Early inflammation dysregulates neural circuit formation *in vivo* via microglial activation and Interleukin-1β

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2016

Integrated Program in Neuroscience (IPN)
McGill University

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

To Maya.

Please don't do a PhD.

If you do, make it a short one.

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ACKNOWLEDGEMENTS

This work would not have been possible without the sacrifice, hard work, love and support of my parents, Hamdah and Shajar Farooqi and my wife, Martina Scotti. They have my eternal gratitude.

I would also like to offer my sincere thanks to:

Past and present members of the Ruthazer lab for their assistance and comradeship:

Dr Martin "Correlation" Munz	Dr Delphine "Martini" Gobert
Dr Lois "YouTube" Miracourt	Dr Marion "Patches" Van Horn
Dr Steven "Electric" Glasgow	Dr Mari "Jazzabel" Sild
Anne "Technik" Schohl	Elena "Professor" Kutsarova
Phil "Dr. Phil" Kesner	Dr Cynthia "Zebrafish" Solek

The members of my committee Dr Sam David and Dr David Stellwagen for their invaluable support and guidance.

Dr Jack Antel for kindly co-supervising this project and for always making time to see me.

Dr Josephine Nalbantoglu who let me into the program and went beyond the call of duty in ensuring the success of my fellowship applications.

Benoit Chambaron and Fernando Chaurand in the animal facility for their kind and expert assistance with fish husbandry.

Very special thanks to Dr. Edward "Great Khan" Ruthazer, the most awesome PI since the original Great Khan united the tribes in 1206 and founded the greatest contiguous empire the world has ever known. May your armies march on to greatness.

This work was supported by generous grants and awards from:

Integrated Program in Neuroscience, McGill University

Faculty of Medicine, McGill University

Canadian Institutes of Health Research (Vanier Canada Graduate Scholarship)

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin releasing hormone
ADH	anti-diuretic hormone
ANOVA	analysis of variance
apoE	apolipoprotein E
ASD	autism spectrum disorder
BBB	blood-brain-barrier
BDNF	brain-derived neurotrophic factor
BMT	bone-marrow transplantation
c1q/c3/c4b	complement proteins
CNS	central nervous system
CNV	copy number variant
CRH	corticotropin-releasing hormone
dpf	days post-fertilisation
dsRed	*Discosoma* sp. red fluorescent protein
E	embryonic day
eNOS	endothelial nitric oxide synthase
GFAP	glial fibrillary acid protein
GFP	green fluorescent protein
GWAS	genome-wide association study
HGF	hepatocyte growth factor
hpf	hours post-fertilisation
HLA	human leucocyte antigen
IFNγ	interferon gamma
iNOS	inducible nitric oxide synthase
IGF-1	insulin-like growth factor
IGFBP-1	insulin-like growth factor binding protein 1
IL-	interleukin
LPS	lipopolysaccharide
LTP	long-term potentiation
MCP-1	macrophage chemoattractant protein 1
MET	mesenchymal-epithelial transition (MET)

MHC	major histocompatibility complex (MHC)
MIA	maternal immune activation
MMP	matrix metalloproteinase (MMP)
MPEG1	macrophage-expressed gene 1
MS	multiple sclerosis
mYFP	membrane-targeting yellow fluorescent protein
NGF	nerve growth factor
NMDA	n-methyl d-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NPC	neuronal progenitor cell
NT-	neurotrophin
OPC	oligodendrocyte precursor cell
P	postnatal day
PBMC	peripheral blood monocyte
PirB	Paired immunoglobulin-like receptor B
poly I:C	Polyinosinic:polycytidylic acid
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RGC	retinal ganglion cell
SCID	severe combined immunodificiency
SNP	single nucleotide polymorphism
TLR	toll-like receptor
TGF-	Transformational Growth Factor
TNF-	tumour necrosis factor

ABSTRACT

Maternal infection and inflammation during foetal development are implicated in the pathogenesis of autism spectrum disorders (ASD) and schizophrenia. The mechanisms by which infection and inflammation impact the developing nervous system remain to be adequately established. The precise effects on neural circuit structure and function have also yet to be well described. Recent work suggests that microglia – the macrophages and immune modulators of the CNS – play a critical role, perhaps by physically 'pruning' synapses or releasing inflammatory signals. This may be a feature of normal development and the process may go awry under pathological conditions.

Using a zebrafish model to visualise circuit remodelling in early development, we found that microglia did not physically interact with developing axons in the visual system. They did however alter their morphology in response to acute inflammation. We found that inducing inflammation by applying bacterial components upregulates the dynamic remodelling of axons *in vivo* within hours. Further, a single brief inflammatory insult impairs normal arborisation for days. These effects were absent in PU.1 morphant fish lacking microglia. The pro-inflammatory cytokine interleukin-1β (IL-1β) was necessary and sufficient to induce these neurodevelopmental effects, even in the absence of microglia.

