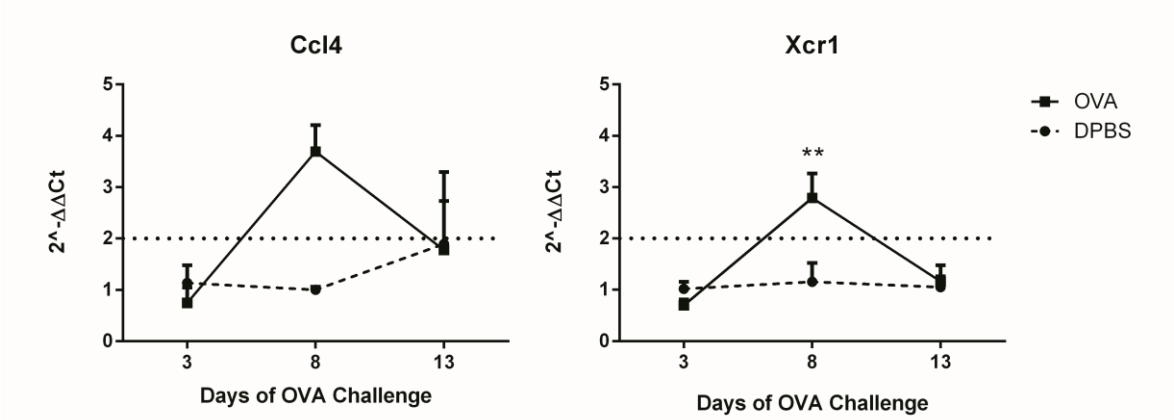

Figure 5.11- The effect of OVA challenge on expression of Ccl4 and Xcr1 in the brainstem. A terminal brainstem sample was collected two days after the final OVA/DPBS challenge and snap-frozen. Gene expression was measured using the RT^2 PCR Profiler^{TM} Array for inflammatory genes. Values are represented as mean fold change from control (2^ΔΔCt) ± SEM using HPRT and HSP90AB1 as reference genes. **p<0.01 vs. control using two way ANOVA followed by Sidak's post hoc test. n=3

The expression of four genes was found to be elevated following 13 days of OVA challenge, as compared to control (Figure 5.12). OVA challenge had a significant effect on the expression of IL1a ($F_{1,11}$=6.106, p=0.0311), which was associated with a significant interaction between number of days of challenge and challenge treatment ($F_{2,11}$=6.129, p=0.0163). OVA challenge was also found to have a significant effect on the expression of Il1b in the brainstem ($F_{1,12}$=5.495, p=0.0371), where expression levels were also

significantly dependent on the number of days of OVA challenge ($F_{2,12}$=5.814, p=0.0172). OVA challenge had no significant effect on the expression of Il1f8 ($F_{1,12}$=1.758, p=0.2095) or Tnf ($F_{1,12}$=0.0033, p=0.9549), though the expression of these genes was substantially elevated in the brainstem of animals challenge with OVA for 13 days, as compared to controls.

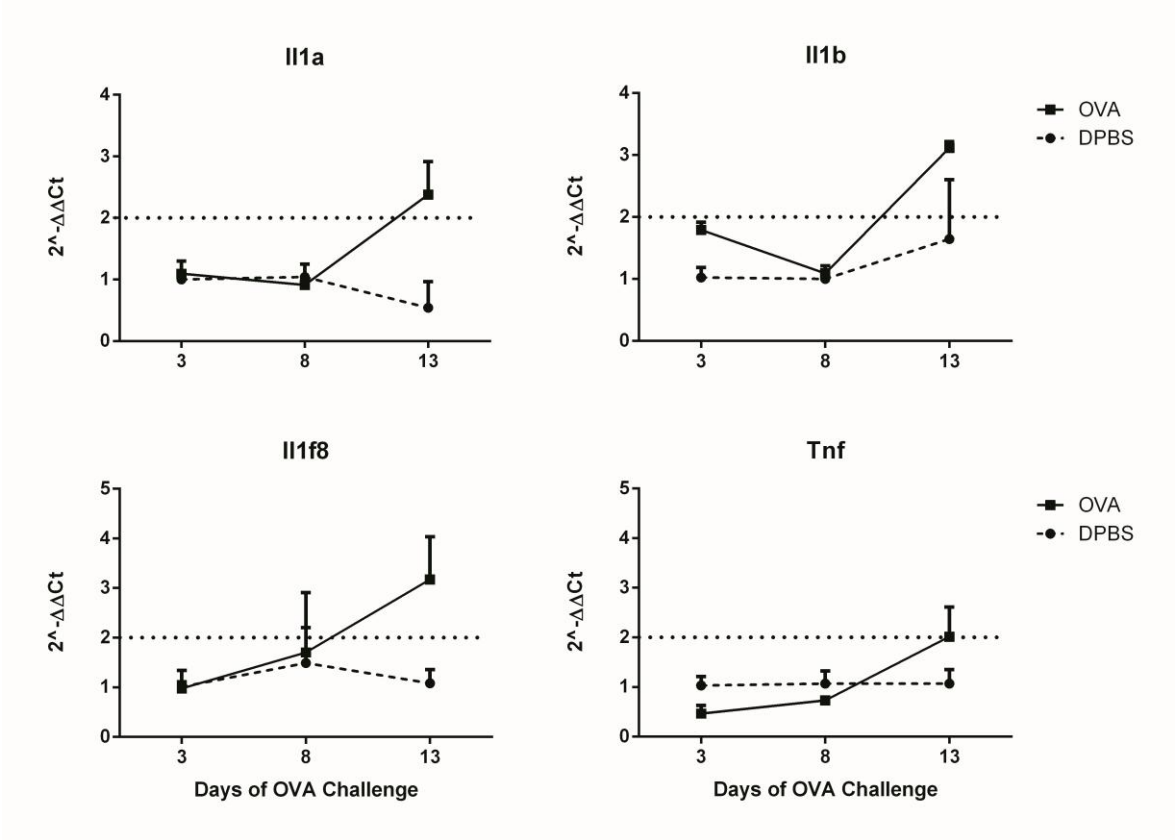

Figure 5.12- The effect of OVA challenge on expression Il1a, Il1b, Il1f8 and Tnf in the brainstem. A terminal brainstem sample was collected two days after the final OVA/DPBS challenge and snap-frozen. Gene expression was measured using the RT^2 PCR Profiler^{TM} Array for inflammatory genes. Values are represented as mean fold change from control (2^ΔΔCt) ± SEM using HPRT and HSP90AB1 as reference genes. Statistical analysis was carried out using two way ANOVA. n=3

A trend was also apparent in the change in expression of Ccl1, with a trend towards an increase in expression with increasing OVA challenges (Figure 5.13). OVA challenge, however, had no significant effect on expression of Ccl1 ($F_{1,12}$=1.111, p=0.3126). OVA challenge was also found to significantly affect the expression of Il4 in the brainstem ($F_{1,12}$=5.528, p=0.0366), with elevated levels of Il4 in the brainstem of animals challenged with 3, 8 or 13 days of OVA, as compared to controls (Figure 5.13).

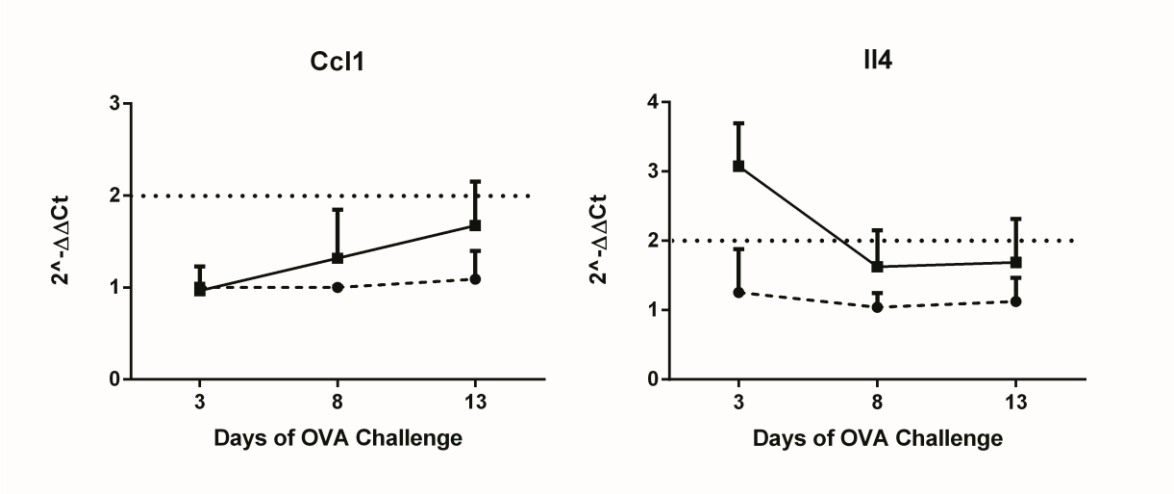

Figure 5.13- The effect of OVA challenge on expression of Ccl1 and Il4 in the brainstem. A terminal brainstem sample was collected two days after the final OVA/DPBS challenge and snap-frozen. Gene expression was measured using the RT^2 PCR Profiler™ Array for inflammatory genes. Values are represented as mean fold change from control (2^ΔΔCt) ± SEM using HPRT and HSP90AB1 as reference genes. Statistical analysis was carried out using two way ANOVA. n=3

5.3.3.5 The effect of sub-chronic allergic lung inflammation on microglial phenotype in the hippocampus and brainstem

The increased cytokine expression in the brainstem following in animals with lung inflammation may be due to microglial activation in the brainstem. Therefore, the next step was to investigate evidence for microglial activation by measuring phenotype and cell number changes by immunohistochemistry. Immunoreactivity for CD11b and FcγRI was investigated to determine change in number and/or morphology of microglia. The expression of MHC Class II and Dectin-1 was additionally examined to assess a change in phenotype (M1 vs. M2) of microglia.

There were no visible changes in the expression of CD11b (Figure 5.14) or FcγRI (Figure 5.15) in the hippocampus or caudal brainstem of animals challenged with OVA for 8 days, as compared to controls. In addition, no morphological changes in microglia were detectable in the brains of animals challenged with OVA for 8 days, as compared to controls. Expression of MHC Class II and Dectin-1 was not detectable in the hippocampus or caudal brainstem of animals challenged with OVA or saline for 8 days.

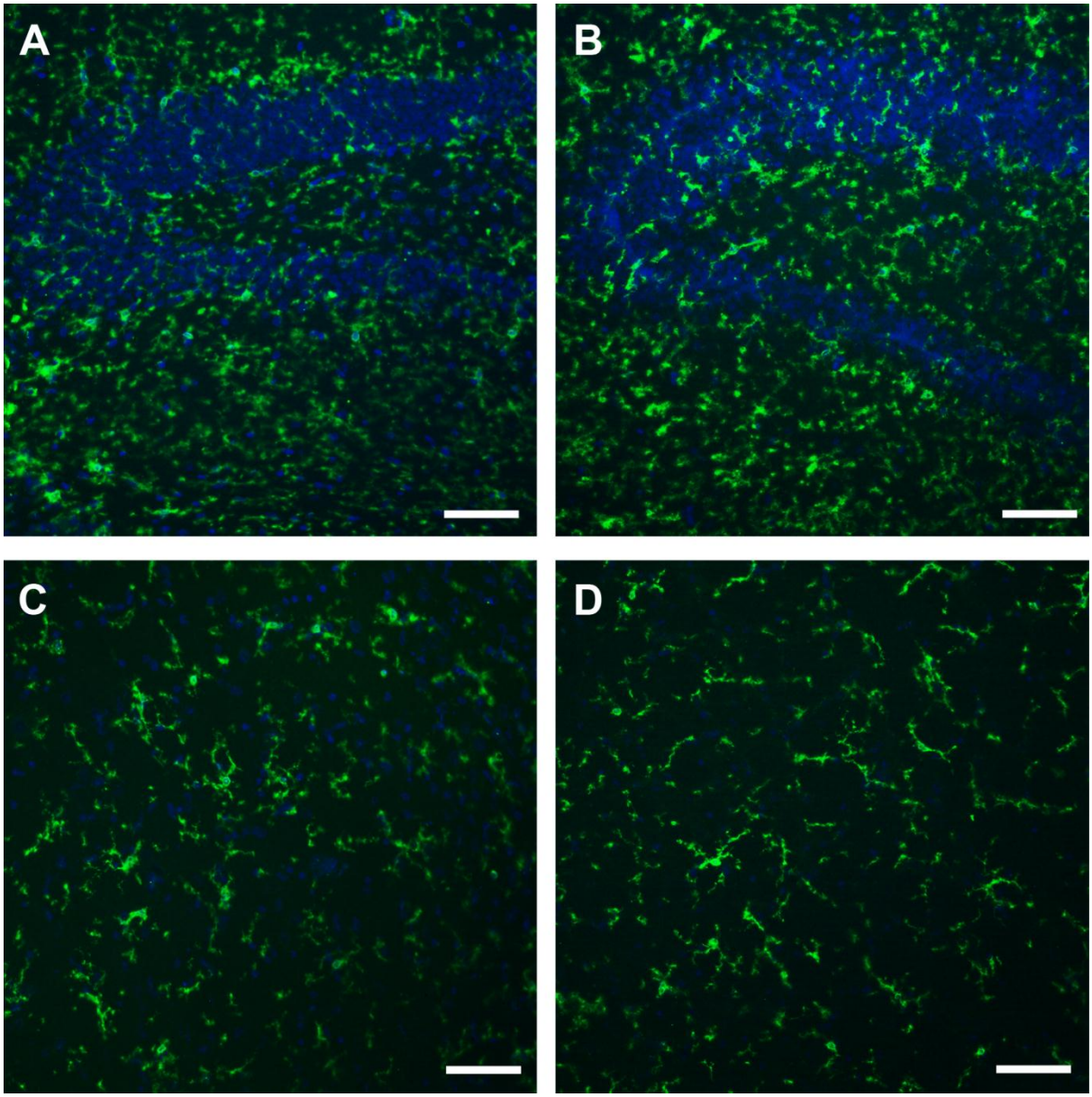

Figure 5.14 – Representative immunohistochemical stain of CD11b in the hippocampus (A-B) and NTS (C-D). Animals were challenged with saline (A, C) or OVA (B, D) for 8 days (sub-chronic inflammation) and culled two days after the final challenge; animals were transcardially perfused with heparinised saline and brains were excised and frozen in OCT in isopentane on dry ice. Representative of n=3-5. Scale bar: 75µm

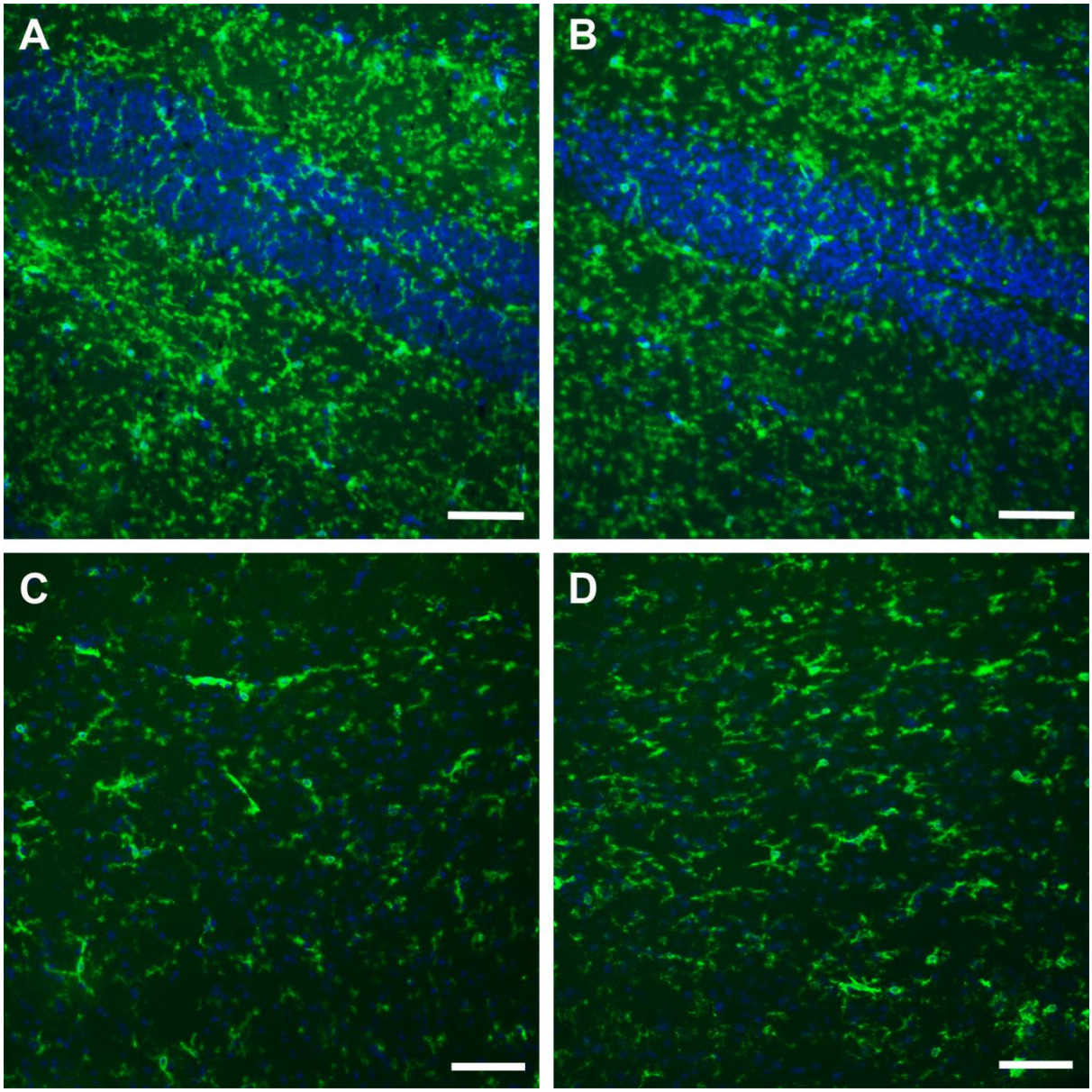

Figure 5.15 – Representative immunohistochemical stain of FcγRI in the hippocampus (A-B) and NTS (C-D). Animals were challenged with saline (A,C) or OVA (B, D) for 8 days (sub-chronic inflammation) and culled two days after the final challenge; animals were transcardially perfused with heparinised saline and brains were excised and frozen in OCT in isopentane on dry ice. n=3-5. Scale bar: 75µm

5.3.3.6 The effect of sub-chronic allergic lung inflammation on infiltration of IgG in the hippocampus and brainstem

It has previously been reported in the literature that chronic allergic lung inflammation is associated with increased permeability of the BBB and infiltration of IgG and IgE into the brain parenchyma[246]. The expression of IgG in the hippocampus and brainstem was consequently investigated following sub-chronic allergic lung inflammation.

The hippocampus and brainstem from animals challenged with 8 days of OVA were found to have increased IgG immunoreactivity, specifically localised to the cerebral vasculature (Figure 5.16). IgG immunoreactivity was most prominent in the AP, NTS and lateral reticular nucleus (LRt) of the brainstem.

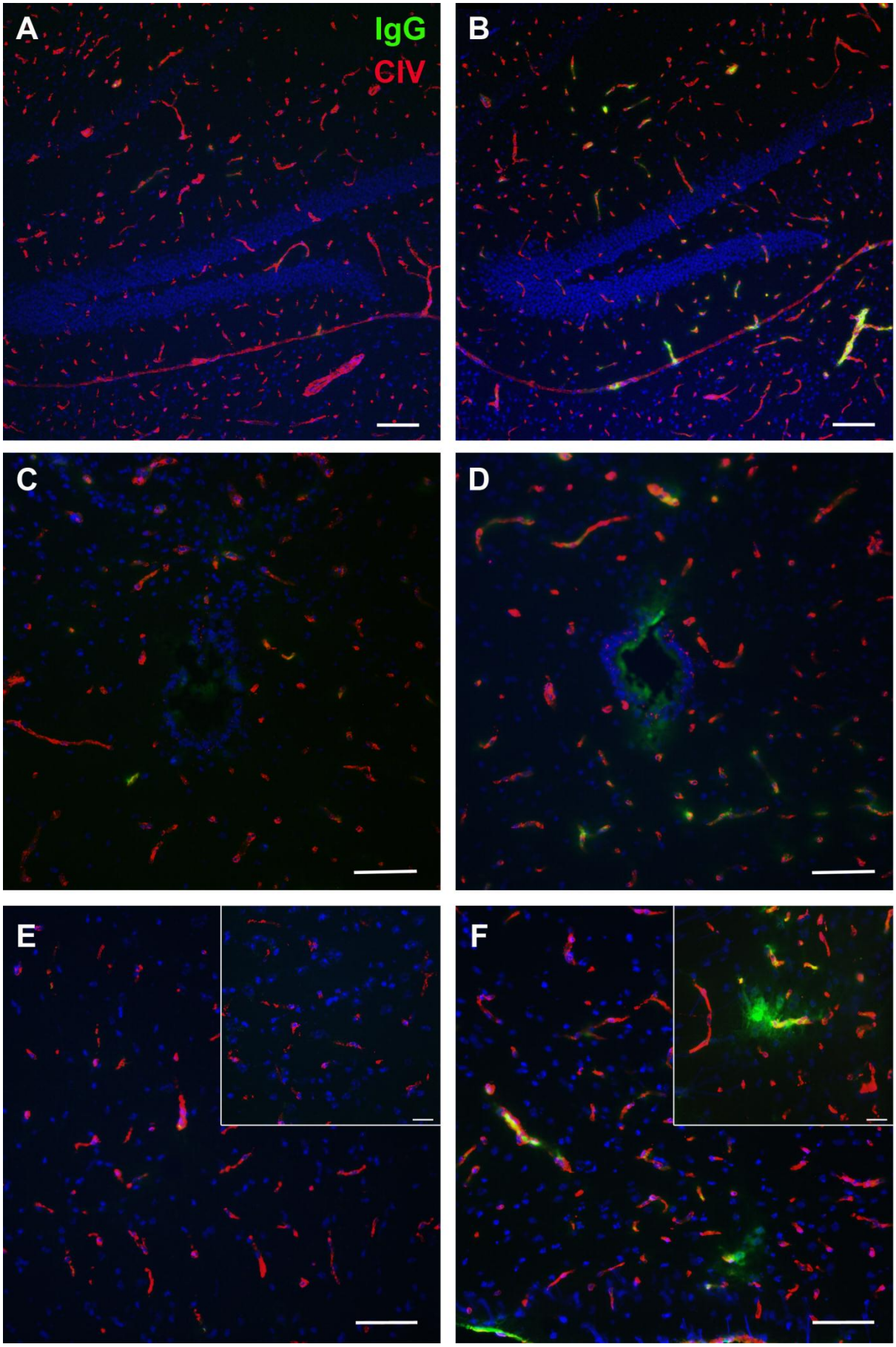
A
IgG
CIV
B
C
D
E
F

Figure 5.16 – Representative immunohistochemical stain of IgG (green) and Collagen IV (red) in the hippocampus (A-B), NTS (C-D) and lateral reticular nucleus (E-F). Animals were challenged with saline (A, C, E) or OVA (B, D, F) for 8 days (sub-chronic inflammation) and culled two days after the final challenge; animals were transcardially perfused with heparinised saline and brains were excised and frozen in OCT in isopentane on dry ice. n=3-5. Scale bar A-B: 100µm, C-F: 75µm, inset: 25µm.