Our data suggest that interleukin-1β released from microglia may underlie aberrant circuit formation induced by inflammatory insult. These studies suggest blockade of inflammatory pathways such as interleukin-1β signalling may be promising interventions to prevent neural circuit dysfunction following maternal infection in pregnancy.

RESUMÉ

L'infection maternelle et l'inflammation pendant le développement du fœtus sont impliqués dans la pathogenèse des troubles du spectre autistique (TSA) et de la schizophrénie. Les mécanismes par lequels l'infection et l'inflammation perturbent le système nerveux en développement restent à être révélée. Les effets précis sur la structure des circuits neuronaux et la fonction restent également à être bien compris.

Des travaux récents suggèrent que les cellules microglie – des macrophages et modulateurs immunitaires du système nerveux - jouent un rôle essentiel, peut-être par une 'élagage' des synapses ou en libérant des signaux inflammatoires. Cela peut être une caractéristique du développement normal, et ce processus peut etre affecté dans des conditions pathologiques.

Par l'utilisation du poisson zèbre (un modèle de remodelage des circuits neuronaux pendant le développement) nous avons constaté que les microglies n'interagissent pas physiquement avec le développement des axones dans le système visuel. Cependant, ils modifient leur morphologie en réponse à une inflammation aiguë. Nous avons constaté que l'induction de l'inflammation (en appliquant des composants bactériens) augmente le remodelage dynamique des axones *in vivo* en quelques heures. De plus, une seule et brève agression inflammatoire est suffisante pour alterer l'arborisation des axones pendant des jours. Ces effets étaient absents dans les poissons qui n'ont pas des cellules microglie.

La cytokine pro-inflammatoire interleukine-1β (IL-1β) était nécessaire et suffisante pour induire ces effets neurodéveloppementaux, même dans l'absence de la microglie. Nos données suggèrent que l'interleukine-1β libérée par la microglie peut causer une formation aberrante des circuits neuronaux, à cause de l'insulte inflammatoire. Nos études suggèrent qu'une intervention précoce visant à reduire l'inflammation, tel que l'utilisation d'agents inhibiteurs de la voie de signalisation de l'interleukine-1β, pourrait avoir un potentiel dans le but de prévenir le dysfonctionnement des circuits neuronaux après une infection maternelle pendant la grossesse.

I. INTRODUCTION - Contributions

Text was written by Nasr Farooqi with corrections and suggestions from Dr. Edward Ruthazer, Dr. Leanne Godinho and Dr. David Stellwagen. Experiments were conceived and designed by Nasr Farooqi and Dr. Edward Ruthazer with input from Dr. Jack Antel.

I. INTRODUCTION

I.i. Neuro-immune interaction: history and overview

This thesis concerns the broad area of neuro-immune interaction in development. The immune and nervous systems are two highly complex and dynamic physiological systems. Historically, they have been considered independent under normal circumstances. In the late 19th and early 20th century, physicians and neuropathologists including Paul Ehrlich, Max Lewandowsky, Lina Stern and Edwin Goldmann experimented with parenteral injection of dyes, toxins and heavy metals into living animals (for detailed historical reviews see e.g. Saunders et al., 2014; Solomos and Rall, 2016; Liddelow, 2011). They quickly noted that at least in adult animals, brain parenchyma did not usually take up these substances. This gave rise to the concept of the "Blut-Hirnschranke" or "barrière hémato-encéphalique": the "Blood-Brain-Barrier".

In the 1920s, seminal work by Shirai demonstrated that rat sarcoma cells grew well when implanted in central nervous system (CNS) parenchyma but not when implanted peripherally (Shirai, 1921). Other studies extended these findings to bacteria and viruses (reviewed in Galea et al., 2007). Peter Medawar showed in the 1940s that homologous adult rabbit skin grafts into brain parenchyma were better tolerated than peripheral grafts. Extending Shirai's work, Medawar demonstrated that sensitising the rabbit immune system by peripheral grafting enabled an immune response to CNS grafts (Medawar, 1948). This implies a specific deficit in

the afferent wing of the immune system (i.e. in detecting an immune insult). Along with the apparent paucity of lymphatic vessels and immune cells such as T- and B-lymphocytes in the brain, the view took hold that the brain parenchyma is an "immune-privileged" site. Although this notion has remained somewhat ill-defined, it is broadly accepted that under normal conditions, innate and adaptive immune responses are muted or absent in CNS parenchyma.

Fascinatingly, contemporary research is challenging the concept of "immune-privilege" and unveiling profound functional, cellular and molecular links between the nervous and immune systems, particularly in development (Boulanger, 2009; Filiano et al., 2015; Louveau et al., 2015a). Some of these findings will be reviewed in section I.iii.

It may be useful here to briefly note the differences between the innate and the adaptive immune system(see e.g. Murphy and Weaver, 2016 for further details). The innate immune system is evolutionarily older and is found to some degree in all classes of plant and animal life, including simple multicellular organisms. The innate immune system provides immediate, unlearned, generic responses to known pathogens which are recognised by genetically encoded pattern recognition receptors (PRRs). Components of the innate immune system include the complement system, generic inflammatory responses, macrophages and neutrophils. The adaptive immune system evolved in Gnathostomata (jawed

vertebrates) around 500 million years ago. It is able to respond to novel pathogens and provides specific responses to them. Key components of the adaptive immune system include T and B-Lymphocytes. Somatic hypermutation and genetic recombination allow a near-infinite diversity of T-cell receptors and B-cell antibodies to be produced to recognise specific pathogens and toxins. These responses are stored as 'immunological memory' in the form of 'memory' T-cells and 'memory' B-cells and can be rapidly accessed if the same pathogen is encountered again.

From an evolutionary and functional perspective, parallels could be drawn between the nervous and immune systems. Both systems have inputs, processing and outputs. In the nervous system, sensory inputs are processed using both innate and adaptive strategies and decisions are made on appropriate motor responses. The immune system has a sensory wing performing continual surveillance for potential threats, decisions are made (self or non-self; benign or dangerous) using innate and adaptive strategies, and appropriate immune responses are carried out. These may include concerted cellular and molecular attacks on invading micro-organisms and to clear dead or dying tissue. As noted, the immune system is also capable of learning and memory. Co-option or co-evolution of mechanisms across the two systems remains an intriguing possibility.

Indeed, some links between the nervous and immune systems have long been known or suspected. Immune and inflammatory activation induces "sickness behaviour" including lethargy, depression, anorexia and reduced grooming (Hart, 1988). This may serve an evolutionary purpose by limiting social contact and transmission of infectious micro-organisms. Activation of the neuroendocrine stress axis, particularly through glucocorticoids (Besedovsky et al., 1986) and catecholamines (Kipnis et al., 2004) can influence the immune system.

Breaking with the longstanding consensus, recent seminal studies have demonstrated that the CNS may indeed have its own lymphatic system, with a network of fine lymphatic vessels at the dural sinuses (Louveau et al., 2015b). The profound contribution of glial cells to the interface between CSF and lymphatics have given rise to the term "glymphatic system" (Jessen et al., 2015). Clearly, we are only beginning to understand the extent and complexity of neuro-immune interaction in health and disease.

In the next section, we will discuss one reason for which these interactions are particularly fascinating and potentially clinically relevant: their increasing implication in the aetiology of neuropsychiatric disease.

I.ii. **Neuro-immune interaction and neuropsychiatric disorders**

Neuro-immune interaction is increasingly implicated in the aetiology of neuropsychiatric disorders. I will focus chiefly on autism spectrum disorder (ASD). Notably, mounting evidence suggests a role for neuroimmune interaction in other disorders including schizophrenia (see e.g. reviews by García Bueno et al., 2016; Khandaker et al., 2015; Leboyer et al., 2016; Müller et al., 2015; Stuart et al., 2015).

ASD is an umbrella term for a heterogenous group of developmental neuropsychiatric disorders characterised by deficits in social communication and repetitive/restricted interests and behaviours (APA, 2013). In the United States, recently reported estimates of prevalence are remarkably high: around 1 in 68 children and 1 in 42 boys (Christensen et al., 2016), a greater than seven-fold increase from a reported 1 in 500 children in 1992 (Estes and McAllister, 2015). Increased public awareness, loosening of the diagnostic criteria, improvements in screening programs and altered reporting practices may account for some of this increase (Hansen et al., 2015). Nonetheless, "the extent to which the continued rise represents a true increase in the occurrence of autism remains unclear." (Hertz-Picciotto and Delwiche, 2009). Regardless of the origins, ASD now constitutes a multi-billion dollar disease burden in North America (Gottfried et al., 2015).

The genetic contribution to ASDs has been intensively studied. Early familial and twin studies demonstrated high heritability, up to 90% in some studies; a recent estimate from a study of over 2 million families is around 50% (Sandin et al., 2014). The broad clinical spectrum of ASDs is reflected in the spectrum of genetic variants which can contribute to it: autosomal recessive, autosomal dominant, X-linked and additive risk variants have been described, as well as chromosomal translocations and triplet repeats. Rare genetic syndromes with high penetrance may account for around 5% of individuals with ASDs (de la Torre-Ubieta et al., 2016).