5.4 Discussion

In this study, it was found that chronic forms of allergic lung inflammation were associated with a patterned increase in the transcriptional activity of glutamate receptors, intracellular signalling molecules, genes involved in synaptogenesis and tachykinins, indicative of increases in central synaptic plasticity. In addition, central inflammatory changes were evident, with increased BBB permeability and IgG infiltration and subsequent pro-inflammatory cytokine expression at more chronic stages of inflammation.

5.4.1 Acute, Sub-chronic and Chronic Allergic Lung Inflammation

To induce chronic TH2 lung inflammation, a series of protocols were developed based on the well-established model of allergic inflammation used in the previous chapter. An initial model of severe lung inflammation was used as a proof of principle experiment, to detail if synaptic and inflammatory changes were evident in the brainstem following allergic lung inflammation. Once these changes had been established, models of acute, sub-chronic and chronic lung inflammation were developed, to investigate the impact of physiological models of inflammation on the brain. As already described, this involved the immunisation against OVA in conjunction with aluminium hydroxide, followed by a series of repeated OVA challenges. However, as compared to the previous model, which involved intranasal instillation of OVA, these protocols involved chronic challenge with aerosolised OVA, a preferred method of delivery as it did not require the use of volatile anaesthetics, such as isoflurane. In a previous experiment, which involved chronic intranasal challenge with house dust mite (HDM) as a model of chronic allergic inflammation, it was found that this protocol induced changes in microglial morphology in the AP of both HDM-treated and saline-treated control animals (Appendix 9.6), an effect attributed to the use of isoflurane. Indeed, isoflurane has previously been shown to activate the vagus nerve[252], activate and sensitise TRP receptors of the vagus nerve[318],

induce pro-inflammatory cytokine production[253,319] and increase permeability of the BBB[254]. Chronic use of isoflurane would therefore be a confounding factor when examining the effects of chronic allergic lung inflammation on the brain, and a model using aerosolised OVA was consequently developed.

By increasing the number of days of OVA challenge, models for acute, sub-chronic and chronic allergic lung inflammation were developed, in which eosinophillic inflammation peaked at the sub-chronic stage.

5.4.2 Sub-chronic Lung Inflammation Impacts on Neuronal Transmission in the Brainstem

In Chapter 4, acute allergic lung inflammation was found to increase neuronal activity in the cmNTS in the brainstem, as visualised by an increase in the expression of the IEG c-fos. As previously noted, c-fos is an indicator of acute changes in activity and is therefore not detectable under chronic conditions of inflammation. In this study, the investigation focused instead on genes involved in synaptic plasticity, a process by which a synapse alters its strength as a direct consequence of repeated activation (excitatory or inhibitory)[316]. Repeated excitation of a synapse by, for example, the excitatory neurotransmitter glutamate can lead to increases in synaptic efficacy, an NDMA receptor-dependent mechanism known as long-term potentiation (LTP)[320].

In this study, sub-chronic lung inflammation was found to induce glutamate receptor expression in the brainstem, significantly increasing the expression of the NMDA receptor subunit ζ-1 (NR1, Grin1) and ε-4 (NR2d, Grin2d), and clearly, but not significantly, increasing the expression of the NMDA receptor subunit ε-1 (NR2a, Grin2a), the AMPA receptor 3 (Glur3, Gria3) and 4 (Glur4, Gria4) and the metabotropic glutamate receptor 1 (mGlur1, Grm1). AMPA and NMDA glutamate receptors are fundamental in the initiation of LTP. At a resting state, Ca^{2+}-permeable NMDA receptors bind glutamate, but do not allow influx of Ca^{2+}, as they are blocked by physiological levels of Mg^{2+}. However, upon continuous activation, AMPA receptors open, resulting in neuronal depolarisation and expelling Mg^{2+} from the NMDA receptor pore, thus allowing influx of Ca^{2+} [316]. This Ca^{2+} influx instigates a series of intracellular signalling events, involving among other proteins, Ca^{2+}/calmodulin-dependent protein kinase (CaMKII)[321] and protein kinase B (AKT)[322],

and also increases levels of the IEG JunB[323], all of which were found to be upregulated at the mRNA level following sub-chronic lung inflammation. This intracellular signalling cascade eventually results in increased expression and sensitivity of AMPA receptors, increased NMDA receptor expression and consequently increased glutamate sensitivity[316].

In addition to changes in receptor expression and intracellular signalling molecules, sub-chronic lung inflammation was associated with upregulation of genes involved in synaptogenesis. Allergic inflammation led to increases in Dlg4 (which encodes PSD-95), Homer-1 and Nptx (which encodes neuronal pentraxin-2) in the brainstem, all of which are associated with the anchoring and clustering of AMPA receptors at the synaptic membrane[324-326]. In addition, Kif17, which was significantly increased followed sub-chronic lung inflammation, is an important microtubule-based motor protein, which aids the transport of NMDA receptor subunits to the synapse[327]. The upregulation of these genes alongside the elevated mRNA levels of NMDA receptor subunits and AMPA receptors suggest an increased density of glutamatergic receptors at the synapse, resulting long-term plastic changes through increased sensitivity of neurons to glutamate.

Glutamate is a key neurotransmitter in the synapse between first and second order cmNTS neurons extending from the lung[198]. NMDA receptors (specifically NR1) are highly expressed on neurons in the cmNTS synapsing with afferent neurons from the lung, both synaptically and within cytoplasmic organelles[328] and have been shown to play a key role in the central cough reflex[315,329,330]. The significant upregulation of glutamate receptors, intracellular signalling molecules and genes involved in synaptogenesis in the brainstem during sub-chronic lung inflammation suggest the initiation of plasticity in the cmNTS. It is probable that the continuous firing of afferent neurons from a sustained inflammatory event in the lung becomes integrated into patterned, but amplified efferent firing from second or third order neurons back to the lungs, through the increased sensitivity to glutamate. The upregulation of glutamate receptor expression and downstream signalling molecules may be a response to cope with and integrate the repetitive bursts from first order afferents. This may, in turn lead, to a stronger efferent output, leading to an exaggeration of reflex responses including increased bronchoconstriction, apnoea and cough.

5.4.2.2 Sub-chronic allergic lung inflammation impacts on tachykinin expression in the brainstem – a role for substance P?

Sub-chronic lung inflammation was additionally found to significantly increase Tac1 expression, the gene encoding the protachykinin-1 protein, a precursor to the tachykinins neuropeptide K, neuropeptide-γ, neurokinin A and substance P (SP). SP plays a vital role in both lung inflammation in asthma, as well as central cough reflexes. It is key in the process of neurogenic inflammation in asthma, being released from lung terminals of vagal bronchopulmonary c-fibres in response to inflammatory stimulation, contributing to further inflammation in the lungs[210]. Centrally, SP, like glutamate, is released from bronchopulmonary c-fibre afferents in the cmNTS and has been shown to modulate respiratory reflex out. It plays a role in the central cough reflex[315,330], having both excitatory effects on second order neurons[331], depressive effects on synaptic transmission to second order neurons[332] and plastic effects in response to lung inflammation, resulting in increased efferent output, bronchoconstriction or cough[235,333]. The increase in Tac1 expression in the brainstem is, like increase in glutamate receptor signalling, suggestive of an initiation of plasticity in the brainstem, resulting in an augmentation of the central reflex.

Collectively, sub-chronic TH2 lung inflammation results in upregulation of genes involved in NMDA-dependent LTP and genesis of AMPA and NMDA-receptor rich synapses, as well as upregulation of tachykinins, key players in synaptic transmission in the cmNTS and modulators of the airway reflex output. These changes imply that TH2 lung inflammation results in structural, phenotypical and functional changes of neurons in the brainstem, which would lead to plastic changes, and an exaggerated efferent output to the airways. This validates and provides a molecular clarification for previous studies, which have shown that lung inflammation induced by extended allergen exposure (in primates) and extended tobacco smoke (ETS) leads to plasticity in the NTS[207,235,236,239]. This alteration in neuronal firing in the NTS could result in exaggeration of central reflex, ultimately contributing to symptoms of TH2 lung inflammation, such as dyspnoea and cough.

It should be noted that the alterations in gene expression found in these studies were apparent in an entire brainstem region, and not specifically localised to the cmNTS. However, with the knowledge that lung inflammation leads to plastic changes in the

cmNTS, that glutamate and SP expression in the cmNTS are key contributors to exaggerated central reflexes in allergic lung inflammation, as well as the finding in Chapter 4 that acute allergic lung inflammation impacts only the cmNTS, it is reasonable to speculate that the transcriptional changes observed occur specifically in this subdivision of the NTS. Nevertheless, localisation of changes through qPCR analysis of microdissected areas of the NTS or immunohistochemical or immunohybridisation studies would be necessary to confirm this supposition.

5.4.2.3 How functionally relevant are the genomic changes?

The results suggest that neuroplastic changes are most pronounced under sub-chronic conditions of allergic inflammation, as gene expression of the aforementioned genes returns to control levels during chronic inflammation. However, several factors must be taken into account when before concluding that these changes are functional relevant. The SABiosciences software provided for analysis of gene expression considered a two-fold up- or downregulation of gene expression functionally relevant. In reality, it is impossible to determine the magnitude of change in gene expression necessary for functional relevance, without carrying out secondary functional analysis, such as measurement of changes in airway reactivity.

It must be taken into account that only mRNA, and not protein levels were measured, implying only that transcriptional activity was at its height during sub-chronic inflammation. It is likely that the increase in transcriptional activity at this stage of inflammation results in increased levels of protein, which are then sustained resulting in long-term and fixed plastic changes. An attempt to measure protein levels of the NR1 subunit of the NDMA receptor were carried out using immunohistochemistry, although this was unsuccessful. Further experiments are necessary to fully understand the proteomic and sustained changes in the brainstem neurons following sub-chronic and chronic lung inflammation. It would also be necessary to study the kinetics of receptor turnover in these samples.

It should also be pointed out that simultaneous analysis of 84 genes in one assay can infer unwanted noise in analysis of results. It would therefore have been beneficial to validate the changes seen with individual qPCR analysis.

5.4.3 Increased BBB Permeability and Progressive Pro-inflammation with Chronic Allergic Inflammation

At sub-chronic stages of lung inflammation, where transcriptional changes in genes associated with synaptic plasticity were at their height, increases in BBB permeability were apparent, associated with increased infiltration of IgG into the brain parenchyma. This finding corroborates previously published data, which showed widespread increases in IgG and IgE centrally following chronic allergic lung inflammation[246]. Central IgG is known to bind microglial FcγRs, inducing microglial activation[334] and pro-inflammatory cytokine production[335]. Although neither of these changes were apparent at this stage of allergic inflammation, more chronic stages of inflammation were associated with clear transcriptional increases in members of the IL-1 family (IL-1α, IL-1β, IL-1F8) and TNF, suggestive of central glial activation. This could indicate that continually infiltrating immunoglobulins may interact with microglial FcγRs and induce innate inflammation. However, further investigations into the phenotype and activation status of glial cells, as well as expression of glial FcγRs at stages of chronic inflammation would be necessary to confirm this postulate.

Increased BBB permeability and glial activation have additionally been previously associated with alterations in neuronal function, particularly neuropeptide production. Neuropeptides, such as SP, which is transcriptionally upregulated during sub-chronic inflammation, are well-known to impact on inflammation, both peripherally (contributing to neurogenic inflammation) as well as centrally. SP is known to increase vascular permeability[210], and has also been shown to interact directly with glial cells via the NK-1 receptor, resulting in production of pro-inflammatory cytokines such as IL-1[336], providing a link between alterations in central neuronal function and subsequent inflammatory changes.

Allergic lung inflammation was additionally associated with increases in T-lymphocyte chemoattractants, with significant transcriptional increases in CCL4 and XCR1 at sub-chronic stages of inflammation, as well as progressive increases in CCL1, also known as T-cell activation-3 (TCA3). Allergic inflammation was additionally found to impact levels of central IL-4, a cytokine associated with TH2 cell differentiation. Together this would

suggest central T-cell infiltration following TH2 lung inflammation, possibly in association with the increases in BBB permeability. Indeed, CCL4 has previously been shown to enhance T-lymphocyte adhesion to the cerebral endothelium[337]. However, further investigations would be necessary to confirm this postulate.

5.4.4 The Effect of Allergic Lung Inflammation on the brain – a similar phenomenon to neuropathic pain?

Central changes which lead to unwanted or exaggerated reflexes may be a relatively unstudied concept in airway or asthma research, but it is a well-established phenomenon in the pain community. It is well-known that injury and peripheral inflammation leads to hypersensitisation of nociceptors in the periphery, through mechanisms such as neurogenic inflammation, resulting in hyperalgesia, phenomena which are mirrored in the lung parenchyma in asthmatic inflammation[199]. However, continuous stimulation of peripheral nociceptors in pain can occasionally lead to a phenomenon known as central sensitisation in which second-order neurons in the spinal cord become hypersensitive through central neuronal or inflammatory changes. This can result in painful sensations from non-painful stimuli, termed allodynia, or ultimately painful sensations without a stimulus, termed neuropathic or chronic pain. Central sensitisation involves the increased excitability of second order neurons in the dorsal horn, through upregulation NMDA and AMPA receptors, increased sensitivity of neurons to glutamate, increased production of SP and activation of glia, which secrete immune mediators and other proteins that contribute to pain[338]. These changes mirror what is seen in the brainstem following sub-chronic and chronic TH2 lung inflammation. Molecular changes in the brainstem following allergic lung inflammation are indicative of central sensitisation and this phenomenon would provide an explanation for the numerous symptoms of asthma.

5.4.5 The Impact of Allergic Lung Inflammation on the Brain – the Importance of Severity and Chronicity of Inflammation

The initial pilot study, which used a protocol in which animals were hyperimmunised, resulted in a severe form of TH2 lung inflammation. Though this study was initially intended to find prospective gene targets to study further, it proves a useful model in understanding the effect of severe TH2-type inflammatory events on the brainstem. When comparing effects of this model with those of the acute, sub-chronic and chronic models, it

is noticeable that the effect of TH2 inflammation on plasticity in the brainstem seems to be highly dependent on the severity of inflammation. Changes in expression of neuroplasticity-associated genes, including AMPA, NMDA and metabotropic glutamate receptors, intracellular signalling molecules associated with NMDA receptor activation and genes associated with the formation of a NMDA and AMPA-rich postsynaptic densities were most apparent in the hyperimmunised, as well as the sub-chronic model, where inflammation was at its peak. For example, expression of Grin1, Grin2d, Gria3, Camk2g and Dlg4 were all highly and significantly upregulated following severe lung inflammation, a pattern that was also apparent following sub-chronic lung inflammation. This suggests that robust peripheral lung inflammation has a strong impact on the state of plasticity in the brainstem, and may be an inducer of gene expression. Indeed, inflammation has previously shown to induce NMDA receptor and substance P expression, both peripherally[339] and centrally[340].

Whereas severity of inflammation is an indicator of the induction of plastic changes in the brain, chronicity of an inflammatory event seems to be related to the inflammatory status in the brain. Severe, acute and sub-chronic inflammation had little impact on the inflammatory gene expression in the brain, with only a clear, though non-significant, upregulation in the macrophage chemokine CCL4 after severe and sub-chronic inflammation. Contrastingly, chronic inflammation, in which the inflammatory event in the lungs lasted almost three weeks, was associated with changes in the expression of cytokines and chemokines, particularly associated with a pro-inflammatory event. Though a rather unstudied topic, long-term peripheral inflammatory events have previously been shown to induce a delayed onset of cytokine expression in the brain[128,132].

It is essential to note at this stage that the data from this series of experiments clearly accentuates the importance in investigating the effect of physiological models of inflammation on the brain. Though acute models, such as single challenges with LPS, or acute models of allergic lung inflammation, provide significant insight into the mechanisms by which peripheral inflammation communicates with the brain, they may not reveal the full spectrum of central changes associated with inflammation. Development of chronic models of allergic inflammation, which more closely mirror human conditions, have revealed changes in plasticity that were not apparent at acute stages, as well as changes in central inflammation that were not apparent at even sub-chronic stages. In

addition, studies investigating chronic peripheral TH1 type inflammation are currently revealing increases in central cytokines, alterations in the phenotype of the cerebral endothelium and changes in neurotransmitter metabolism, changes that have otherwise been masked with the use of acute models of inflammation[127,132]. It is clear that the use of physiological and chronic inflammatory models is key in future research into immune to brain communication.

5.5 Conclusions

In this study it was found that chronic allergic lung inflammation, which has previously been associated with alterations in the activity of peripheral neuronal fibres, results in transcriptional changes indicative of plastic changes of neuronal fibres in the brainstem. Not only does this reflect previous studies which show evidence for synaptic plasticity in the brainstem following chronic lung inflammation, but also provides an explanation for the increased CNS reflex and the numerous symptoms associated with asthma, including chronic cough, dyspnoea and breathlessness. Allergic lung inflammation was also associated with increased BBB permeability, IgG infiltration and delayed increases in pro-inflammatory mediator production. The plastic and inflammatory changes associated with chronic peripheral inflammatory changes mirror events in a phenomenon known as neuropathic pain, providing an additional explanation for the hypersensitivity of neuronal fibres in allergic lung inflammation. This study accentuates the importance in investigating the effect of chronic physiological models of inflammation on the brain, uncovering key central changes that would have been otherwise undetectable in an acute model.

Chapter 6: The impact of the TLR7 agonist resiquimod on the CNS in a model of pre-established lung inflammation

6.1 Introduction

For the past 50 years, treatment of allergic asthma has principally focused on the use of short- or long-acting β_2 agonists and inhaled corticosteroids (ICS)[341]. Though proving widely beneficial in the relief of asthma symptoms and control of underlying asthmatic inflammation, multiple concerns regarding the failure for β_2 agonists to treat the underlying cause of the disease and the numerous side effects of ICS has resulted in the development of a new class of drugs for treatment of allergic respiratory disease. These new drugs, known as immunomodulators, instead act by altering the inflammatory events which underlie asthma pathology.

Considerable effort has been devoted to exploring the effectiveness of TLR agonists as an immunomodulatory treatment for allergic asthma, a treatment idea rooted in the known inverse correlation between exposure to TLR agonists such as endotoxin and incidence of asthma[342]. As detailed in Chapter 1, TLR2, 4, 7 and 9 agonists have repeatedly been shown to reduce allergic inflammation and impact on pathological features of asthma such as airway remodelling and airway hyperresponsiveness in animals models of allergic rhinitis and asthma[343]. Recent reports have also demonstrated success in industrially-developed TLR agonists in Phase I, II and III clinical trials[171].

TLR7 is an intracellular TLR, highly expressed in the airways, that recognises single-stranded viral RNA. Activation of TLR7 results in expression of pro-inflammatory cytokines and interferons[16] and synthetic TLR7 ligands, such as the imidazoquinolines imiqiumod (R837) and resiquimod (R848), has repeatedly been shown to reduce airway inflammation, airway remodelling and AHR in chronic OVA immune complex models of asthma[172-174,178,179,344,345]. In addition, the industrially-developed TLR7 agonists GSK2245035 and AZD8848 have recently passed Phase I and II clinical trials, respectively[181].

Despite the pre-clinical and clinical success in the treatment of allergic lung disease with TLR agonists, a potential and critical consequence of TLR agonist administration has been largely ignored. It is well established that challenge with TLR4 (LPS) and TLR3 (poly I:C) agonists induces sickness behaviour, increased central cytokine expression and glial priming[61,346-348]. Fever, sickness behaviour and central cytokine expression have more

recently also been attributed to challenge with the TLR7 agonist imiquimod in a rat model[133] and are highly undesirable side-effects in terms of patient well-being. Prior to further investigation into the efficacy of TLR7 agonists in asthma being carried out, it is essential to understand the potential negative impact this novel therapy can have on the CNS.

In this chapter, we investigate how the TLR7 agonist resiquimod impacts on the brain in the model of sub-chronic lung inflammation developed in Chapter 5, by exploring the effect on sickness behaviour and central cytokine expression.

6.2 Methods

To determine the impact of resiquimod on the brain, control animals were treated with resiquimod alone i.p. or i.n. at 5mg/kg, as detailed in section 2.1.3.6, and results were compared to mice with sub-chronic lung inflammation treated resiquimod, as detailed in section 2.1.3.7. Behavioural assessment, involving the open field assay, was carried out as detailed in section 2.1.4.2, 2.5 hours after immune stimulation with resiquimod. Serum, BALF and brain samples were collected 2.5 hours after immune stimulation, as detailed in sections 2.1.5.2, 2.1.5.3 and 2.1.5.4. Cytokine analysis and analysis of BALF cells was performed as described in sections 2.2.2.3 and 2.2.4.4, respectively. qPCR analysis of brain tissue was carried out as detailed in sections 2.2.3.1 (Protocol B), 2.2.3.3, 2.2.3.4 and 2.2.3.6.

6.3 Results

6.3.1 The Effect of Resiquimod Administration on Peripheral Inflammation

To determine the effect of systemic resiquimod administration on the brain, it was first necessary to first confirm if resiquimod induced peripheral inflammation. Previous studies have shown that systemic and topical administration of TLR7 agonists induces pro-inflammatory cytokine (IL-6, TNF-α) and type I interferon expression in the circulation[133], and additionally reduces cellular lung inflammation in models of allergic lung inflammation[172-174,178,179,344,345] . To determine the biological effects of resiquimod (5mg/kg)

in our model, levels of circulating inflammatory mediators and cells in the BALF were measured.

6.3.1.1. Systemic cytokines

To confirm that resiquimod induced a pro-inflammatory immune response, IL-6, TNF-α, IL-1β and IFN-α levels were measured in the serum of animals with sub-chronic lung inflammation treated with resiquimod i.p. or i.n. (Figure 6.1). Resiquimod administration significantly increased levels of IL-6 (H=26.21, p<0.0001), TNF-α (H=26.60, p<0.0001), IL-1β (H=16.82, p=0.0008) and IFN-α (H=24.88, p<0.0001). Serum levels of IL-6 (i.n.:p=0.0049) and TNF-α (p=0.0020) were significantly elevated in animals treated with resiquimod i.p. (IL-6: p=0.0049; TNF-α: p=0.0020) and i.n. (IL-6: p=0.0007; TNF-α: p=0.0013), as compared to saline treated control mice, whereas serum levels of IL-1β (p=0.0002) and IFN-α (p=0.0124) were significantly elevated in animals treated with resiquimod i.n. only, as compared to control.