Confusion frequently arises from imprecise use of the terms "inherited", "heritable" and "genetic". ASDs may be strongly genetic but not inherited: a large proportion of these individuals have *de novo* copy number variations (CNVs) or single-nucleotide polymorphisms (SNPs) which are likely gene-disrupting. As they are *de novo* they are not inherited but may be heritable by subsequent generations. The statistics involved in comparing entire genomes of ASD individuals and unaffected controls, while correcting for the many thousands of individual comparisons, make it very difficult to obtain significance for any individual mutation. Considered singly, each mutation is rare. For these reasons, despite huge genome-wide association studies (GWAS) no single gene mutation has as yet reached statistical significance for association with ASD (de la Torre-Ubieta et al., 2016).

To put it another way, practically all ASD-associated mutations can also be found in "normal" individuals. The somewhat arbitrary definition of normality reflects one of the key difficulties in neuropsychiatric diagnosis. Differences in diagnostic criteria and in their application complicate the task of comparing studies in ASD at different centres and different times (and sometimes even within centres and studies). It may make sense to consider that a continuum of genetic and environmental risk influences a continuum of behavioural and developmental traits. At the severe end of this continuum, where signs and symptoms are clinically pathological, a diagnosis of ASD or other neuropsychiatric disorder is made (Robinson et al., 2016).

Monozygotic twin concordance for ASD has been reported as high as 90%, though more recent studies using stricter diagnostic criteria have reported rates around 60%, dropping to around half that for dizygotic twins (Hallmayer J et al., 2011). This implies that approximately half the risk for ASD comes from environmental factors and gene-environment interaction. For over 50 years, neuro-immune pathways have been proposed as central to gene-environment interaction in the patho-aetiology of ASD. Below, we will briefly examine the following 5 main lines of evidence:

1. Epidemiology
    a) Maternal infection
    b) Association with autoimmune disease

2. Post-mortem brain analysis

3. Immune profiling
4. Genetic profiling
5. Animal models

A sixth emerging line of evidence involves interactions between the gut microbiome, the immune system and the CNS. (For useful reviews see e.g. Rescigno, 2014; Rook et al., 2014; Wang and Kasper, 2014; Carabotti et al., 2015).

Epidemiology: some of the earliest studies in this vein examined children of mothers exposed to Rubella in a US pandemic of 1964. These studies showed that exposure was associated with an increase in incidence of ASDs from 0.05% to 8-13% (Chess, 1971; Estes and McAllister, 2015). Subsequent studies have shown associations with ASD and other viral (mumps, varicella, cytomegalovirus, herpes simplex, influenza), bacterial (syphilis) and parasitic (toxoplasma) infections suggesting that maternal immune activation (MIA), rather than a specific pathogen is the common aetiological thread (Patterson, 2012, 2011). In 2010, a major study used the Danish health registry to examine the records of over 1.6 million children born between 1980 and 2005 and demonstrated an association between ASD and viral infection in the first trimester (hazard ratio 2.98) or bacterial infection in the second trimester of pregnancy (hazard ratio 1.42, Atladóttir et al., 2010). More recently, a study of the Kaiser Permanente database for Northern California demonstrated that any maternal infection during hospital admission, but particularly bacterial infection (odds ratio 1.58), was associated with an increased

risk of delivering a child with ASD (Zerbo et al., 2015). Notably, no association was found for infections outside hospital, perhaps reflecting that a threshold of severity must be crossed for immune activation to impact brain development.

Strengthening the immune link, autoimmune and allergic disorders including atopy (eczema, asthma and rhinoconjunctivitis) are associated with ASD individuals and their families (Keil et al., 2010). Parental history of an autoimmune disorder such as inflammatory bowel disease or type-1 diabetes increases the risk of ASD in the offspring and individuals with ASD are more likely than the general population to suffer from a concurrent autoimmune disorder (McDougle et al., 2015; Kohane et al., 2012). Intriguingly, gestational immune-tolerance (suppression of the maternal immune system to prevent attack on the foetus) may be disturbed in mothers with auto-immune disorders.

Post-mortem brain studies constitute a second line of evidence for neuro-immune interaction in ASD. Two caveats should be noted with such studies. As the relevant patient population is young, studies are hampered by a (fortunate) paucity of brain tissue. Any conclusions remain somewhat tentative on the basis of small sample sizes. Secondly, visualising histological changes does not tell us whether such changes are a cause, consequence or epiphenomenon of aberrant neural circuit formation or function.