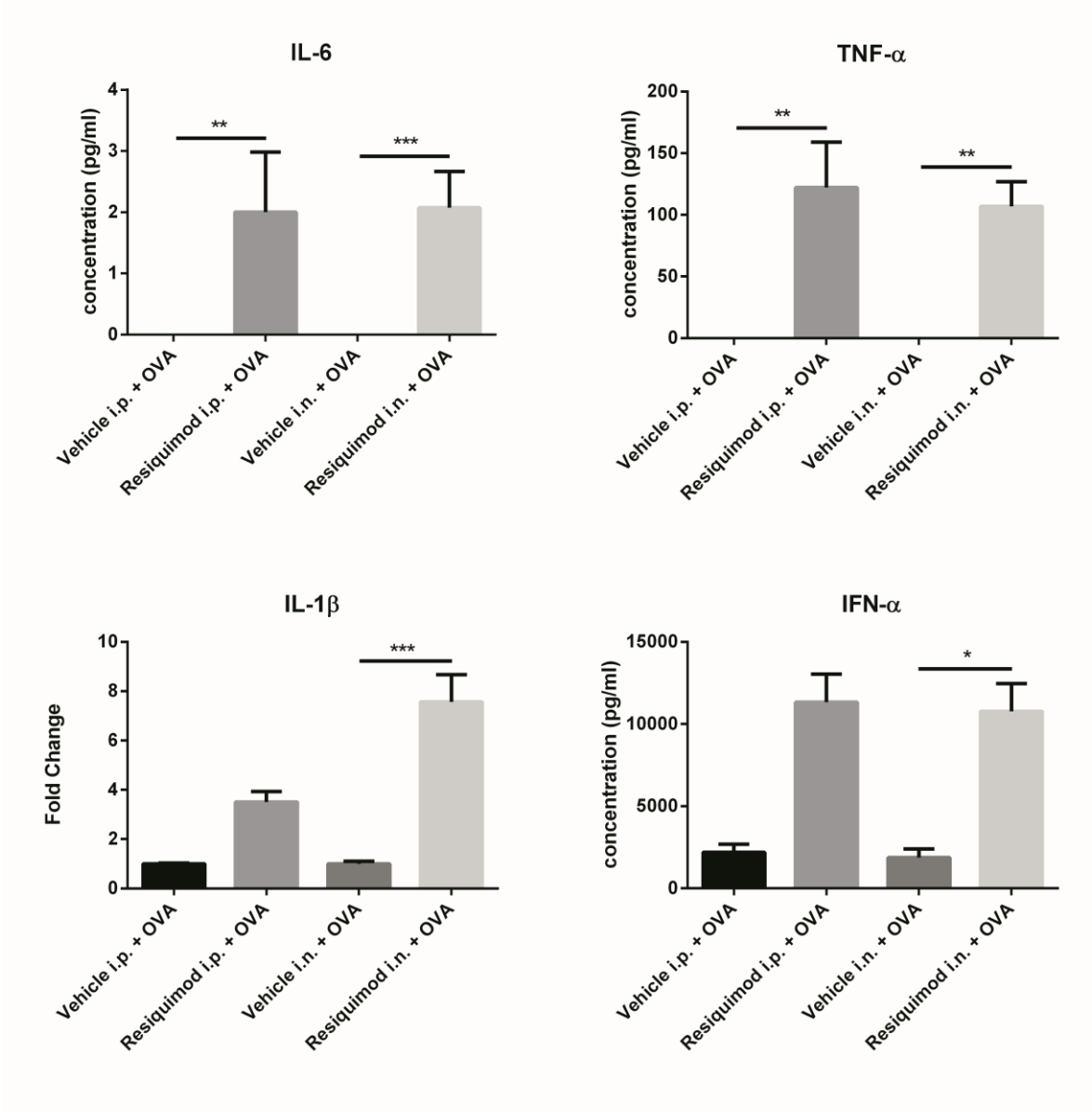

Figure 6.1 - The effect of i.p. or i.n. resiquimod administration (5mg/kg) on serum levels of IL-6, TNF-α, IL-1β and IFN-α in a model of pre-established lung inflammation. Animals were immunised against OVA in conjunction with $Al(OH)_3$ on days 0 and 14; from day 21, animals were challenged with aerosolised OVA for 8 days. On the final day of OVA challenge, animals were treated with resiquimod 0.5 hour prior to OVA challenge. Terminal blood samples were collected 2.5 hours after resiquimod challenge and analysed using single-plex (IL-6, TNF-α, IFN-α) and multiplex (IL-1β) ELISAs. Values are expressed as mean pg cytokine per ml serum (IL-6, TNF-α, IFN-α) or mean fold change from vehicle (IL-1β) ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. vehicle control using the Kruskal-Wallis test followed by the Dunn's post-hoc test. n=8

6.3.1.2. BAL cells

To determine if resiquimod had an immunomodulatory effect on allergic lung inflammation, as previously demonstrated in house (GSK), levels of inflammatory cells in the BALF were measured in naive animals or animals immunised and challenged with OVA, treated with vehicle or resiquimod i.p. or i.n. (Figure 6.2). OVA immunisation and challenge (I+O) had a significant effect on levels of macrophages (H=17.76, p=0.0014), lymphocytes ($F_{4,26}$=29.40, p<0.0001), neutrophils ($F_{4,26}$=17.22, p<0.0001) and eosinophils ($F_{4,26}$=41.85, p<0.0001), where levels of lymphocytes, neutrophils and eosinophils in BALF were significantly elevated in all I+O groups, as compared to naive animals, and levels of macrophages were significantly elevated in I+O animals treated with vehicle i.n., as compared naive animals. I+O animals treated with resiquimod i.n. had substantially lower levels of macrophages and neutrophils in BALF and significantly lower levels of lymphocytes (p=0.0008) and eosinophils (p=0.0094) in BALF, as compared to the BALF of I+O animals treated with vehicle i.n. No difference in cell numbers in BALF was evident in I+O animals treated with resiquimod i.p., as compared to those treated with vehicle i.p.

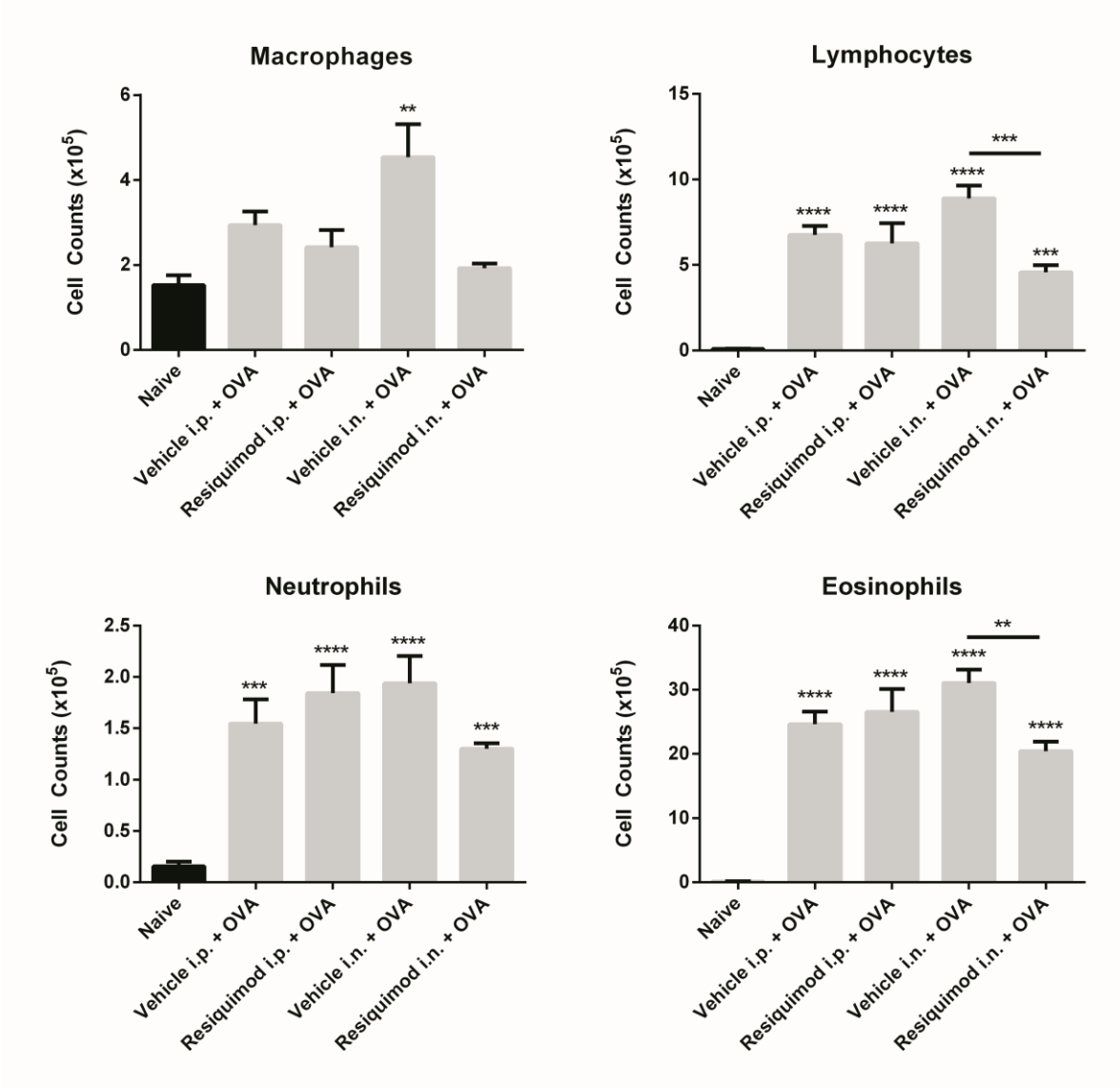

Figure 6.2 - The effect of i.p. or i.n. resiquimod administration (5mg/kg) on numbers of BALF macrophages, lymphocytes, neutrophils and eosinophils in a model of pre-established lung inflammation. Animals were immunised against OVA in conjunction with $Al(OH)_3$ on days 0 and 14; from day 21, animals were challenged with aerosolised OVA for 8 days. On the final day of OVA challenge, animals were treated with resiquimod 0.5 hours prior to OVA challenge. BALF was collected 2.5 hours after resiquimod challenge and analysed using Sysmex Technology. Values are expressed as total mean cell counts ($x10^5$) ± SEM., **p<0.01, ***p<0.001, ****p<0.0001 vs. naive control unless otherwise specified using the Kruskal-Wallis test followed by the Dunn's post-hoc test (macrophages) or one way ANOVA followed by the Tukey post-hoc test. n=5-8

6.3.2 The Effect of Resiquimod Administration on Behavioural Changes in the Open Field

To determine the effect of resiquimod treatment on the brain in a model of sub-chronic lung inflammation, behavioural changes in the open field assay were measured (Figure 6.3). The assay focused on the measurement of rears, as changes in locomotor activity were found to be highly variable in repeated assays using BALB/C mice[349] (unpublished observations). Resiquimod given to naïve animals was associated with a significant downregulation of rears when administered i.p. (p=0.0039), but had no effect when administered i.n. (p=0.1833). Comparatively, when administered to I+O animals, resiquimod was associated with a significant downregulation of rears when administered i.p. (p=0.0010) and i.n. (p=0.0024), as compared to vehicle control. When administered to I+O animals, i.p. resiquimod treatment induced a 6.6 fold downregulation of rears, as compared to vehicle control, as opposed to 2.6 fold downregulation in naïve animals. The same was true for intranasal resiquimod challenge, where resiquimod administered to I+O animals resulted in a significant 5.2 fold downregulation of rears as compared to vehicle control, whereas resiquimod to naïve animals resulted in a 3.1 fold downregulation as compared to vehicle control. These results suggest more profound behavioural changes in mice with sub-chronic lung inflammation; however, it should be noted that behavioural changes were already reduced prior to resiquimod treatment.

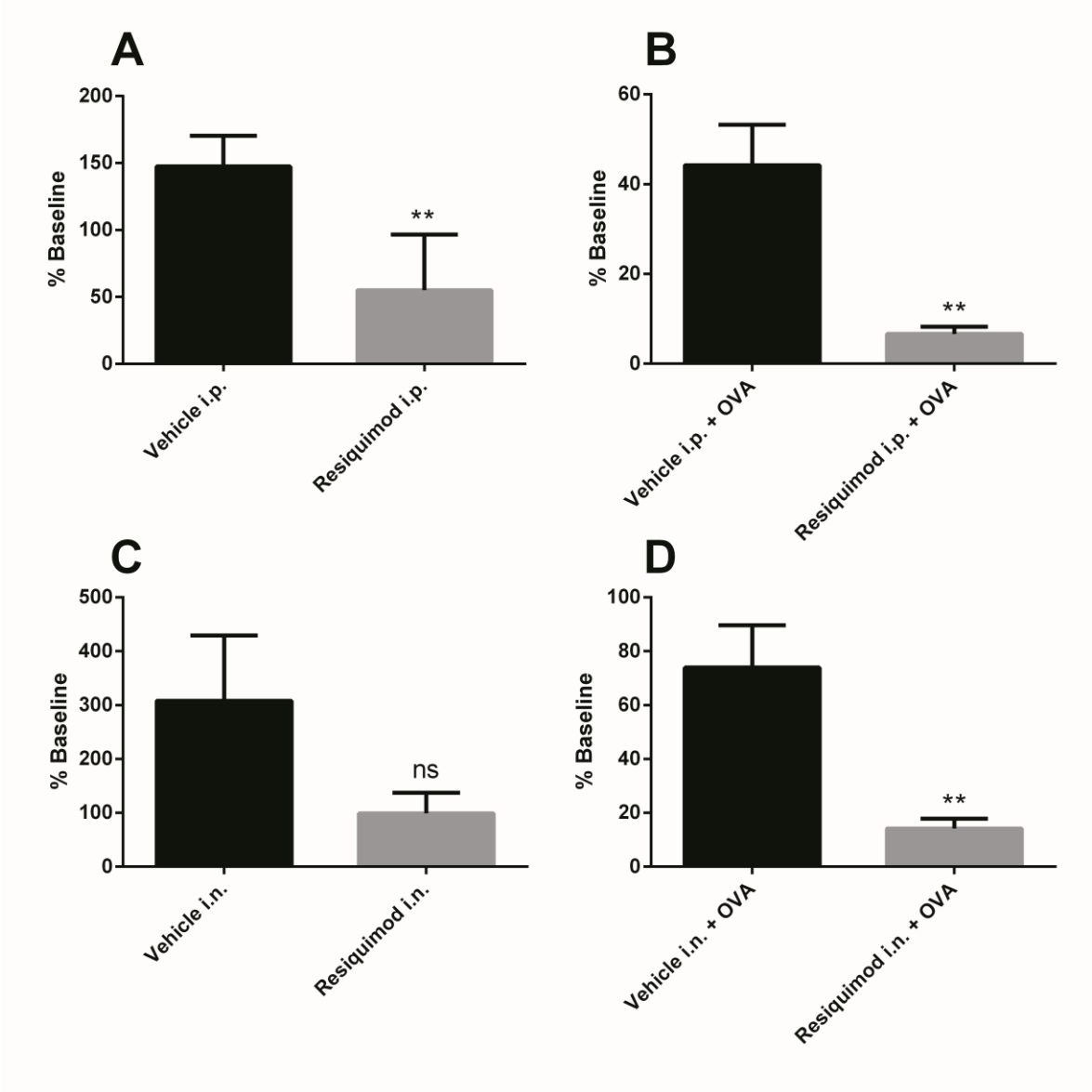

Figure 6.3 - The effect of i.p. (A,B) or i.n. (C,D) resiquimod administration (5mg/kg) on rears in the open field in mice with (B,D) or without (A,C) pre-established lung inflammation. Behaviour was assayed between 2.5 and 3 hours after immune stimulation. A baseline measurement was taken one day prior to resiquimod treatment. Values are represented as mean percent of the baseline measurement ± SEM. **$p<0.01$ vs. vehicle control using the Student's t-test (C,D) or the Mann-Whitney test (A,B). n=8-9

6.3.3 The Effect of Resiquimod Administration on Central Pro-inflammatory Cytokine Expression

To further determine the central effect of resiquimod administration in a model of sub-chronic lung inflammation, central expression of IL-1β (Figure 6.4) and TNF-α (Figure 6.5) was measured in the hippocampus and brainstem. When given to naïve mice, resiquimod

treatment significantly increased levels of IL-1β in the hippocampus, when administered i.p. (H=12.77, p<0.0001) and i.n. (H=0.0102, p=0.102), as well as in the brainstem, when administered i.p. ($F_{3,12}$=27.46, p<0.0001) and i.n. ($F_{3,12}$=15.31, p=0.0002) (Figure 6.4). Levels of IL-1β in the brainstem were significantly elevated following resiquimod administration i.p. (p<0.0001) and i.n. (p=0.0004), and substantially elevated in the hippocampus following i.p. and i.n. resiquimod challenge. When administered to I+O animals, i.p. (p=0.0078), resiquimod treatment was associated with significantly increased levels of IL-1β in the brainstem, but these changes were not seen after i.n. administration (p=0.2546). Furthermore, levels of IL-1β in the hippocampus of I+O animals was only marginally elevated following resiquimod treatment. The results show that levels of IL-1β in the brainstem of I+O animals treated with resiquimod were significantly lower than levels in the brainstem of naïve animals challenged with resiquimod (i.p.: p=0.0153; i.n.: p=0.0083). The same was true in the hippocampus, although it did not reach significance (i.p.: p=0.5674; i.n.: p>0.999).

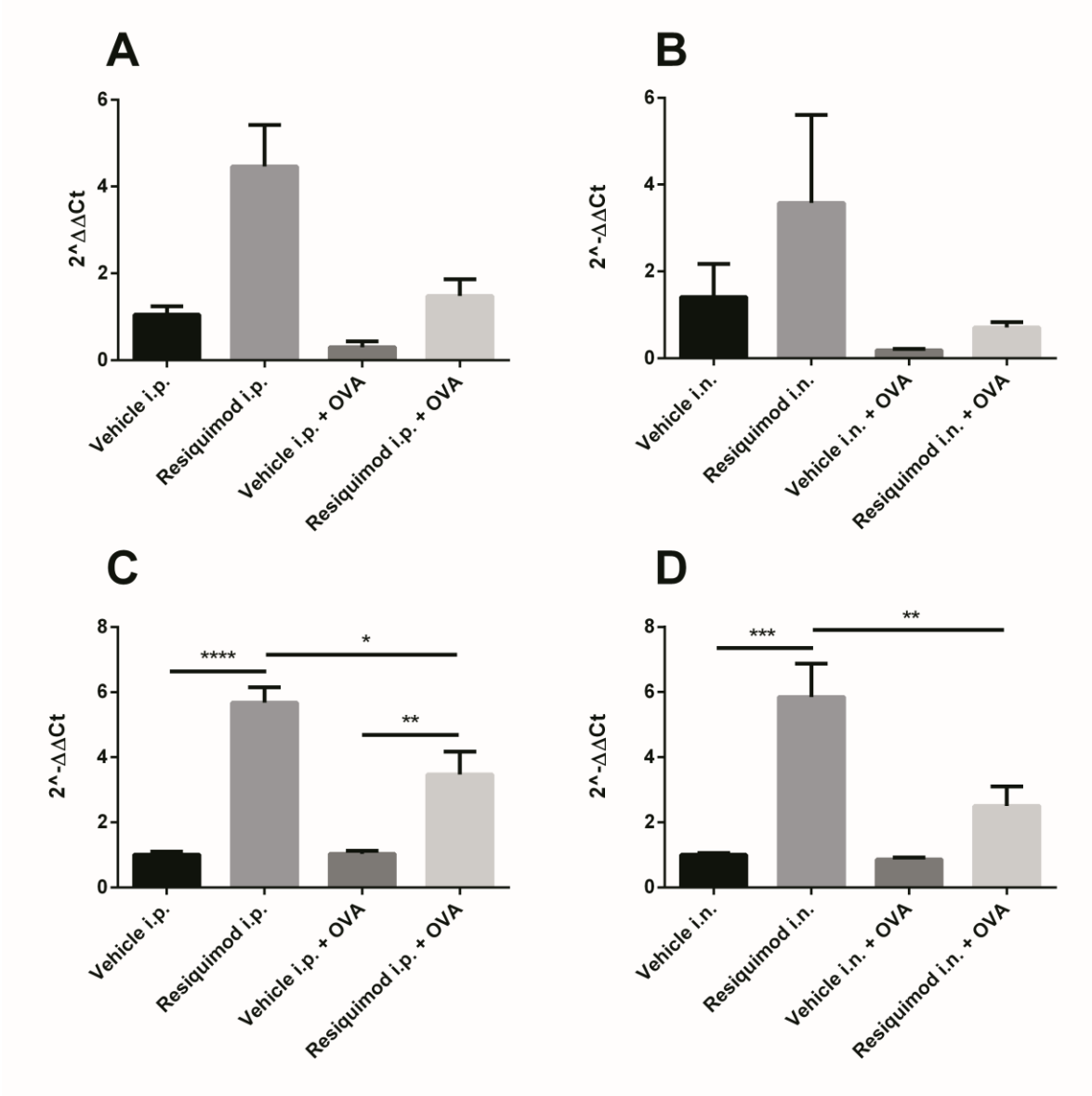

Figure 6.4 - The effect of i.p. (A,C) or i.n. (B, D) resiquimod administration (5mg/kg) on mRNA levels of IL-1β in the hippocampus (A,B) and brainstem (C,D) of mice with or without pre-established lung inflammation. Brain tissue was collected 2.5 hours after immune stimulation with resiquimod; IL-1β was measured using SYBR Green qPCR. Values are expressed as mean fold change (2^-ΔΔCt) from vehicle, relative to HPRT expression ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle control using one way ANOVA followed by a Tukey post-hoc test (C,D) or Kruskal-Wallis test followed by a Dunn's post-hoc test (A,B). n=4

Resiquimod treatment had a significant effect on levels of TNF-α in the hippocampus and brainstem of naïve mice when administered i.p. (hi: H=13.06, p<0.0001; bs: H=13.50, p<0.0001) and i.n. (hi: H=10.27, p<0.0001; bs: H=13.26, p<0.0001) (Figure 6.5). Levels of TNF-α were significantly elevated in the hippocampus of naïve animals treated with resiquimod i.n. (p<0.0122), and in the brainstem of naïve animals treated with resiquimod

i.p. (p=0.0022) and i.n. (p=0.0050) as compared to respective brain areas of control animals. In contrast, levels of TNF-α were not significantly elevated in the brainstem or hippocampus of I+O animals treated with resiquimod i.p. or i.n. Crucially, levels of TNF-α in the hippocampus and brainstem of I+O animals treated with resiquimod were substantially lower than levels in the brainstem and hippocampus of naïve animals treated with resiquimod.

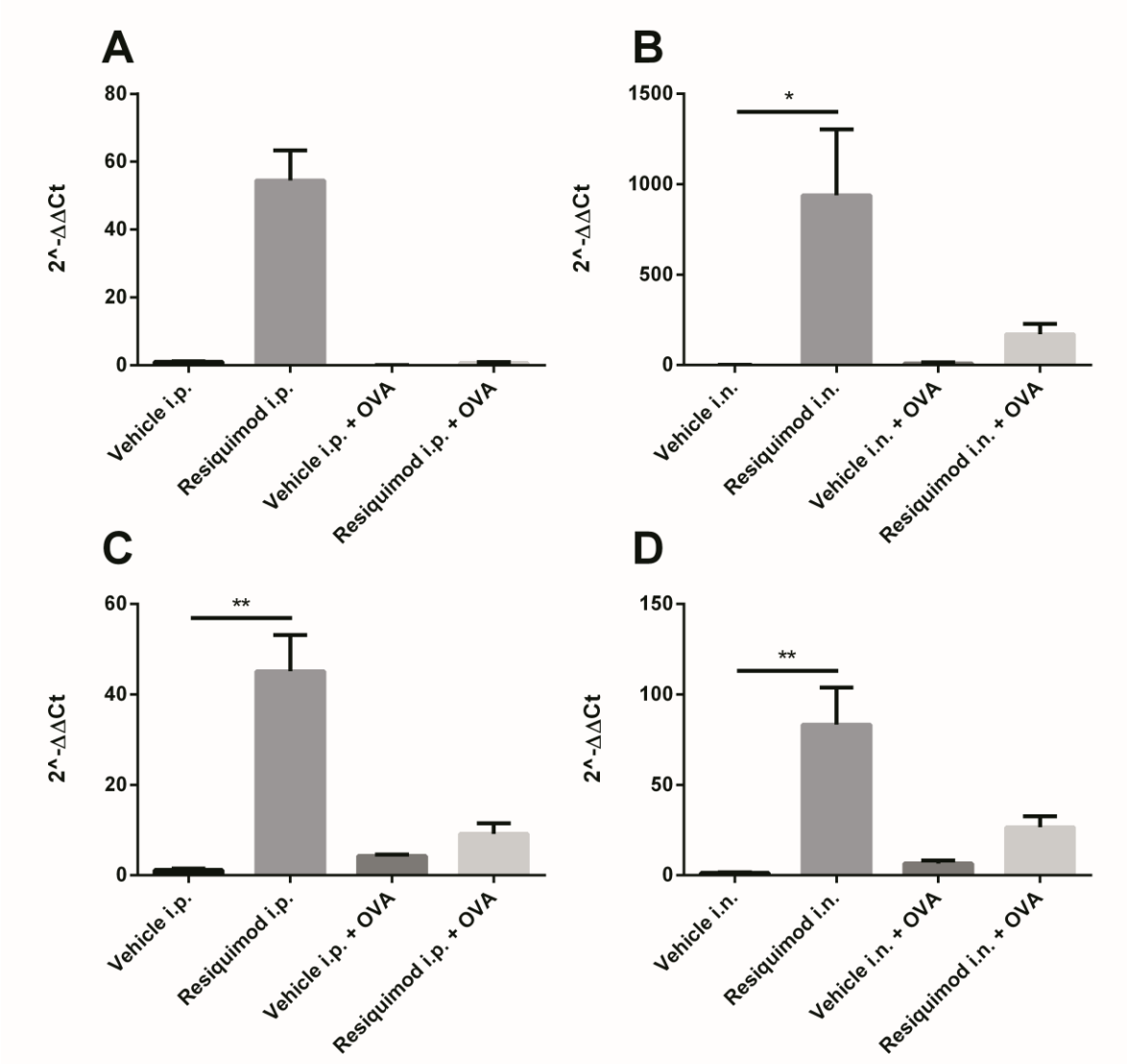

Figure 6.5 - The effect of i.p. (A,C) or i.n. (B, D) resiquimod administration (5mg/kg) on mRNA levels of TNF in the hippocampus (A,B) and brainstem (C,D) of mice with or without pre-established lung inflammation. Brain tissue was collected 2.5 hours after immune stimulation; TNF was measured using SYBR green qPCR. Values are expressed as mean fold change (2^-ΔΔCt) from vehicle, relative to HPRT expression ± SEM. *p<0.05, **p<0.01 vs. vehicle control using the Kruskal-Wallis test followed by a Dunn's post-hoc test. n=4

6.3.4 The Effect of Resiquimod Administration on Central COX-2 Expression

COX-2 has previously been shown to be upregulated in the cerebral endothelium in response to inflammation[64]. To assess whether the attenuated central response to resiquimod in animals with sub-chronic lung inflammation was due to altered responsiveness of the endothelium, levels of COX-2 were measured in the brainstem and hippocampus of naive animals or I+O animals treated with resiquimod (Figure 6.6). Treatment was found to significantly affect expression of COX-2 in the hippocampus following i.p. (H=11.78, p=0.0004) and i.n. challenge ($F_{3,11}$=9.441, p=0.0022), as well as in the brainstem following i.p. ($F_{2,13}$=16.11, p=0.0002) and i.n. ($F_{2,13}$=16.06, p=0.0002) challenge. Levels of COX-2 were significantly elevated in the brainstem of naive (p=0.0009) or I+O animals (p=0.0040) treated with resiquimod i.p., as well naive (p=0.0009) or I+O animals (p=0.0041) treated with resiquimod i.n, as compared to respective vehicle controls. No difference in levels of COX-2 was apparent in the brainstem of I+O animals or naïve animals treated with resiquimod. In the hippocampus, levels of COX-2 were significantly elevated following i.p. (p=0.0254) or i.n. (p=0.0036) resiquimod challenge in I+O animals. These changes were not seen in naïve mice treated with resiquimod. However, I+O animals treated with vehicle also showed substantially higher levels of COX-2 in the hippocampus, suggestive of an effect of sub-chronic lung inflammation alone on central COX-2 expression.

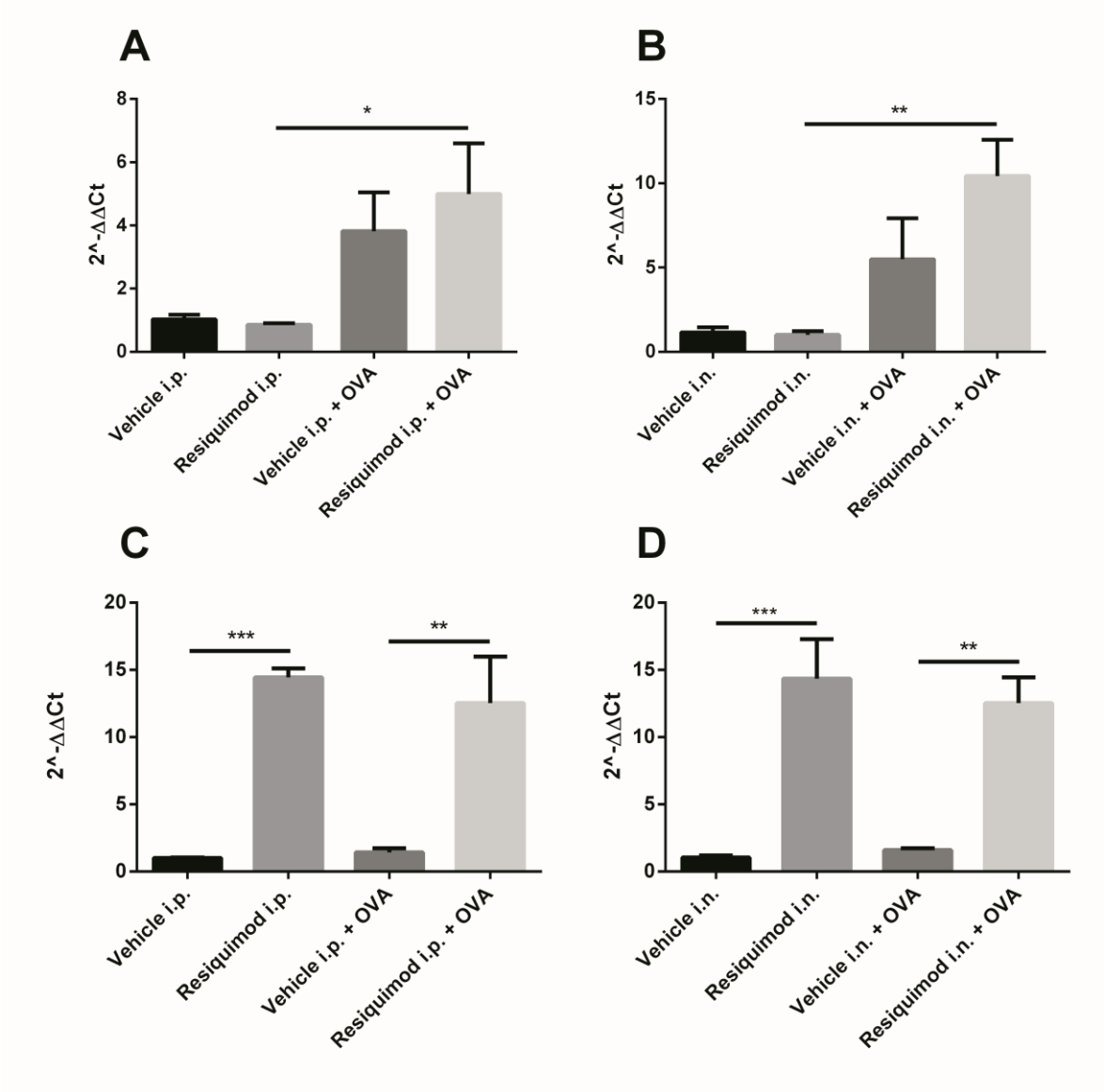

Figure 6.6 - The effect of i.p. (A,C) or i.n. (B, D) resiquimod administration (5mg/kg) on mRNA levels of COX-2 in the hippocampus (A,B) and brainstem (C,D) of mice with or without pre-established lung inflammation. Brain tissue was collected 2.5 hours after immune stimulation; COX-2 was measured using SYBR green qPCR. Values are expressed as mean fold change (2^-ΔΔCt) from vehicle, relative to HPRT expression ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle control using one way ANOVA followed by a Tukey post-hoc test (B,C,D) or Kruskal-Wallis test followed by a Dunn's post-hoc test (A). n=4

6.3.5 The Effect of Resiquimod Administration on Central Expression of Anti-inflammatory Cytokines

COX-2 has been shown to have anti-inflammatory properties centrally, particularly when expressed in neurons of the hippocampus[350,351]. Because COX-2 was elevated in the brains of I+O animals, expression levels of the anti-inflammatory cytokine transforming growth

factor β (TGF-β) were measured in the hippocampus and brainstem in naive animals or I+O animals treated with resiquimod (Figure 6.7). Levels of TGFβ mRNA were found to be substantially higher in the hippocampus and brainstem of I+O animals, as compared to the respective areas in naïve controls, where treatment significantly affected levels of TGF-β in the hippocampus (i.p.: $F_{3,12}$=53.76, p<0.0001; i.n.: H=10.64, p=0.0017) and brainstem (i.p.: $F_{3,12}$=5.015, p=0.0176; i.n.: $F_{3,12}$=5.084, p=0.0169). Levels of TGFβ mRNA were significantly elevated in the hippocampus of I+O animals treated with resiquimod i.p. (p<0.0001) as compared to naïve animals treated with resiquimod i.p. Similar findings were obtained in the brainstem of I+O animals treated with resiquimod i.n. (p=0.0478) as compared to naïve animals treated with resiquimod i.n.. In addition, the brainstem of I+O animals treated with vehicle i.p. were found to have higher levels of TGFβ mRNA than naïve animals treated with vehicle i.p. (p=0.0144), suggesting elevated baseline levels of TGF-β in I+O mice..

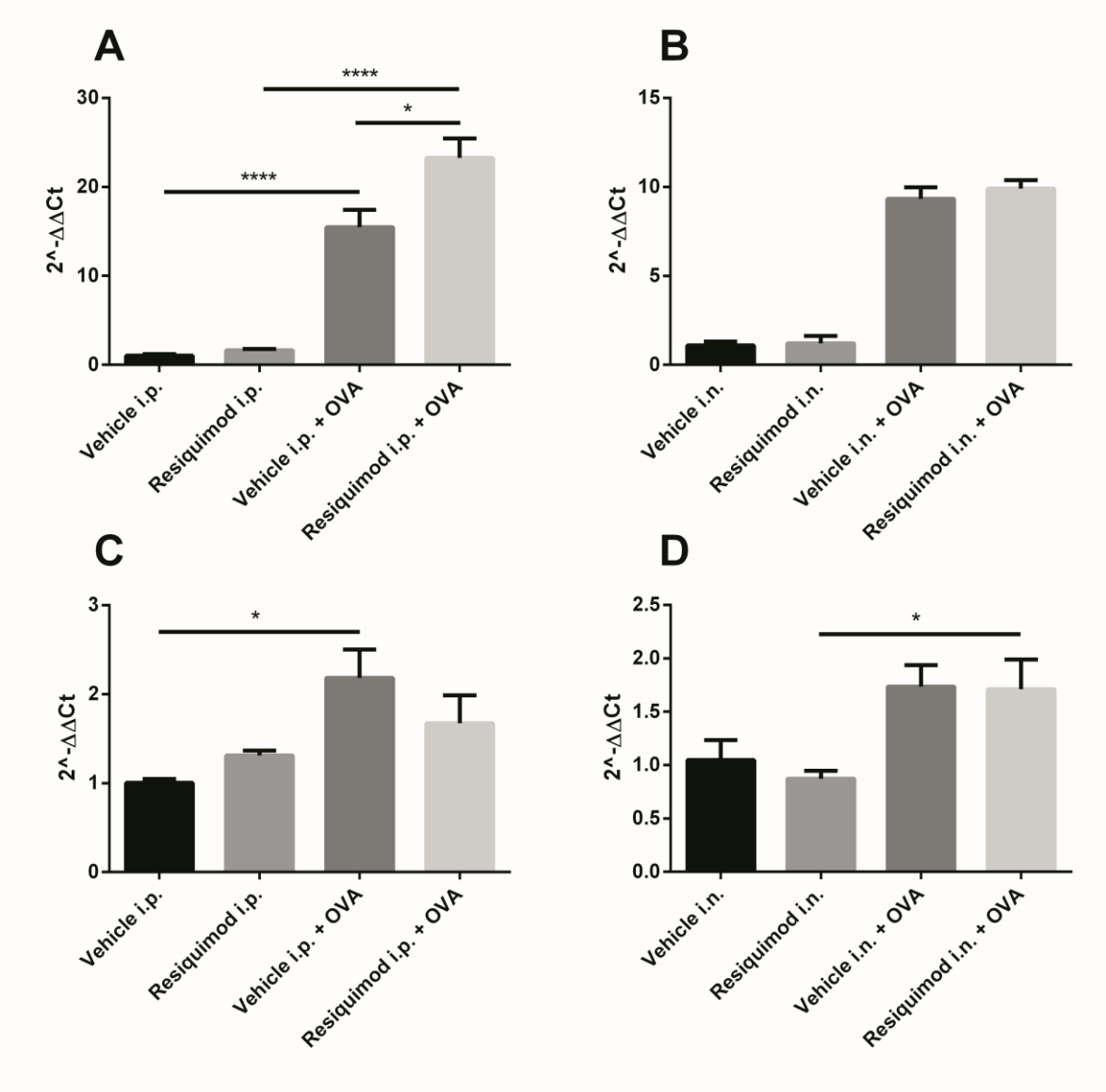

Figure 6.7 - The effect of i.p. (A,C) or i.n. (B, D) resiquimod administration (5mg/kg) on mRNA levels of TGF-β in the hippocampus (A,B) and brainstem (C,D) of mice with or without pre-established lung inflammation. Brain tissue was collected 2.5 hours after immune stimulation; TGF-β was measured using SYBR green qPCR. Values are expressed as mean fold change (2^-ΔΔCt) from vehicle, relative to HPRT expression ± SEM. *p<0.05, ***p<0.001 vs. vehicle control using one way ANOVA followed by a Tukey post-hoc test (A,C,D) or Kruskal-Wallis test followed by a Dunn's post-hoc test (B). n=4

6.3.6 The effect of Resiquimod Administration on Expression of GABABR1 and BDNF mRNA

The above results suggested that expression levels of the anti-inflammatory cytokine TGF-β are upregulated in animals with sub-chronic lung inflammation, associated with a reduced central pro-inflammatory cytokine response to resiquimod challenge. Expression

of the $GABA_B$ receptor on the surface of microglia has been associated with a reduced cytokine response to pro-inflammatory stimuli[352], a receptor that is also known to be involved in anxiety behaviour[353], a mood disorder associated with asthmatic inflammation[299]. The expression of $GABA_{B(1)}$ receptors was consequently evaluated in the hippocampus and brainstem (Figure 6.8).

Treatment had a significant effect on levels of $GABA_{B(1)}R$ mRNA in the hippocampus (i.p.: $F_{3,12}$=60.22, p<0.0001; i.n.: $F_{3,12}$=53.17, p<0.0001) and brainstem (i.p: H=12.64, p<0.0001; i.n.:$F_{3,12}$=12.16, p=0.0006). Levels of $GABA_{B(1)}R$ were substantially elevated in the hippocampus of I+O animals, as compared to naïve controls, reaching significance in the hippocampus of animals treated with vehicle (p=0.0001) or resiquimod i.p. (p<0.0001) and vehicle (p<0.0001) or resiquimod i.n. (p<0.0001), as compared to respective naïve controls. The effect was also apparent in the brainstem, although to a lesser degree as compared to the hippocampus, where levels of $GABA_{B(1)}R$ was significantly elevated in I+O treated with vehicle i.p. (p=0.0085) or with vehicle (p=0.0022) or resiquimod i.n. (p=0.0241), as compared to respective non-I+O controls.

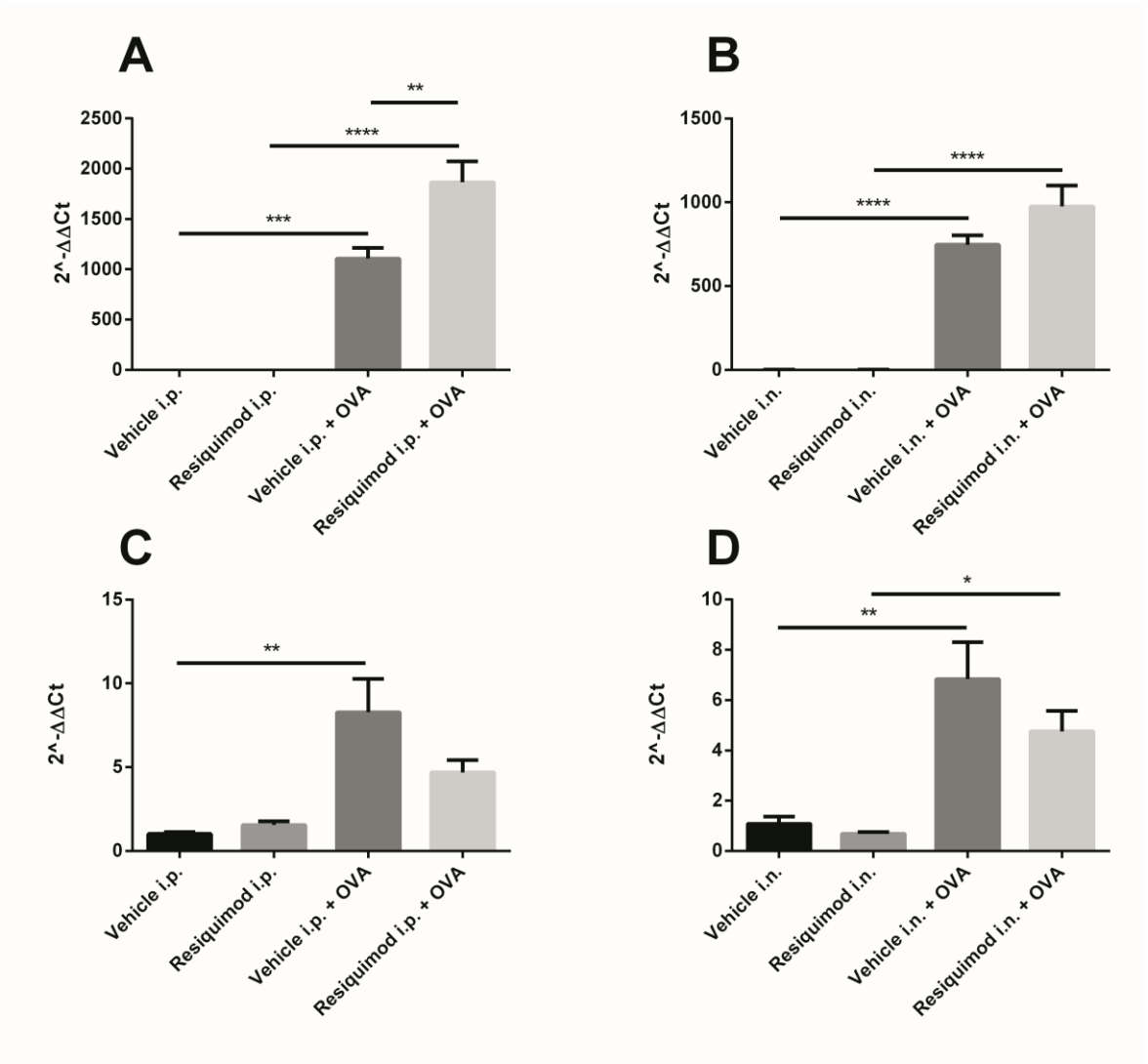

Figure 6.8 - The effect of i.p. (A,C) or i.n. (B, D) resiquimod administration (5mg/kg) on mRNA levels of $GABA_{B(1)}R$ in the hippocampus (A,B) and brainstem (C,D) of mice with or without pre-established lung inflammation. Brain tissue was collected 2.5 hours after immune stimulation; GABABR1 was measured using SYBR green qPCR. Values are expressed as mean fold change (2^-ΔΔCt) from vehicle, relative to HPRT expression ± SEM. *p<0.05, ***p<0.001 vs. vehicle control using one way ANOVA followed by a Tukey post-hoc test (A,B,D) or Kruskal-Wallis test followed by a Dunn's post-hoc test (C) n=4

$GABA_B$ receptor stimulation has previously been shown to reduce levels of brain-derived neurotrophic factor (BDNF)[354]. Decreased levels of this neurotrophin have been shown to play an important role in depression and anxiety[355], disorders which are prevalent in the asthmatic population[356]. As a consequence, expression levels of BDNF were measured in the hippocampus and brainstem of animals with or without pre-established lung inflammation, treated with vehicle or resiquimod (Figure 6.9).

I.p. or i.n. resiquimod treatment did not have a significant effect on levels of BDNF in the hippocampus or brainstem of naïve control mice. Contrastingly, levels of BDNF were

significantly downregulated in the hippocampus of I+O animals treated with vehicle i.p. (p=0.0220) or i.n. (p=0.0059), as compared to non-I+O controls treated with vehicle, as well as in the hippocampus of I+O animals treated with resiquimod i.p. (p=0.0379) or i.n. (p=0.0313), as compared to respective non-I+O controls. A similar phenomenon was apparent in the brainstem, where levels of BDNF were significantly downregulated in I+O animals treated with vehicle i.n., as compared to non-I+O controls treated with vehicle i.n. (p=0.0066), suggesting decreased baseline levels of BDNF.

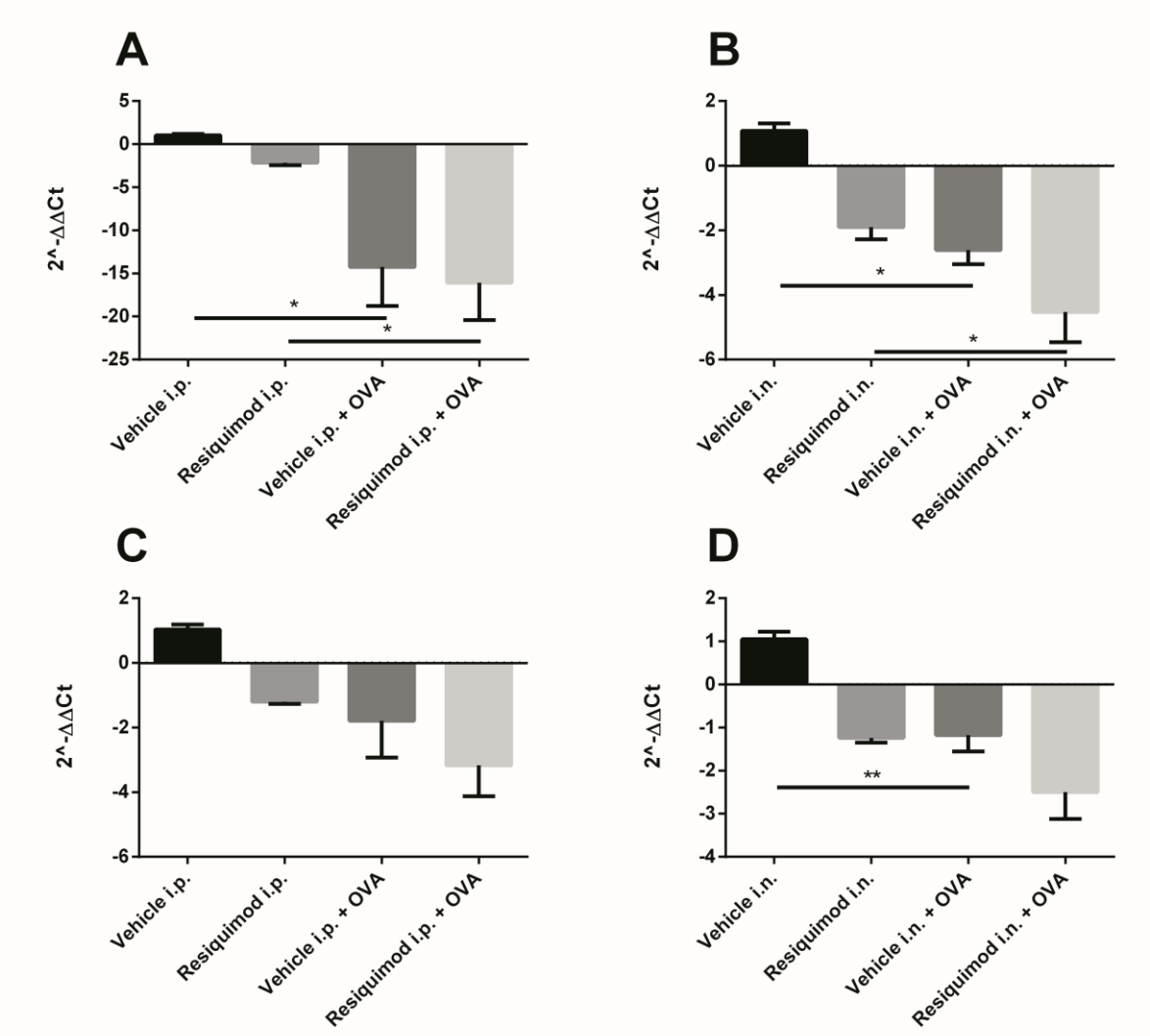

Figure 6.9 - The effect of i.p. (A,C) or i.n. (B, D) resiquimod administration (5mg/kg) on mRNA levels of BDNF in the hippocampus (A,B) and brainstem (C,D) of mice with or without pre-established lung inflammation. Brain tissue was collected 2.5 hours after immune stimulation; BDNF was measured using SYBR green qPCR. Values are expressed as mean fold change (2^-ΔΔCt) from vehicle, relative to HPRT expression ± SEM. *p<0.05, **p<0.01 vs. vehicle control using one way ANOVA followed by a Tukey post-hoc test. n=4

6.4 Discussion

Pharmaceutical companies and universities are currently exploring the use of immunomodulating TLR agonists as novel treatments for allergic respiratory diseases, such as allergic rhinitis and asthma[171]. This is surprising and somewhat counterintuitive, when considering the knowledge that real-live TLR agonists like influenza and rhinovirus have deleterious and exacerbating effects on allergic lung inflammation[160]. Similarly, TLR agonists, such as LPS or poly I:C, are well-known to impact on the CNS, inducing sickness

behaviour and central cytokine expression, which are as of yet unexplored, but undoubtedly unwanted consequences of immunomodulation in asthma patients. In this study we demonstrate that treatment of allergic animals with the TLR7 agonist resiquimod does result in central expression of IL-1β and TNF-α, but the levels were significantly lower than seen with resiquimod administration in healthy controls. In contrast $GABA_BR$ and BDNF levels were changed in mice with allergic inflammation, and further altered after immunomodulation, changes which coincided with exaggerated anxiety behaviour. Together, these observations suggest increased depressive or anxiety behaviours in mice with lung inflammation.

6.4.1 Sub-chronic Lung Inflammation Attenuates the effect of Resiquimod on Central Cytokine Expression – an Event Dependent on Microglia?

In this study it was found that the effect of resiquimod on central expression of IL-1β and TNF-α was significantly attenuated in animals with allergic lung inflammation. In terms of patient well-being, this result is encouraging, suggesting that immunomodulation of allergic asthma may have little effect on innate inflammatory pathways in the CNS. Previous studies, such as by Damm et al[133] found that imiquimod alone (often used topically in cancer treatment) significantly increased central expression of pro-inflammatory cytokines, similar to our control naïve animal group. However, this study failed to examine the effect of drug treatment on the CNS in a disease model, ignoring the impact that a disease state can have on the brain. This study is the first to investigate the central impact of drug therapy on a diseased background, highlighting the importance of investigating the CNS effect of pharmaceutical treatment in the disease to which it is targeted.

The attenuated effect of resiquimod on central cytokine expression may reflect an allergic inflammation-dependent dampening of immune to brain communication pathways, particularly a blunting of typical pro-inflammatory microglial responses to circulating inflammatory mediators or PAMPs. Multiple immune to brain communication pathways may be responsible for the expression of central cytokines following resiquimod administration, including interaction of resiquimod or circulating pro-inflammatory cytokines with respective receptors expressed on the cerebral endothelium or on microglia. The elevation in COX-2, an enzyme that is preferentially upregulated in the cerebral

endothelium in response to LPS-induced inflammation[64], was unaffected by sub-chronic lung inflammation, suggesting microglia, as opposed to endothelial cells, are less responsive to resiquimod in animals with sub-chronic lung inflammation.

The increase in central TGF-β expression following sub-chronic lung inflammation substantiates a dampening of microglial pro-inflammatory responses. TGF-β is an anti-inflammatory cytokine which induces and is expressed by alternatively activated (M2) macrophages[1,17,18]. These polarised macrophages are induced by TH2 cytokines (IL-4 and IL-13) and are characterised by high expression of anti-inflammatory cytokines (IL-10, TGF-β and IL-1ra) and low levels of pro-inflammatory cytokines[17,18]. In response to IL-4 and TGF-β, microglia have been shown to polarise to an M2 phenotype *in vitro*, characterised by increased expression of YM1 and Arginase I[357]. The significantly high levels of TGF-β centrally following allergic lung inflammation would suggest the development of M2 microglia, a phenotype that exhibits lower levels pro-inflammatory cytokine expression in response to TLR agonists such as LPS[358]. Allergic lung inflammation is associated with elevated levels of circulating IL-4 and IL-13[6], cytokines that, in this model, may filter into CVOs and interact with resident microglia, resulting in polarisation to an M2 phenotype, expression of TGF-β and a dampened response to a secondary stimulation with TLR agonists.

Recent studies have additionally shown that $GABA_A$ and $GABA_B$ receptors are expressed in microglia and impact on microglial associated inflammation. GABA, as well as the $GABA_B$ receptor agonist baclofen, has been shown to attenuate induction of NFκB- and p38 MAPK-dependent inflammatory pathways, and in consequence reduce the release of LPS-induced TNF-α and IL-6 by microglia[352]. In addition to possible microglial polarisation, increased expression of the $GABA_B$ receptor in both the hippocampus and brainstem of animals with sub-chronic lung inflammation may contribute to a dampening of central pro-inflammatory cytokine production, through inhibition of cytokine or resiquimod-induced inflammatory pathways in microglia.

It should be noted that the effect of resiquimod on the peripheral inflammatory response, when administered alone or in animals with sub-chronic lung inflammation, was never directly compared. In animals with allergic lung inflammation, where the inflammatory response is skewed to a TH2-like immune response, it is likely that the ability for

resiquimod to induce peripheral pro-inflammatory cytokine production is somewhat blunted. Indeed, in a model of allergic asthma, macrophages have been shown elevate their expression of triggering receptor expressed on myeloid cells (TREM)-2 and endogenous TLR inhibitors, resulting in a blunted immune response to subsequent bacterial challenge[359]. The dampening of the central inflammatory response in response to resiquimod may in fact be a direct reflection of an attenuation of the peripheral pro-inflammatory response. However, further investigations into peripheral cytokine expression would be necessary to validate this postulate.

6.4.2. The Effect of Sub-chronic Inflammation on Levels of $GABA_B$ receptor and BDNF – a link with mood disorders

In attempting to elucidate the mechanism behind attenuated central cytokine responses to resiquimod, further investigations revealed that sub-chronic lung inflammation was additionally associated with increased transcript levels of the $GABA_{B(1)}$ receptor and reduced levels of BDNF. GABA, the chief inhibitory neurotransmitter in the CNS, and the neurotrophin BDNF are both well-described for their role in mood disorders, such as anxiety and depression[355], disorders that are highly prevalent in the asthmatic population[356]. Correspondingly, sub-chronic lung inflammation was associated with an enhanced behavioural response to resiquimod, with an exacerbated decline in rears, a behavioural readout for anxiety[306,349], suggesting an elevation in anxiety-related responses in animals with sub-chronic lung inflammation.

The vast majority of research investigating the role of GABA in mood disorders has focused on the involvement of GABA-$GABA_A$ receptor interactions in anxiety. Decreased expression or activity of the $GABA_A$ receptor has anxiogenic consequences, justifying the role for benzodiazepines, positive allosteric modulators of the $GABA_A$ receptor, as anti-anxiety medication[353]. The role of the $GABA_B$ has, contrastingly, been widely ignored. However, recent investigations have shown that positive allosteric modulation of the $GABA_B$ receptor is anxiolytic[360] and, counter-intuitively, inhibition has anti-depressant action[361]. $GABA_{B(1)}$ receptor KO mice show anti-depressant like activity in the forced swim test (FST), effects that are recapitulated in pharmacological studies using the $GABA_B$ receptor antagonist CGP56433A[361]. Further corroborating these findings, a recent study by Khundakar and Zetterström[354] (2011) demonstrated that stimulation of $GABA_B$ receptors by the agonist baclofen resulted in a decreased expression of BDNF in the

hippocampus, a neurotrophin that has repeatedly shown to be downregulated in animal models of depression[362,363], as well as in serum of depressed patients[364,365]. However, decreased BDNF has also been shown to be anxiogenic, via decreased activity of the $GABA_A$ receptor[366], despite studies demonstrating that $GABA_B$ receptor stimulation decreases BDNF[354] and is anxiolytic[360]. To provide further confusion, a study by Bravo et al[367] demonstrated that reduction in anxiety and depressive behaviour following ingestion of *Lactobacillus rhamnosus* was associated with reduced hippocampal $GABA_B$ receptor expression.

The vast and somewhat contradictory data makes interpretation of the changes in $GABA_B$ and BDNF expression following sub-chronic lung inflammation the hippocampus challenging. However, collectively these changes could indicate the development of an anxiety- or depressive-like state in these animals, as decreased hippocampal BDNF has been solidly associated with both moods[354,368]. The intensified decline in rears following resiquimod treatment in animals with sub-chronic lung inflammation would suggest enhanced anxiety behaviour, correlating with epidemiological studies showing a link between panic and anxiety disorders and asthma[299]. However, further behavioural assessment of the animals, for example using a FST and EPM would be necessary to confirm mood-related changes. In addition, expression levels of the $GABA_A$ receptor and IDO in the hippocampus, which has been more concretely associated with anxiety and depressive behaviour, would provide additional evidence to suggest the development of mood changes in these mice.

The mechanism by which allergic lung inflammation induces changes in $GABA_B$ receptor expression and BDNF was not elucidated in this chapter. A number of studies have shown the systemic inflammation is associated with a reduction in central BDNF[369,370], but the mechanism by which this occurs is, as of yet, unknown. Some suggest that systemic inflammation and production of central pro-inflammatory cytokines reduces central BDNF expression by neurons[371]. Indeed, resiquimod treatment alone induced peripheral and central pro-inflammatory cytokines and resulted in a small reduction in BDNF expression levels. However, reduced BDNF was not associated with increased pro-inflammatory cytokine production in animals with sub-chronic lung inflammation, suggesting an alternative pathway. It has additionally been suggested that vagus nerve stimulation can alter central expression of $GABA_B$ and BDNF in forebrain regions[367,372,373]. Indeed, in the

previous chapter alterations in excitatory glutamate receptor expression were found (in the brainstem), an affect that was most likely an adaptation to repetitive stimulation of the vagus nerve. Alterations in other neurotransmitter receptors may also occur as an adaptive, homeostatic mechanism, in order to adjust central signalling pathways, in response to continuous stimulation.

It should also be noted that the significant regulation of BDNF and $GABA_B$ receptor expression occur in animals that were repeatedly confined to an aerosolisation chamber in a fume hood, daily events that the naive mice (exposed to vehicle or resiquimod) did not encounter. Noise, vibrations and restricted floor space associated with the aerosolisation procedure are likely to be stressors for animals and chronic stress has repeatedly been associated with increased anxiety and decreased BDNF mRNA expression in the hippocampus[362,374].

6.4.3 The Effect of Resiquimod on the CNS – dependent on chronicity and strain?

It is necessary to appreciate that the effect of resiquimod on the CNS in animals with allergic lung inflammation may be dependent on the chronicity of peripheral inflammation. In this study, resiquimod was administered to animals with sub-chronic lung inflammation, as this was found, as seen in the previous chapter, to be the peak of eosinophilia. Contrastingly, alterations in inflammatory genes (e.g. IL-1) in the CNS were only evident with increasing chronicity of peripheral lung inflammation. This is key, as it suggests that animals with chronic inflammation, that demonstrate significant upregulation of pro-inflammatory cytokines centrally, may in fact respond differently to resiquimod treatment. It was suggested above that, due to the significant upregulation of TGF-β, microglia in animals with sub-chronic lung inflammation have adopted an M2 phenotype, thus being less responsive to secondary pro-inflammatory insults. However, the rise in central IL-1 in animals with chronic lung inflammation would suggest a switching to an M1 phenotype, which may result in a heightened response to resiquimod.

A similar factor may be relevant with the choice of mouse strain. Initial experiments examining the impact of resiquimod alone on the CNS used two disparate mouse strains: C57BL/6 and BALB/C. It was found that the impact of resiquimod on peripheral and

central cytokine expression was significantly dampened in BALB/C mice, as compared to C57BL/6 (Appendix 9.7), most likely due to the genetic predisposition for BALB/C mice to mount a TH2 type immune response[375]. One could argue that the blunted effect of resiquimod on central pro-inflammatory cytokines in animals with sub-chronic lung inflammation is highly dependent on strain, as macrophages or microglia in BALB/c mice may more readily adopt an M2 phenotype. However, one must also take into account that BALB/C mice were chosen for their predisposition in the development of TH2 type immune response and may, in fact, be an apt representation of atopic allergic patients, which likewise have a genetic and environmentally-dependent predisposition for developing allergic responses[22].

6.4.4 The Impact of Resiquimod Administration on Peripheral Inflammation

In this study, both systemic and topical (intranasal) administration of resiquimod was associated with a significant upregulation of systemic pro-inflammatory cytokines, and numbers of eosinophils and lymphocytes were significantly reduced following topical administration. This is interesting, revealing that TLR agonists alone can attenuate allergic inflammation, whereas real-live TLR agonists, such as influenza or rhinovirus have an exacerbating effect[160]. This is probably due to the fact that influenza and rhinovirus also stimulate alternative inflammatory pathways, such as through interaction with RIG-I and MDA-5[161]. However, it should be noted that in contrast to previous studies, resiquimod administration was not associated with a complete abrogation of allergic inflammation. This is most likely due to the protocol employed, as previous studies demonstrating successful immunomodulation have involved repeated resiquimod challenge[172,173,344,345], where, in most circumstances, resiquimod was administered prior to OVA challenge[172-174,344,345]. In addition, in this study, samples were collected 2.5 hours after resiquimod administration, too early to see clear changes to cell numbers in the lungs. To fully understand the impact of immunomodulation on the CNS, it would of course be necessary to investigate the impact of repeated resiquimod challenges on the brain of animals with allergic lung inflammation. However, for the purpose of this study, to investigate the central impact of acute TLR stimulation in animals with allergic lung inflammation, a single resiquimod challenge, which induced significant increases in peripheral pro-inflammatory cytokines, was sufficient.

6.5 Conclusion

The results in this chapter demonstrate that the systemic administration of the TLR7 agonist resiquimod induces central cytokine expression, but this effect is significantly attenuated in animals with sub-chronic lung inflammation. Contrastingly, the effect of resiquimod on rearing behaviour, a determinant for anxiety, was exacerbated, not encouraging in terms of patient well-being. This phenomenon may, however, have been due to pre-established alterations in behaviours of animals with allergic lung inflammation, as insinuated by the decreased levels of BDNF. This study is the first to demonstrate the effect of TLR-dependent immunomodulation of allergic lung disease on the brain, and specifically, the CNS effect of therapeutic drug treatment in a disease model. It highlights the significance of investigating immunomodulatory treatment on the brain, due to the well-established impact the immune system has on the CNS, but also the central impact and influence the underlying disease can have. Further work is necessary to understand the multiple mechanisms behind attenuation of central cytokine expression and exacerbation of anxiety-related behaviour in animals with sub-chronic lung inflammation. However, collectively the study demonstrates that although TLR agonists are known to impact on the innate inflammatory response on the brain, using these agonists as immunmodulatory treatment may not be as detrimental to the CNS inflammatory state of a patient, but may be undesirable in terms of a patient's emotional well-being.

Chapter 7: General Discussion

For the past 25 years, research in the field of immune to brain communication has focused primarily on the impact of TLR agonists and pro-inflammatory mediators on the brain. Though this has undoubtedly provided useful insight into the mechanisms and pathways by which the immune system can affect the brain, it has generated little understanding into the impact of chronic infectious conditions or inflammatory diseases on the CNS. Asthma, a chronic inflammatory disease of the airways, directly affects 300 million people worldwide[139], and is associated with alterations in the activity of peripheral neurons[199], as well as mood disorders, such as anxiety and depression[349]. Despite this, the direct effect of allergic asthma on the CNS has only recently been recognised[108,246,376]. Understanding the effect on the CNS would not only contribute to understanding the pathology and psychopathology of the disease, but also becomes crucial knowledge when taking into consideration the effect that developing therapies, namely immunomodulating TLR agonists, can have on the brain. In light of this, the main aim of this thesis was to investigate and understand the impact of allergic lung inflammation on the brain, and determine the secondary impact of immunomodulation on the CNS.

In the collection of studies reported here, it was found that allergic lung inflammation does communicate with the brain, likely mediated by the vagus nerve, leading to a distinctive pattern of neuronal activity and behavioural changes, as compared to those induced by systemic pro-inflammatory mediators. Chronic lung inflammation was not only associated with changes in synaptic plasticity in the brainstem, a CNS-dependent mechanism that would explain the AHR in asthma, but also alterations in $GABA_{B(1)}$ receptor and BDNF in the hippocampus, providing a biological justification for the prevalence of anxiety and depression in asthmatic patients. Immunomodulatory treatment with the TLR7 agonist resiquimod was associated with attenuated central pro-inflammatory responses in animals with pre-established lung inflammation, but an exaggeration in anxious or depressive behaviour, not encouraging in terms of patient well-being.

The initial investigations into the impact of acute allergic inflammation on the brain were fundamental in providing an explanation for the mechanism or pathway by which localised TH2-mediated inflammation impacted on the brain. In Chapter 3, an acute model of granulocytic TH2-like inflammation in the peritoneum was developed using the TLR2/Dectin-1 agonist zymosan. The lack of circulating pro-inflammatory cytokines and prostaglandins initially suggested that the effect on the CNS was not dependent on humoral

immune to brain communication pathway, a postulate that was further corroborated by the lack of c-fos immunoreactivty (neuronal activity) in the CVOs. The discrete neuronal activity in specific subnuclei of the NTS, as well as the LC and PVN, which receive direct projections from the NTS, undoubtedly suggested that immune to brain communication following localised granulocytic inflammation was dependent on a neuronal route of communication, likely via the vagus nerve. The role of the vagus nerve in localised acute allergic lung inflammation was recapitulated in Chapter 4, where discrete patterns of neuronal activity in the cmNTS, the location of the first central synapse of bronchopulmonary c-fibres of the vagus nerve, were evident.

These chapters, along with only a handful of other studies[108,244,246,290], demonstrate that immune to brain communication is evident in the absence of circulating pro-inflammatory cytokines, and that neuronal routes of communication are dominant in localised inflammatory conditions. Previous studies have similarly reported vagus-dependent changes in neuronal activity in the CNS following local inflammation, though these studies focused only on gut inflammation[112,248,301]; the first two chapters in this thesis are the first studies to show that inflammation in the lungs follows the same pattern of vagal immune to brain communication. Although the mechanism by which localised inflammation induced neuronal immune to brain communication was not elucidated in these studies, local production of IL-1β (in Chapter 3) or PGE_2 (induced in the early phase of an allergic reaction in the lungs) may have contributed, as respective receptors for these immune mediators have been shown to be expressed on vagal paraganglia[94,95]. This is substantiated by a previous study investigating the impact of allergic gut inflammation on the CNS, which reported that mast cell degranulation (which results in the release of lipid metabolites such as PGE_2) plays a central role in vagus-mediated immune to brain communication[245]. These chapters, along with previous studies, also revealed that vagus nerve signalling results in an atypical behavioural response, with no changes in typical sickness behaviours such as lethargy or anhedonia. This is not surprising, as localised inflammatory conditions, like asthma, are not typically associated with clinical signs of illness, such as fever or lethargy. However, here we provide a biological mechanism for this phenomenon. Collectively, these chapters crucially outline that the vagus nerve plays a central role in mediating immune to brain communication in granulocytic TH2-mediated inflammation.

The role of the vagus nerve in neuroimmune signalling becomes that much more significant when examining the impact of ***chronic*** lung inflammation on the brain. Following sub-chronic allergic lung inflammation (equivalent to 8 days of allergen challenge), upregulation of genes involved in synaptic plasticity was evident in the brainstem; notably genes involved in the formation of NMDA and AMPA receptor- rich synapses. This was associated with an upregulation of the expression of TAC1, the gene which encodes a family of tachykinins, including SP. Although time constraints did not allow us to specifically locate these changes to the cmNTS, a necessary future direction, the localised activation of these neurons following acute allergic lung inflammation in Chapter 4 undoubtedly suggest that the changes in plasticity occur in this area.

It is well-established that asthmatic inflammation is associated with a hyperreactivity of both smooth muscle and neuronal fibres innervating the airways, resulting in exaggerated central reflex activation, and subsequently exaggerated cough and mucous production, as well as dyspnoea and apnoea[199]. Many studies have demonstrated that inflammation-induced alterations in receptor expression on bronchopulmonary c-fibres is a key factor in airway hyperreactivity[149,216,223,224], but few have recognised that genotypical changes in axons that innervate the lungs may be mirrored by their central projections. Previous studies have recognised that chronic inflammation in the lung parenchyma, such as induced by extended tobacco smoke or ozone exposure, can result in plasticity in the first and second central synapses of bronchopulmonary c-fibres in the NTS[207,235,236,239,241,332], resulting in hyperreactivity, a phenomenon that is reproduced by the administration of SP to the central synapses. Studies have also demonstrated that AMPA and NMDA receptors, which are crucial to the development of synaptic plasticity, are expressed at these synapses and play a central role in the cough reflex[377], already alluding to a mechanism by which lung inflammation-induced plasticity in the NTS is achieved. In Chapter 5, the increase in genes involved in synaptic plasticity in the brainstem, as well as the upregulation of TAC1 insinuates that synaptic plasticity is evident in the NTS following allergic lung inflammation and underpins a biological mechanism for this phenomenon. These changes suggest that chronic inflammation in the lung parenchyma leads to chronic firing of the vagus nerve, which triggers an adaptive mechanism, or "coping strategy" in the NTS, leading to increased synaptic strength, increased firing of the efferent neuronal fibres and ultimately exaggerated lung reactivity. The results provide a biological mechanism,

dependent on the neuronal immune to brain communication and the CNS, for pathological changes in asthma, as well as key symptoms such as increased cough and dyspnoea.

Increased excitability of neurons in the CNS in response to peripheral changes, known as central sensitisation, is not an uncommon biological phenomenon. Chronic pain is associated with continuous inflammation in peripheral c-fibres, resulting in plasticity of central synapses in the dorsal horn of the spinal cord and unwanted and unnecessary pain sensation[378]. In addition, more recent studies have suggested a similar mechanism for tinnitus, where inflammation and plastic changes in the auditory cortex leads to unprovoked perception of sound[379]. These diseases are partially dependent on on-going inflammation around peripheral nerve endings, particularly c-fibres, leading to exaggerated signalling at the first order synapse in the CNS and amplified reciprocal reflexes. Though vagus nerve signalling is traditionally seen as an adaptive phenomenon, providing the brain with an early and rapid warning of the presence of infection, continuous signalling via the vagus nerve during excessive chronic peripheral inflammation can have detrimental consequences, resulting in aberrant changes in the CNS. The plastic changes identified in Chapter 5 may be reflected in other forms of localised and chronic inflammation (e.g. inflammatory bowel disease) and may be key in understanding the pathology of other inflammatory diseases.

Asthma has long been associated with other forms of CNS-dependent changes, specifically alterations in mood, such as depression and anxiety[356]. In Chapter 6, increases in the expression levels of $GABA_B$ and decreased expression of BDNF in the hippocampus following sub-chronic lung inflammation alluded to the development of mood changes in the animals. Although this was not corroborated through behavioural assays such as the FST or EPM, a necessary future direction, exaggerated decreases in rears following resiquimod challenge would suggest a predisposition for anxiety-related responses in these animals. The mechanism by which alterations in expression levels of $GABA_B$ and BDNF occur was not determined in these studies, though several factors point to a dependence on the vagus nerve. In initial studies in Chapters 3 and 4, where specific factors of neuronal activity suggested that immune to brain communication was vagally mediated, a trend towards a decrease in the amount of time spent in the open arms of the EPM was evident, suggestive of increased anxiety behaviour. Similar studies investigating acute localised inflammation in the gut have solidly determined that changes in anxiety behaviour are

vagus-dependent[290,380]. Indeed, numerous studies have demonstrated that stimulation of the vagus nerve can alter levels of both $GABA_B$ and BDNF in the hippocampus, both through inflammation-induced stimulation of vagal fibres in the gut[367,373], as well as through electrical chronic vagus nerve stimulation (VNS)[372]. A vagus-dependent mechanism in anxiety behaviour not only provides a biological explanation for the prevalence of mood disorders in asthmatic patients, where inflammation is localised and results in chronic stimulation of the vagus nerve, but also underpins the comorbidity between anxiety or depression with numerous other diseases characterised by localised inflammation, including irritable bowel syndrome, chronic pain, heart disease and arthritis[292].

It is, as of yet, unknown how stimulation of the vagus nerve alters expression levels of neurotransmitter receptors and neurotrophins in forebrain regions, and thus contributes to changes in behaviour, but several papers suggest that it is purely dependent on neural messages conveyed by the vagus to forebrain regions[381,382], and not dependent on alterations in peripheral cytokines or corticosteroids. Interestingly, however, results in Chapter 4 demonstrated that acute allergic lung inflammation does not induce changes in neuronal activity in forebrain regions. However, this may be an artefact of the chronicity of inflammation, as more chronic forms of inflammation did result in alterations in neurotransmitter receptors in forebrain regions, such as the hippocampus. One can speculate the chronic inflammation, and consequently chronic stimulation of the vagus nerve, results in increased activity of hindbrain regions resulting in the progression of signals to the forebrain. However, further work into the mechanism of how this occurs is necessary to fully understand vagus-dependent alterations in central biochemistry.

It cannot be ignored that depressive behaviours are also known to be correlated with increased levels of systemic pro-inflammatory cytokines. A plethora of studies have, for example, identified strong correlations between increased systemic IL-6 and C-reactive protein and Major Depression (as reviewed by Krishnadas and Cavanagh[123]). In addition, circulating IFN-γ and IFN-α are known to contribute the depressive-like behaviours[124-126]. However, the studies in this thesis, along with a collection of other studies[108,290], strongly suggest that localised inflammation, where circulating pro-inflammatory cytokines are absent, also result in mood disorders, such as anxiety and depression.

One of the main aims of this thesis was to investigate the impact of current immunomodulatory therapies in development for treatment of allergic asthma on the CNS. TLR agonists, such as those against TLR4, 7 and 9 have been shown in both pre-clinical and clinical models to be successful in immunomodulating and reducing asthmatic inflammation, as detailed in Chapter 1. However, the effect on the brain has been widely ignored, despite the wealth of evidence demonstrating that TLR3 and 4 agonists influence behaviour, innate inflammation and biochemistry in the CNS, as well as increasing severity of chronic neurodegeneration[348,383]. Results in Chapter 6 are the first to demonstrate the effect of a TLR agonist, specifically the TLR7 resiquimod, on the brain in a model of pre-established lung inflammation, revealing that resiquimod had little impact on innate inflammation on the brain. Resiquimod alone significantly elevated expression of IL-1β and TNF-α in the brainstem and hippocampus, but this effect was significantly attenuated in animals with sub-chronic lung inflammation. This phenomenon was most probably due to the expression of TGF-β in the brains of animals with lung inflammation, suggestive of alternative microglial activation. It should be mentioned that animals with pre-established lung inflammation showed an exaggerated behavioural response to resiquimod treatment. Though a previous study has demonstrated that TLR7 agonists (e.g. imiquimod) induce central cytokine expression[133], this study is the first to show the impact in a pre-established disease model. The results suggest that animals or individuals with allergic asthma are tolerant to the central inflammatory effects of TLR agonists, but may experience unwanted decline in mood. Immunomodulation may be beneficial in terms of lung inflammation, and safe in terms of central innate inflammation, but the association with mood decline makes this treatment undesirable in terms of patient well-being.

The attenuation of central innate inflammation is a particularly interesting and somewhat unexpected result, when comparing to other studies that have investigated the effect of a secondary inflammatory insult on the brain. In mouse models of ME7 prion disease, central expression of TGF-β is similarly elevated, as are the M2 macrophage markers TREM-2 and Dectin-1[384]. However, in response to secondary systemic insults by TLR agonists, such as LPS or poly I:C, central pro-inflammatory responses are significantly exaggerated, due to "priming" of microglia by on-going neurodegeneration[348,383]. It is still unclear why this switch from an M2 to and M1-like microglial phenotype occurs in response to secondary peripheral inflammatory insults, and it is consequently difficult to determine why responses are different in prion in disease, as compared to following

allergic lung inflammation, when microglial phenotype appears similar. However, these discrepancies highlight the plasticity and polarity of macrophages in the CNS. Though expression of TGF-β was upregulated, no changes in the expression of Dectin-1, an M2 macrophage maker, were evident by immunohistochemical means in Chapter 5, suggesting microglia may not have been fully skewed to an alternatively activated phenotype, but do possess some characteristics of M2 macrophages. The diversity and plasticity of macrophages may clarify the discrepancy in response to secondary inflammatory insults and further investigations into the activation state are necessary to fully understand why responses to resiquimod are attenuated.

It should be noted that the attenuation in the effect of resiquimod on the brain may of course be due to an attenuated peripheral inflammatory response, as animals with pre-established lung inflammation have been shown to be less responsive to secondary pro-inflammatory insults in the periphery[359], but due to time constraints, peripheral inflammatory cytokines could not be compared in naive animals and animals with sub-chronic lung inflammation treated with resiquimod. This is necessary future experiment. However, levels of COX-2, which is upregulated in the cerebral endothelium in response to pro-inflammatory cytokines[63,64], was equivalent in naive animals and animals with allergic lung inflammation treated with resiquimod, disputing this theory.

It must be mentioned that a concrete effect of allergic lung inflammation on the inflammatory pathways on the brain was not clear. Though an upregulation of TGF-β was apparent in the brains of animals with sub-chronic inflammation, chronic inflammation (equivalent to 13 days of challenge) was associated with mild increases in the expression of pro-inflammatory cytokines (IL-1α, IL-1β, IL-1f8 and TNF-α), as seen in Chapter 5. The reason for this is unknown, but may have been in response to IgG infiltration, as discussed in Chapter 5, or upregulation of SP, which has previously been shown to have a pro-inflammatory effect on microglia[385], although this has recently been disputed[386]. These results suggest that inflammatory changes in the brain following allergic lung inflammation are not stable, and are clearly dependent on the chronicity of the peripheral inflammatory event. It may suggest that if resiquimod was given at a later time point, for example after 13 days of OVA challenge, as opposed to 8, the effect on innate inflammation in the CNS may have been different. The variability in central cytokine

expression brings up an interesting and significant question: how does this compare to humans?

In these mouse models of allergic lung inflammation, the inflammatory event was deemed "chronic" if it persisted over a three-week time period. This is undoubtedly a step forward in the field of immune to brain communication, as only very recently have studies investigated the effect of long-term inflammation on the brain[132,136,137]. However, humans with chronic asthma have persistent lung inflammation for years, not weeks. In light of the changes in central innate inflammation in response to only a few extra days of inflammation, it bodes the question as to how the brain responds to allergic inflammation in humans, after years of persistent lung inflammation. It is possible that continuous inflammation could lead to innate tolerance in the CNS, as continuous infiltration of circulating TH2 cytokines into the brain may lead to the restriction of microglia to an M2 phenotype. In addition, upregulation of SP production centrally has recently been shown to induce an M2 phenotype, in the spinal cord[386]. However, it has also been suggested that SP can activate microglia[385]. In addition, it has been shown that repeated infection and chronic increases in cytokines (though pro-inflammatory) can increase the risk of developing Alzheimer's disease (AD) and the severity of cognitive decline, due to increases in central pro-inflammation[387-389]. Indeed, one study has shown that atopy increases the risk of developing AD and a history of asthma is associated with a shorter life expectancy after AD diagnosis[390], suggesting chronic lung inflammation may induce pro-inflammatory pathways in the human CNS.

When attempting to compare central inflammatory changes in a mouse model of allergic lung inflammation to humans with allergic asthma, it must also be mentioned that mouse strain plays a crucial role in the discrepancy immune responses. C57BL/6 mice are known to predominantly develop TH1 type immune responses, whereas BALB/C mice are genetically predisposed to the development of TH2 –like immune responses[375]. In addition, in response to resiquimod, BALB/C mice produce significantly less IL-1β centrally, than C57BL/6 mice (see Appendix 9.7), suggesting that innate microglial responses are similarly skewed dependent on strain. This is corroborated in a study by Lambersten et al[391], which showed that microglia from BALB/C mice produce significantly less TNF-α in response to focal cerebral ischemia, as compared to C57BL/6. The fact that BALB/C mice were used in this study, where macrophages or microglia may be more prone to the

adoption of an M2 phenotype, may skew the results to suggest that central anti-inflammatory responses develop in the brain. Though atopic asthma patients are also genetically pre-disposed to the development of TH2 type immune responses, the macrophage responses in humans may be substantially more complex and intricate, making interpretation from one mouse strain difficult. Further studies would need to investigate changes in more chronic models of inflammation, as well as in different animal models. For example, a model of asthma using IL-13 over-expressing mice (personal communication with Hans-Michael Haitchi) would enable the development of more chronic asthma, without continuous interference with the animals. However, to fully discern the effect of chronic allergic inflammation on inflammatory status in the brain, it would be necessary to look clinically, either through post-mortem examination, or by investigating the correlation between CNS diseases or disorders associated with central inflammation (e.g. neurodegenerative diseases, cognitive decline in ageing, stroke) and allergic asthma.

The results from this study and others also suggest that communication via the vagus nerve plays a role in mood disorders. Numerous epidemiological studies suggest correlations between asthma and mood disorders and the results in this study suggest a biological mechanism by which this may occur. However, clinical studies, through for example post-mortem examination, would be necessary to confirm this. FMRI studies have shown that areas involved in emotional reactivity (insula and anterior cingulate cortex) are active in response to asthma-relevant stimuli and contributes to symptoms exacerbation[300], but future studies would need to determine if these areas are hyperresponsive in asthmatic individuals, as compared to healthy controls. In further pre-clinical models, vagus nerve inhibition would also aid in understanding if and how neuronal immune to brain communication contributes to biochemical changes in the brain (such as changes in expression of BDNF or $GABA_B$ receptor) which lead to mood changes. In addition, mood disorders, specifically anxiety, have been shown to contribute to symptom exacerbation in asthma and, inhibition of vagal signalling may also provide a novel treatment strategy for asthmatic patients.

Though in this study resiquimod was deemed effective in terms of limited acute negative side-effects in the brain, it is necessary to corroborate this finding in humans. As discussed above, further studies in to allergic inflammation in more chronic and other animal models

of asthma are necessary, to understand the full effect of allergic lung inflammation on the central inflammatory responses. The same is true for humans. Though invasive studies are not possible in humans, a recent study by Sudduth et al[392] analysed inflammatory proteins in serum as an indication of M1 or M2 microglial polarisation in AD. Other studies have used PET imaging to image microglia in patients with neurodegenerative disease, providing a means by which one can analyse central inflammatory responses in asthmatic humans. This is a necessary step, as central inflammatory responses play a crucial role in determining the impact of a secondary immune stimulus, such as an immunomodulator or a secondary exacerbating viral infection, on the brain and on patient well-being.

As previously mentioned, the results from this study are some of the first to demonstrate the extraneous effects of a disease treatment in a disease model. Crucially, the investigations identified that resiquimod had a differential effect on the CNS in naive, or healthy, animals as compared to animals with allergic lung inflammation. Currently, clinical trials focus on investigating the safety of developing therapeutics in healthy individuals in Phase I and subsequently investigate their efficacy in diseased individuals in Phase II. However, the safety of the therapy in diseased individuals is completely ignored. The studies in this thesis suggest that the disease itself can modify the effect of a drug, particularly the unwanted and off-target effects and advocates that investigating safety in diseased individuals is equally as important as exploring safety in healthy individuals.

Immune to brain communication is a vital physiological and homeostatic mechanism in response to infection, driving changes in behaviour and peripheral immunoregulation to combat the infectious agent. However, in diseases which are characterised by local, persistent and unnecessary inflammation, such as allergic asthma, continuous immune to brain communication via the vagus nerve still occurs, leading to maladaptive and non-physiological changes in neuronal activity and neurochemistry, possibly contributing to both pathological and psychopathological symptoms associated with the disease. This significantly highlights the importance of investigating immune to brain communication pathways in physiological disease models, opening up a new approach to research in immune to brain communication. All inflammatory diseases are different, characterised by numerous different immune responses (TH1/TH2/TH3/TH9/TH17/TH22), and localised to a multitude of different organs. The results in this study reveal that the impact of lung-based TH2 type immune response on the brain are widely diverse from the published CNS

effects of systemic TH1 type immune responses[132,135,137], suggesting that the diversity of inflammatory disease profiles can have an extensive array of distinct effects and potentially negative side-effects on the CNS. It is abundantly clear the investigating the impact of inflammation on the brain has, until now, been incredibly limited and future work in immune to brain communication must focus on the impact of the diverse inflammatory responses on the CNS.

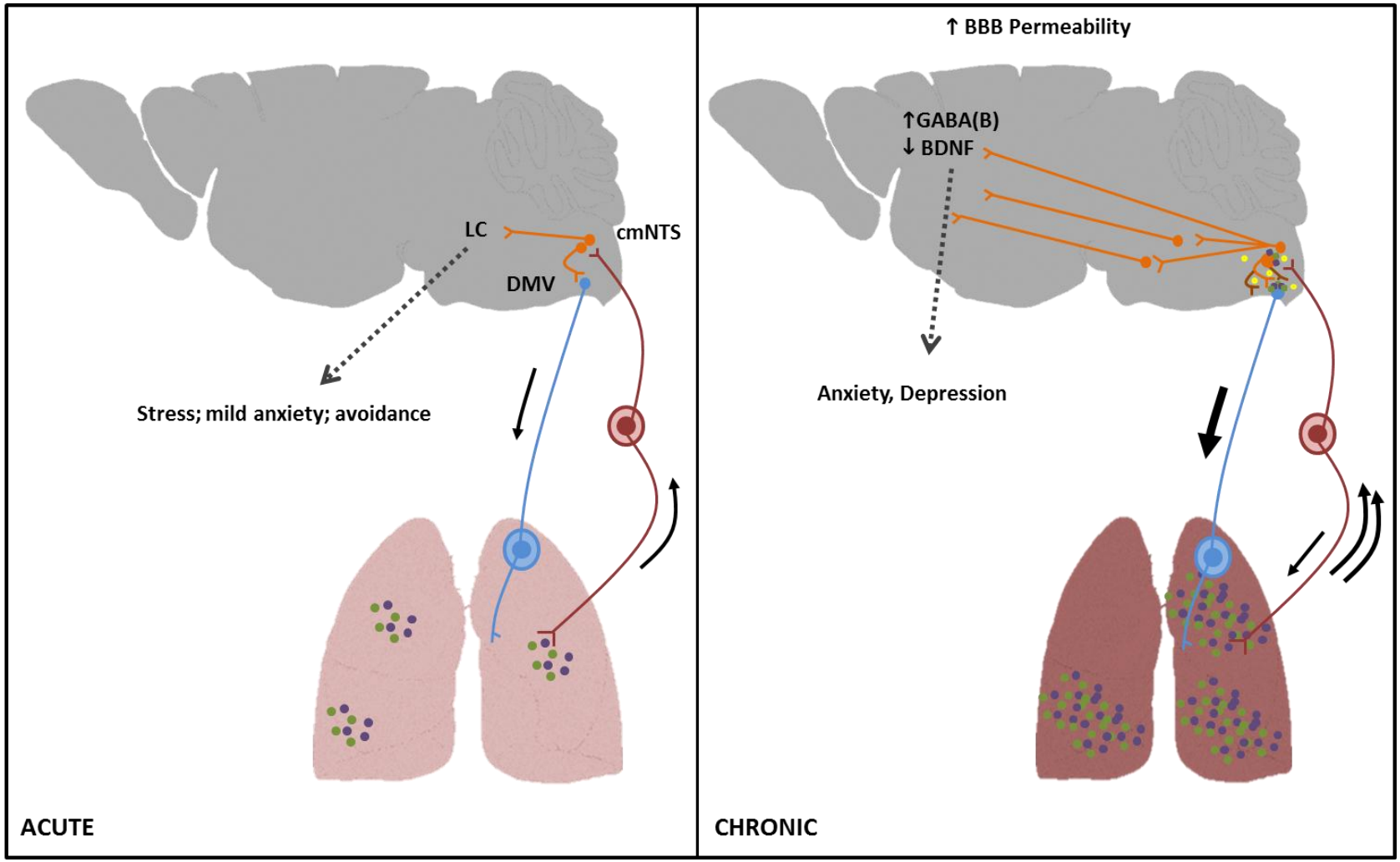
LC
cmNTS
DMV
Stress; mild anxiety; avoidance
ACUTE
↑ BBB Permeability
↑GABA(B)
↓ BDNF
Anxiety, Depression
CHRONIC

Figure 7.1 – Immune to brain communication in allergic lung inflammation. During acute allergic lung inflammation, inflammatory mediators in the lung (e.g. PGE_2) interact with bronchopulmonary c-fibres of the vagus nerve (red), leading to activation of the cmNTS and the LC, but no forebrain regions. This initiates the central reflex, leading to a downstream signal to the lungs via the parasympathetic fibres (blue), leading to bronchoconstriction and cough. The activation of the LC, an area relating involved in stress responses, suggests an early development of anxiety or avoidance behaviour. Chronic allergic lung inflammation leads to continuous signalling via the vagus nerve, which not only alters the sensitivity of the c-fibres, but also induces plasticity in the cmNTS, which leads to an exaggerated downstream signal via parasympathetic fibres and exaggerated bronchoconstriction. Chronic lung inflammation also leads to neuronal changes in the forebrain, such as increased $GABA_B$ expression and decreased BDNF expression, suggestive of the development of anxiety and depressive behaviour.

Chapter 8: Future Directions

The results in this thesis are some of the first to demonstrate that physiological inflammatory diseases, like systemic infections, communicate with the brain. The results highlight the role of the vagus nerve in a localised TH2-mediated inflammatory disease, and show how alterations in the CNS can contribute to pathological and psychopathological symptoms of asthma. They also demonstrate how the new immunomodulatory therapeutic strategies being developed for the treatment of asthma impact on the brain. However, the results in this thesis are only a small fraction of the potential research that can be carried out to understand how asthma effects the brain.

8.1 The Role of the Vagus Nerve

The main result of this thesis suggests that the vagus nerve is of crucial importance in immune to brain communication in localised allergic lung inflammation. Though patterns of neuronal activity following acute allergic inflammation suggest that this is the case, a necessary next step would be to investigate the impact of inhibiting activity of the vagus nerve. Vagotomy, a procedure often carried out in immune to brain communication studies to determine the role of the vagus nerve, was not possible. To determine the role of the neuronal pathway in lung inflammation, the vagus nerve would have to be cut above the diaphragm, which would be fatal. Instead, vagotomy was attempted pharmacologically, using the TRP channel antagonist ruthenium red (RR), but the numerous extraneous effects of RR, such as inhibition of peripheral inflammation, made results inconclusive. However, previous studies have successfully inhibited vagus-dependent central reflexes through micro-injection of the local anaesthetic lidocaine into the NTS[393]. Inhibition of the vagus nerve, through pharmacological or mechanical means would give full insight of the role of the vagus nerve in communication with the brain in allergic lung inflammation. Previous studies have shown that inhibition of vagal signalling, specifically through inhibition of the TRPA1 receptor using the antagonist HC-030031, inhibits the late asthmatic response, as measured by quantifiable changes in lung function changes[149]. Investigation of the effect of vagus nerve inhibition on neuroplastic changes in the brainstem would determine if this form of immune to brain communication does contribute to the pathology and symptoms of asthma. If successful, this may present a novel therapeutic treatment strategy for the pathological features and symptoms of chronic asthma.

Investigations into the activity of vagal ganglia would be a further approach by which to determine the role of the vagus nerve. Upon depolarisation of vagal neuronal fibres, there is an increase in intracellular calcium in the cell bodies located in the vagal ganglia, due to opening of membrane-bound calcium channels and release from intracellular calcium stores[394]. *Ex vivo* imaging of calcium concentrations in vagal ganglia, such as through the use of a fluorescent dye that binds to calcium, in response to pharmacological activation would determine whether vagal neurons projecting to the lungs are hypersensitive due to the increased inflammation in the lung. Increased activity in the cell bodies of these vagal fibres would, as explained in Chapter 1, lead to increased pulmonary release of neuropeptides and neurotransmitters at peripheral afferents, but may also lead to increased central release of neuropeptides and neurotransmitters by central afferents. Increased glutamate or SP release at the cmNTS would provide a biological explanation for the change in glutamate receptor expression and synaptic plasticity detected in the brainstem.

As covered in Chapter 5, the increased synaptic plasticity centrally following inflammation around peripheral c-fibres is a phenomenon mirrored in neuropathic, or chronic, pain. It is well known that inflammation in the sensory ganglia in neuropathic pain, namely the dorsal root ganglia (DRG), play a vital role in the outcome of the disease. Studies have shown increased numbers of lymphocytes, neutrophils, satellite glia and macrophages, as well as increased levels of complement and pro-inflammatory cytokines in the DRG in animal models of neuropathic pain[395]. It has additionally been shown that pharmacological inhibition of inflammation in these sensory ganglia results in an attenuation of pain symptoms[395]. This suggests that further study into the inflammatory state of nodose and jugular ganglia following allergic lung inflammation may provide a novel treatment strategy to target the symptoms of asthma, such as exaggerated bronchoconstriction, apnoea and dyspnoea.

8.2 Functional Relevance

One of the key future experiments to be carried out for this thesis is to determine the functional relevance of the neuronal changes observed in the brainstem. Though the changes in glutamate receptor and SP expression would suggest increased activity of efferent parasympathetic pathways, this should be confirmed with functional studies of airway reactivity, using enhanced pause (Penh) variability measurements or flexiVent™

measurements of AHR in response to metacholine or 5-HT challenge. In demonstrating functional changes in the allergic inflammatory models used, local pharmacological inhibition of glutamate or SP receptors in the cmNTS would be able to confirm or refute the role of central plasticity changes in symptoms of asthma, such as exaggerated bronchoconstriction, apnoea and dyspnoea.

As explained in Chapter 5, a critical limitation to the changes in gene expression observed in the brainstem is the inability to determine its functional relevance. Not only is it difficult to pinpoint the degree of up- or downregulation of expression necessary to be functionally relevant, but we are, at this point, unable to determine whether changes in mRNA expression will have any effect on protein levels and therefore neuronal function. For example, BDNF mRNA is known to encode both pro-BDNF and mature BDNF, two proteins which have opposite effects functionally[396]. Therefore, future studies must be carried out to confirm whether the changes in gene expression are translated into changes in protein expression, changes that may be more relevant to neuronal function.

8.3 Further Inflammatory, Allergic and Immunomodulatory Studies

One of the critical points that was not addressed in this thesis was whether the CNS effect of allergic inflammation was dependent on the type or location of the immune response. Because the CNS effect of both localised innate inflammation in the peritoneum and localised adaptive inflammation in the lungs both induced similarly discrete patterns of c-fos expression, whereas systemic innate inflammation resulted in widespread c-fos activation, one could postulate that the localised aspect of the immune response, and not the type, played a more crucial role in the impact on the CNS. However, to more appropriately confirm this, it would be necessary to design an experiment where the CNS effect an innate inflammatory response in the lungs, such as induced by intranasal challenge with LPS, and adaptive inflammatory response, such as induced by OVA-immune complex formation, would be compared. In addition, the studies in this thesis do not address whether the CNS effect was specific to an adaptive TH2-like immune response; in other words, it is unknown if TH2-associated cytokines and chemokines are responsible for immune to brain communication. To address this issue, and confirm or refute the role of TH2 immune mediators, an experiment could be designed in which the CNS effect of an adaptive TH1-type inflammatory response in the lungs, such as induced

by influenza challenge, and adaptive TH2-type inflammatory response in the lungs, such as induced by OVA-immune complex formation would be compared.

As discussed in Chapter 7, with the limited numbers of models used, it is difficult to determine the true effect of allergic lung inflammation on the brain. Changes centrally were not only highly dependent on strain (C57BL/6 vs BALB/C), but also on the chronicity of inflammation, as a five-day extension of allergic inflammation resulted in a switch from a TGF-β-associated anti-inflammatory response centrally, to a pro-inflammatory response dominated by cytokines of the IL-1 family. Development of further models of allergic lung inflammation, with varying chronicity would be beneficial to determine the true range of effects of allergic lung inflammation on the brain. In addition, using guinea-pig, as opposed to rodent, models would be advantageous, as neuronal airway innervation in the guinea-pig is more similar to human.

In addition to investigation of other models of allergic inflammation on the brain, a necessary future direction would be to further investigate the impact of immunomodulation on the brain. In relation to the above comments, it would of course be necessary to examine the central impact of the immunomodulatory protocol used in different strains, species or models of allergic inflammation. However, the majority of pre-clinical and clinical studies confirming successful dampening of TH2 inflammation following TLR agonist challenge use multiple doses of the immunomodulators. As explained in Chapter 6, for the purpose of this thesis, to examine the immediate CNS effect of a TLR agonist upon a background of allergic lung inflammation, the protocol used was sufficient. However, considering the negative impact that resiquimod was shown to have on behavioural changes in animals with sub-chronic lung inflammation, investigating the impact of multiple doses of the immunomodulator is a necessary future direction.

8.4 Clinical Relevance

The results from this study and others suggest the allergic lung inflammation induces central changes that would underlie mood disorders, such as anxiety and depression, an effect that is exacerbated following treatment with an immunomodulator. A necessary future direction is to corroborate this finding in humans. Numerous epidemiological studies have suggested links between asthma and panic disorders or depression, but few

have examined mechanistic links between allergic inflammation and mood disorders. fMRI studies have shown that areas involved in emotional reactivity are active in response to asthma-relevant stimuli (e.g. allergens)[300]. However, future studies must determine if these areas are hyperresponsive in asthmatics, as compared to healthy controls. Post-mortem examination of brain areas involved in these disorders would determine if biochemical and neurotransmitter changes underlie the prevalence of these disorders. Increased stress is known to reciprocally exacerbate asthmatic inflammation[397]. Understanding the role, cause and consequence of mood disorders in asthmatics may allow the development of therapeutics to target these mood-associated changes and in consequence also treatment asthmatic inflammation.

On a similar line, it would be necessary to determine the central effect of using an immunomodulator in humans, with particular focus on the impact on anxiety and depressive disorders. This could be accomplished using fMRI or simple anxiety and depression questionnaires. The exacerbated decline in rears following resiquimod treatment in animal models may be mirrored in humans, and would be a clearly undesirable consequence of treatment.

As discussed in Chapter 7 and above, it was difficult to determine the concrete effect of allergic lung inflammation on the inflammatory pathways of the CNS. Increasing chronicity of inflammation led to markedly different changes in inflammation, and altering mouse strain had a clear effect on the levels of central pro-inflammatory cytokines. Chronic asthma in humans is experienced over years, whereas rodent models of chronicity last just weeks. To understand the true central effect of chronic asthma, it would be necessary to examine central inflammation using post-mortem samples from humans, or using PET imaging to image microglia in asthma patients. The central inflammatory effect of allergic lung inflammation is likely to play a key role in determining the secondary effect of immunomodulator treatment.

8.5 Conclusion

The results in this thesis represent key steps into understanding how allergic inflammation impacts on the CNS; however further work is necessary to fully appreciate the contribution

of the vagus nerve to pathological and psychopathological changes. Critically, this research highlights the significance of the vagus nerve in both AHR and mood disorders in asthma and identifies the importance of investigating the safety, as well as efficacy, of developing therapeutics in diseased individuals, and, crucially, has translational potential. The impact of allergic lung inflammation on the brain must further be emulated in a clinical setting, through fMRI, psychological or post-mortem studies. Understanding the potential role that immune to brain communication has in allergic lung inflammation and can have on clinical symptoms in humans is a step forward in providing a CNS-dependent therapy to ameliorate pathological symptoms and mood disorders associated with asthma.

Chapter 9: Appendices

9.1 Solution and Buffer Recipes

9.1.1. Cryoprotectant

30% Ethylene Glycol
20% Glycerol

Made up in 0.1M PBS.
This recipe was generously provided by Annick deVries.

9.1.2 Lavage Buffer

10mM EDTA
0.1% BSA
Protease inhibitor tablet (1 per 100ml solution)

Made up in PBS

9.1.3 Immunobuffer

0.1M NaCl
5mM KCl
0.0085M Na_2HPO_4
0.0063M NaH_2PO_4
0.01M Tris-HCl
0.3% Triton X-100

Made up in distilled water

9.1.4 Lysis Buffer

20mM Tris-HCl
150mM NaCl
1% Triton X-100
1 x protease inhibitor tablet

Made up in 50ml distilled water

9.1.5 Substrate Solution for ELISA

9ml 0.05M Phosphate-Citrate Buffer (Sigma, Poole, UK)
1ml DMSO
1 tablet 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma, Poole, UK)
2µl 30% H_2O_2 (Sigma, Poole, UK)

9.1.6 FACS Buffer

1% Heat-inactivated foetal calf serum
Made up in PBS

9.2 Protocols

9.2.1 Gelatinising Slides

1. Wash slides in 10% Deacon 90 (Fisher Scientific, Loughborough, UK) in water for 15-20 minutes.
2. Rinse well in running tap water.
3. Rinse 3 to 4 times in distilled water.
4. Dry overnight at 37°C
5. Make up a gelatine solution
    a. Heat 500ml distilled water to 60°C
    b. Add 2.5g gelatine powder
    c. Stir to dissolve
    d. Cool to 40°C
    e. Add 0.25g chromium potassium sulphate
    f. Stir
    g. Filter
6. Dip slides in gelatine solution for 30-60 seconds
7. Dry at 37°C overnight.

9.2 Resiquimod Dose Response

To determine the time point at which to collect samples following resiquimod treatment, a time course experiment was carried out. This experiment examined the impact of intraperitoneal (i.p.) resiquimod challenge on circulating pro-inflammatory cytokine production and behaviour, as these were the two primary read-outs for future resiquimod studies. Resiquimod was found to induce high levels of IL-6 2.5hr after challenge, and was additionally shown to significantly reduce activity in the open field at this time point (Figure 8.1). As this was the peak of cytokine expression and behavioural changes, behavioural analysis and sample collection was carried out 2.5hr after challenge in future resiquimod studies.

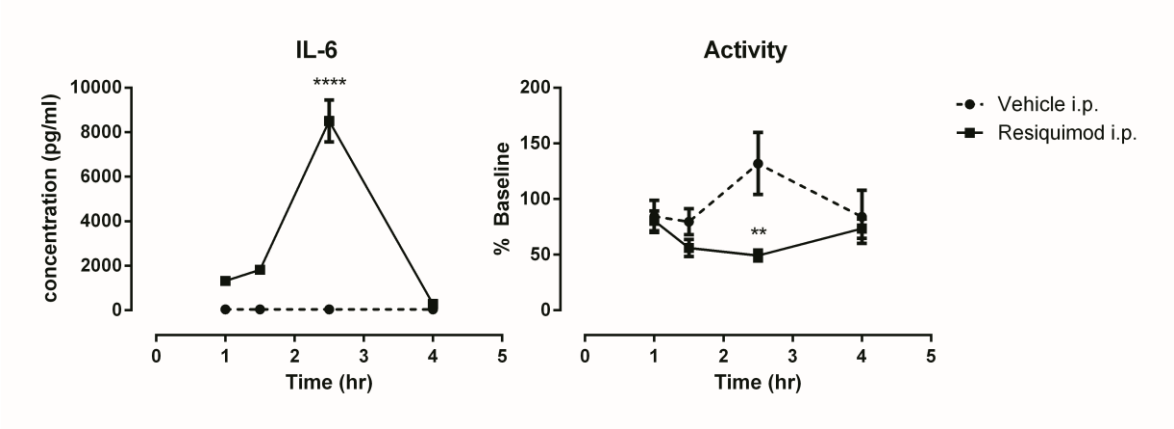

Figure 9.1 –The impact of intraperitoneal (i.p.) resiquimod challenge on levels of IL-6 in the serum and activity in the open field apparatus. Behavioural analysis and sample collection was carried out 1, 1.5, 2.5 and 4 hours after challenge. ****p<0.001, **p<0.01 using two-way ANOVA followed by the Sidak's post-hoct test. IL-6: n=5; activity: n=9.

9.3 Inflammatory Response in the Lung Using An Alternative Protocol for Acute Allergic Lung Inflammation

Data in Chapter 4 revealed that the OVA-immune complex model utilised, in which control animals were not immunised against OVA, but received OVA challenge i.n., induced an inflammatory response, dominated by neutrophils, in the control animals. This was thought to be due to the presence of OVA (or LPS) in the lungs of control animals. As a result, an experiment was carried out in which control animals were immunised against OVA, but were challenged with saline. As shown by the cytospin stains and quantification

below, this protocol completely prevented inflammation on the third day of OVA challenge.

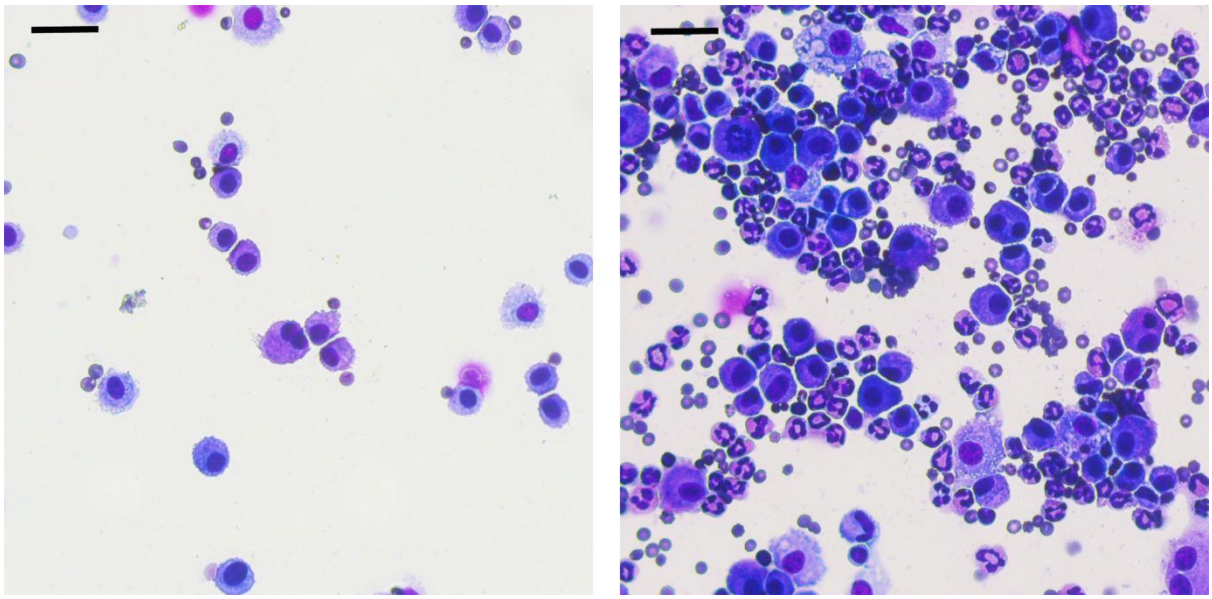

Figure 9.2 – Representative differential Rapid Romanowksy stain of BALF cells from a saline challenged (left) and OVA challenged (right) mouse. Animals were immunised against OVA and challenged with saline or OVA for 3 days. BALF was collected on the final day of OVA challenge. n=2. Scale bar: 20µm

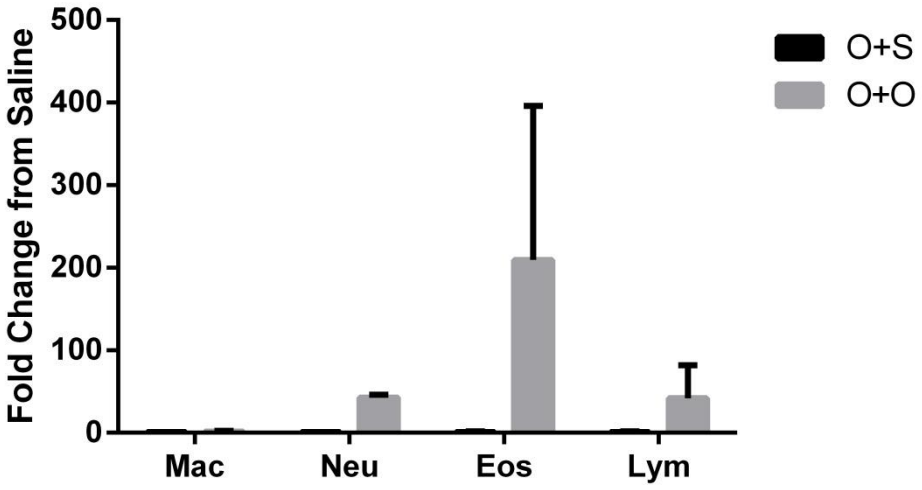

Figure 9.3 – Quantification of differential rapid romanowsky stain of BALF cells from saline challenged (O+S) and OVA challenged (O+O) animals. Animals were immunised with OVA and challenged with OVA or saline for 3 days. BALF was collected on the final day of OVA challenge. n=2

9.4 PCR Array

In Chapter 5, the effect of acute, sub-chronic, chronic and severe allergic lung inflammation on the brain was explored using qPCR Array technology. Due to the magnitude of data acquired, not all results were represented fully represented in the chapter. The disc attached contains files with all data acquired and analysed.

- The file named **Inflammatory Genes** contains the full list of genes analysed using the Mouse Inflammatory Cytokines and Receptors RT^2 Profiler PCR Array (PAMM-011ZE-4), including the Unigene and Refseq codes, a description of the genes and alternative names for the genes.
- The file named **Neuroplasticity Genes** contains the full list of genes analysed using the Mouse Synaptic Plasticity RT^2 Profiler PCR Array (PAMM-126ZE-4), including the Unigene and Refseq codes, a description of the genes and alternative names for the genes.
- The file named **Hyperimmunised Inflammatory** contains raw and analysed data from brainstem samples of hyperimmunised mice (Method Section 2.1.3.5) using the Mouse Inflammatory Cytokines and Receptors RT^2 Profiler PCR Array.
- The file named **Hyperimmunised Plasticity** contains raw and analysed data from brainstem samples of hyperimmunised mice (Method Section 2.1.3.5) using the Mouse Synaptic Plasticity RT^2 Profiler PCR Array.
- The file named **Acute Inflammatory** contains raw and analysed data from brainstem samples from mice with acute allergic lung inflammation (Method Section 2.1.3.3) using the Mouse Inflammatory Cytokines and Receptors RT^2 Profiler PCR Array.
- The file named **Acute Plasticity** contains raw and analysed data from brainstem samples from mice with acute allergic lung inflammation (Method Section 2.1.3.3) using the Mouse Synaptic Plasticity RT^2 Profiler PCR Array.
- The file named **Sub-chronic Inflammatory** contains raw and analysed data from brainstem samples from mice with sub-chronic allergic lung inflammation (Method Section 2.1.3.4) using the Mouse Inflammatory Cytokines and Receptors RT^2 Profiler PCR Array.
- The file named **Sub-chronic Plasticity** contains raw and analysed data from brainstem samples from mice with sub-chronic allergic lung inflammation (Method Section 2.1.3.4) using the Mouse Synaptic Plasticity RT^2 Profiler PCR Array.

- The file named **Chronic Inflammatory** contains raw and analysed data from brainstem samples from mice with chronic allergic lung inflammation (Method Section 2.1.3.4) using the Mouse Inflammatory Cytokines and Receptors RT^2 Profiler PCR Array.
- The file named **Chronic Plasticity** contains raw and analysed data from brainstem samples from mice with chronic allergic lung inflammation (Method Section 2.1.3.4) using the Mouse Synaptic Plasticity RT^2 Profiler PCR Array.
- The above **8 files** are laid out in the same way. The first sheet shows the raw C(t) values for each gene and each sample. The second sheet shows the average C(t) values for each group (control vs. treated) and each gene. The third sheet shows the average ΔC(t) and the standard deviation for each group and each gene. The fourth sheet shows the 2^-ΔΔC(t) for each group and each gene. The fifth sheet shows the fold change, fold regulation and p-value for each gene. The second to fifth sheet contain data analysed using the PCR Array Data Analysis Software. The sixth sheet contains data analysed manually for each sample and gene.

9.5 The Effect of Chronic Isoflurane Exposure on the Brain

As described in Chapter 5, another model of chronic allergic lung inflammation was initially used to investigate the impact on the CNS. This model was deemed more physiological, as it involved repeated exposure to a natural allergen: house dust mite (HDM) and previous studies[251,398] have shown that this model induces chronic eosinophilia and TH2 cytokine production. However, as described above, repeated HDM exposure also involves chronic isoflurane exposure, which can induce peripheral pro-inflammatory cytokine production[253,319], increase the permeability of the BBB[254] and activate the vagus nerve[252]. In experiments for this thesis, it was additionally found that chronic isoflurane exposure resulted in changes to microglial phenotype in the area postrema (Figure 8.3). Due to these confounding factors, further experiments using this model were discontinued.

This is a significant finding as it suggests that isoflurane can have negative consequences on the brain. Several *in vivo* experimental protocols involve chronic use of isoflurane, especially those involving the induction respiratory inflammation. As isoflurane can impact on peripheral vagal fibres and the CNS, both of which have been shown to impact

on inflammation and respiratory function, it is important to recognise that isoflurane may have unwanted consequences in a disease model.

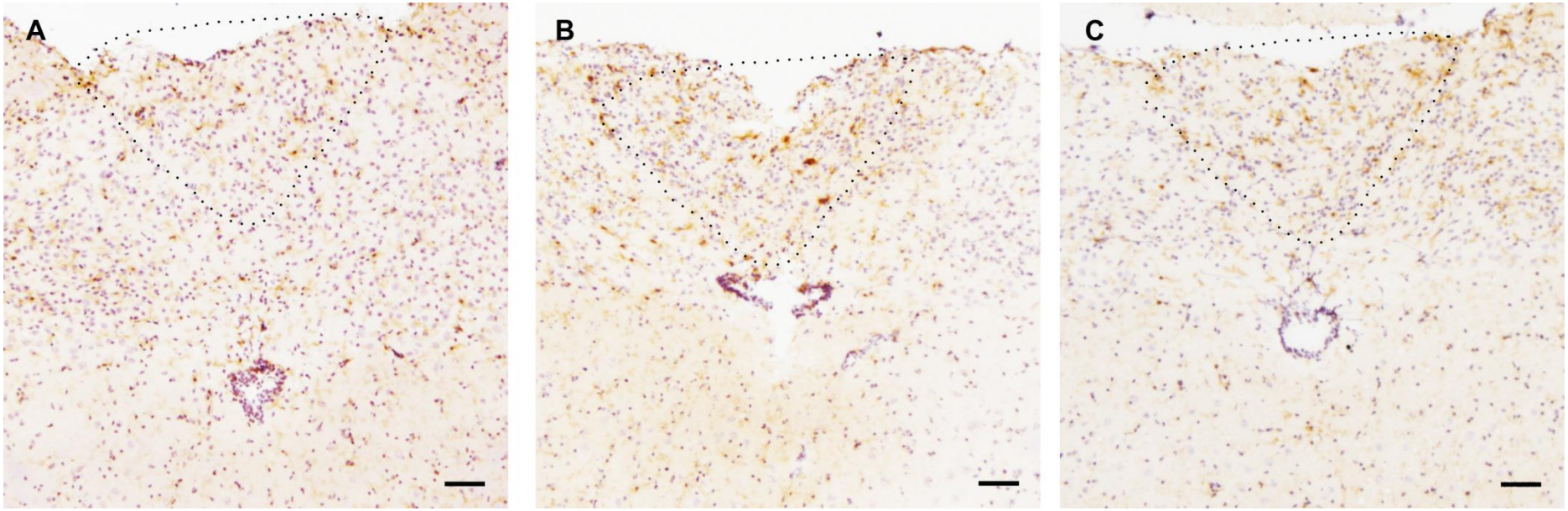

Figure 8.4 – The effect of chronic isoflurane exposure on microglial phenotype in the area postrema. Immunohistochemical stain for CD11b in the area postrema (outlined) in naive mice (A) and mice exposed to isoflurane for 3 weeks and challenged with saline (B) or HDM (C). Increased CD11b immunoreactivity is evident in the area postrema as compared to the surrounding NTS in (B) and (C), but not in (A). Representative of n=2. Scale bar: 100μm

9.6 The Differential Effect of Resiquimod on Systemic and Central Expression and Behaviour in C57BL/6 and BALB/c Mice

Initial experiments investigating the effect of resiquimod on the brain used naïve BALB/C mice. As behavioural changes in the open field were inconclusive in this strain, a phenomenon that has previously been reported[349], C57BL/6 mice were used as a positive control, which are known to exhibit lower emotional reactivity, and therefore be a more reliable strain in behavioural assays. Changes in cytokine expression in the periphery and brain were additionally measured in the strain, to compare these changes with the BALB/C strain.

Significantly, it was found that murine genetic background played a crucial role in both the peripheral and central response to resiquimod, accentuating the importance of strain in studies of immune to brain communication. As compared to C57BL/6 mice, BALB/C mice developed a substantially less pronounced peripheral response, in terms of elevations in IL-6 (Figure 8.4), as well as diminished central inflammatory response, in terms of elevations in central IL-1β (Figure 8.7). Critically, BALB/C mice failed to show any measurable behavioural changes in the open field assay, whereas resiquimod induced a significant reduction in rears and activity in C57BL/6 mice (Figures 8.5 and 8.6).

Genetic background is a vital factor in not only the development of immune responses in mice, but also their behavioural phenotype. BALB/C mice are known to have a genetic predisposition for mounting a TH2 type immune response, whereas C57BL/6 mice more readily mount a TH1 type immune response[375], justifying the low levels of IL-6 in response to resiquimod challenge in BALB/C mice. Low levels of peripheral cytokines may directly contribute to the low levels of central IL-1β, through interaction of peripheral cytokines with the cerebral endothelium, though equivalent levels of COX-2 (preferentially expressed in the endothelium in response to cytokines) dispute this, suggesting variations in C57BL/6 and BALB/C microglial responses. Indeed, strain differences in central microglial responses have been previously reported in a model of focal ischemia[391].

Previous studies have additionally reported strain differences in inflammation-induced fatigue, a behavioural response that was shown to be directly dependent on differing levels

of central pro-inflammatory cytokines[399], suggesting that differences in central inflammation is a determinant for the variability in behavioural responses by BALB/C and C57BL/6 mice. However, it is more likely due intrinsic variability in mouse behaviour. C57BL/6 mice show high levels of locomotion as well as low levels of anxiety-related behaviour[349,400] making this strain highly suitable for this method of behavioural analysis. In contrast, BALB/C mice exhibit low locomotor activity and high emotional reactivity[349,400], particularly with higher levels of anxiety-related behaviour[400], making evaluations in the open field assay inconsistent, accounting for the discrepancies in the effect of resiquimod on open field behaviour in C57BL/6 and BALB/C mice. Collectively, the discrepancies in murine response to TLR7-induced inflammation accentuate the importance of strain choice in immune to brain communication studies.

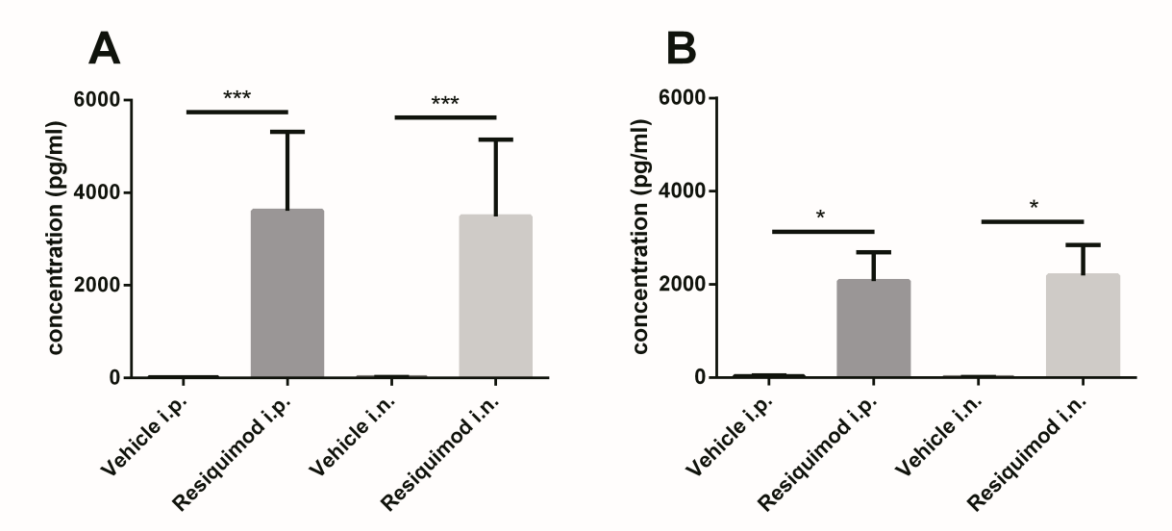

Figure 9.5 - The effect of i.p. or i.n. resiquimod administration (5mg/kg) on levels of IL-6 in the serum of C57BL/6 (A) or BALB/C (B) mice. A terminal blood sample was collected 2.5 hours after immune stimulation; cytokine levels were measured using a single-plex IL-6 ELISA. Values are expressed as mean pg per ml serum ± SEM. (A) ***$p<0.001$ vs. vehicle control after logarithmic transformation of data using a one way ANOVA followed by a Tukey post-hoc test (B) *$p<0.05$ using the Kruskal-Wallis test followed by the Dunn's post-hoc test. n=3-6

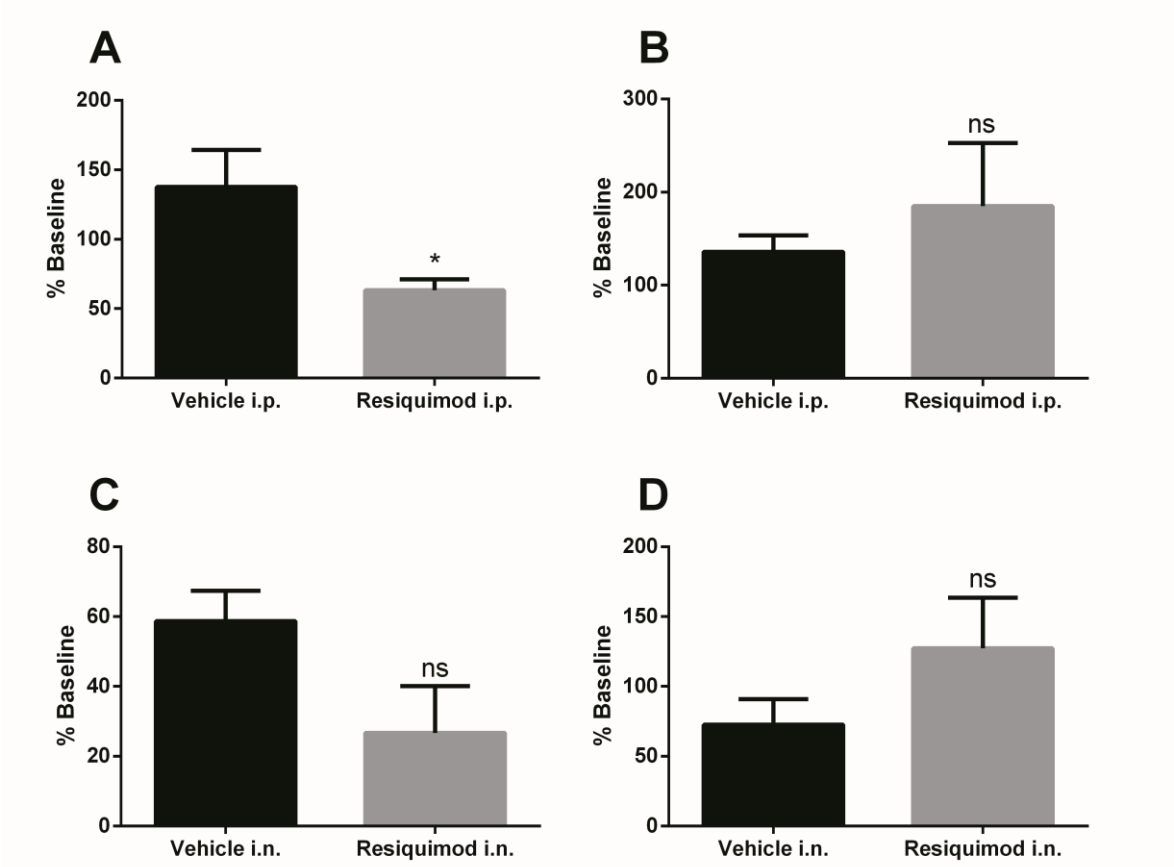

Figure 9.6 - The effect of i.p. (A-B) or i.n. (C-D) resiquimod administration (5mg/kg) on locomotor activity of C57BL/6 (A,C) and BALB/C (B,D) mice in the open field. Behaviour was assayed between 2.5 and 3 hours after immune stimulation. A baseline measurement was taken one day prior to immune stimulation. Values are represented as mean percent of the baseline measurement ± SEM. *$p<0.05$ vs. vehicle control using the student's t-test (A, C, D) or the Mann-Whitney test (B) n=5-7

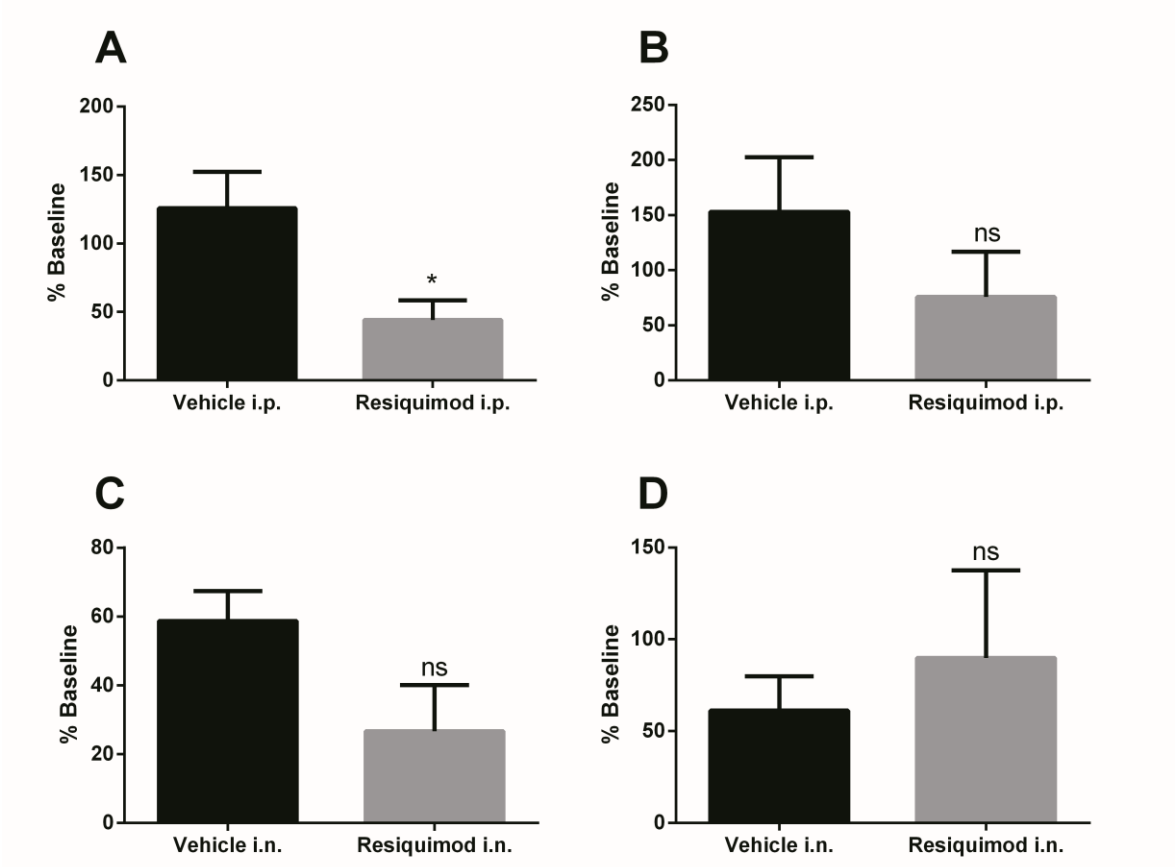

Figure 9.7 - The effect of i.p. (A-B) or i.n. (C-D) resiquimod administration (5mg/kg) on rears of C57BL/6 (A,C) and BALB/C (B,D) mice in the open field. Behaviour was assayed between 2.5 and 3 hours after immune stimulation. A baseline measurement was taken one day prior to immune stimulation. Values are represented as mean percent of the baseline measurement ± SEM. *$p<0.05$ vs. vehicle control using the student's t-test (A,C) or the Mann-Whitney test (B,D). n=5-7

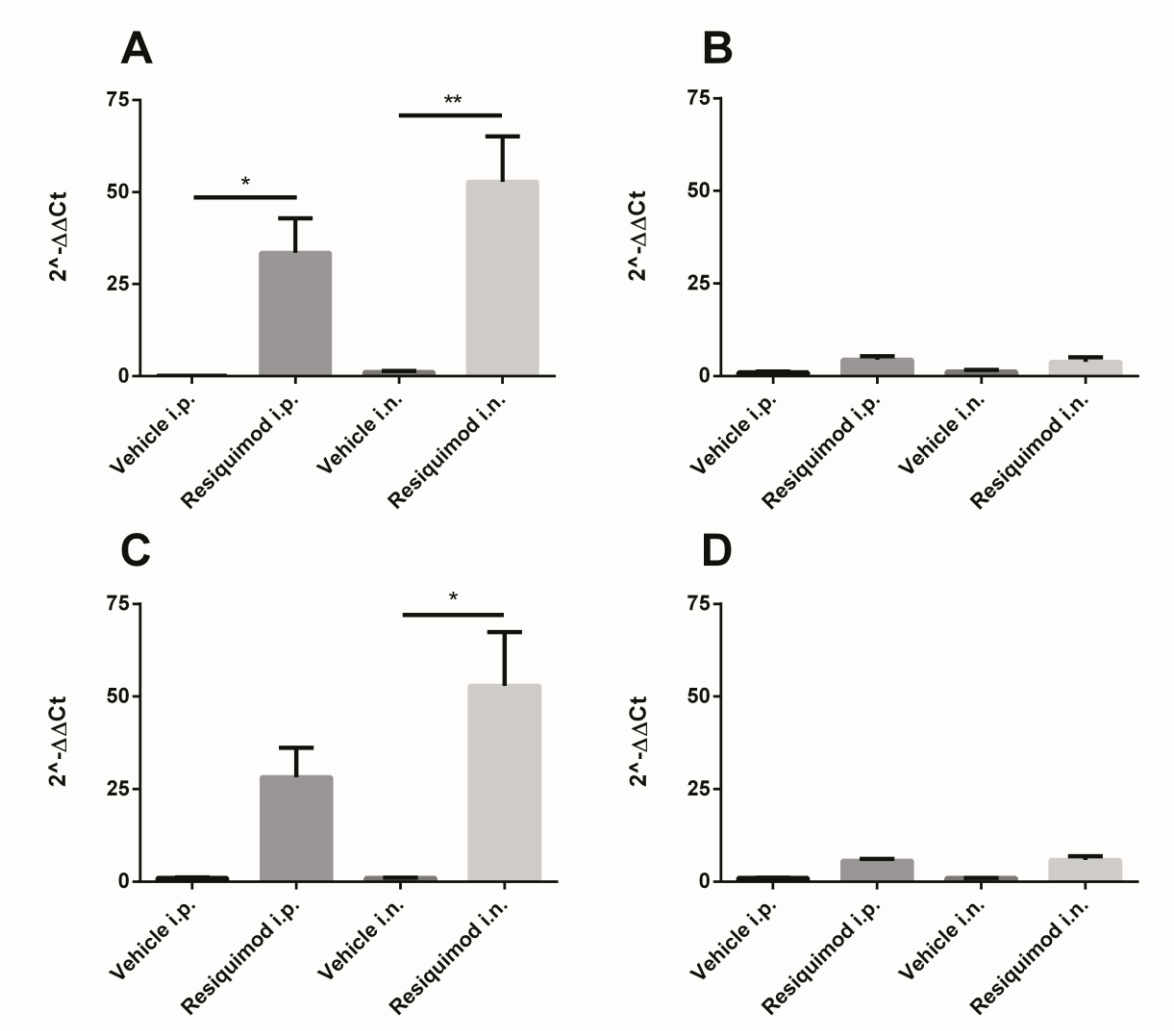

Figure 9.8 - The effect of i.p. or i.n. resiquimod administration (5mg/kg) on mRNA levels of IL-1β in the hippocampus (A,B) and brainstem (C,D) of C57BL/6 (A,C) and BALB/C (B,D) mice. Brain tissue was collected 2.5 hours after immune stimulation; IL-1β was measured using SYBR green qPCR. Values are expressed as mean fold change (2^-ΔΔCt) from vehicle, relative to HPRT expression ± SEM. *p<0.05, **p<0.01 vs. vehicle control using one way ANOVA followed by a Tukey post-hoc test (A,B) or a Kruskal-Wallis Test followed by a Dunn's post-hoc test (C,D). n=4-5

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