With this in mind, it remains interesting to see that such studies have reported pathological features consistent with CNS inflammation or immune activation in the brains of ASD individuals. In the dorsolateral prefrontal cortex for example - an area particularly associated with complex cognitive processing and behaviour – microglia have a more activated (amoeboid) morphology, increased density and upregulated MHC-II expression (Morgan et al., 2010). Similar changes were observed in microglia in insula and visual cortex (Tetreault et al., 2012).

Dysregulation of astrocytes also appears to be a feature of the ASD brain with an increased overall number of astrocytes, increased Glial Fibrillary Acid Protein (GFAP) expression and decreased astrocyte branching (Cao et al., 2012; Edmonson et al., 2014). Finally, increased levels of cytokines and other inflammation-associated signals including Transforming Growth Factor 1 (TGF-1), Macrophage Chemoattractant Protein 1 (MCP-1), Insulin-like Growth Factor Binding Protein 1 (IGFBP-1), and Interleukin-6 (IL-6) have been seen in ASD brain tissue (Vargas et al., 2005). (For further introduction on cytokines see section I .iii. b below). Notably, these studies demonstrated changes consistent with an innate rather than an adaptive immune response.

Immune profiling has also been performed on living ASD patients and their families. Such studies have reported features of immune activation such as increased levels of pro-inflammatory cytokines and decreased levels of anti-

inflammatory ones (Estes and McAllister, 2015). ASD patients have increased numbers of circulating blood monocytes (Sweeten et al., 2003). Stimulation of peripheral blood monocytes (PBMCs) from ASD individuals produced higher levels of pro-inflammatory cytokines than PBMCs from unaffected controls (Enstrom et al., 2010; Molloy et al., 2006). Measurement of cytokine levels in plasma from ASD individuals revealed increases in interleukins IL-1β, IL-6, IL-8 and IL-12p40 over controls (Ashwood et al., 2011). This study also demonstrated that patients with elevations of cytokine expression were more likely to have severe forms of ASD and that the level of cytokine elevation correlated to the clinical severity.

A recent major meta-analysis of studies comparing blood and serum levels of 19 cytokines in ASD individuals and controls found significantly elevated levels of interleukin (IL)-1beta, IL-6, IL-8, interferon-gamma (IFN-γ), eotaxin and monocyte chemotactic protein-1 (MCP-1) in ASD individuals. The concentration of the anti-inflammatory factor transforming growth factor-β1 (TGF-β1) was significantly lower in ASD individuals. Interestingly, this is the opposite of the increase in TGF-β1 observed in post-mortem brain tissue from ASD individuals (Vargas et al., 2005). This may reflect differences in inflammatory responses between CNS and peripheral blood. No significant change was demonstrated for several other cytokines including tumour necrosis factor alpha (TNF-α). The strongest effect sizes were for IFN-γ and TGF-β1 (Masi et al., 2015).

Adult patients with severe ASD have been shown to have higher blood levels of bacterial endotoxin: lipopolysaccharide (LPS), its soluble receptor and typical pro-inflammatory cytokines suggesting an innate immune response (Emanuele et al., 2010). Again, levels of inflammatory markers correlated with severity of ASD.

The presence of autoantibodies against CNS components including myelin basic protein, GFAP and multiple proteins associated with neuronal progenitor cells (NPCs) has also been identified in ASD individuals at higher rates than controls (Gottfried et al., 2015; Mazur-Kolecka et al., 2014).

Genetic profiling constitutes a fourth line of evidence. Aspects of the genetic contribution to ASD have been discussed above. Some of the genes most strongly associated with ASD encode immune-related proteins. A prime example is the mesenchymal-epithelial transition (MET) gene which encodes a hepatocyte growth factor (HGF) receptor. In addition to being a proto-oncogene, MET expression downregulates immune activation (Estes and McAllister, 2015). An SNP in MET (the 'MET-C allele') which decreases MET signalling is strongly associated with ASD (Campbell et al., 2006). Post-mortem brain analysis of individuals with ASD reveals decreased MET expression in the temporal lobe (Campbell et al., 2007). Concomitance of ASD and the MET-C allele is associated with hypo-connectivity in tempero-parietal lobes and reduced cortical thickness in multiple brain areas (Hedrick et al., 2012).

Other gene areas associated with ASD include the human leucocyte antigen (HLA) locus which encodes proteins of the major histocompatibility complex (MHC) family' and the complement c4b null allele (in the MHCIII region). Several members of the IL-1 receptor family have also been associated, including the IL-1β decoy receptor IL-1R2 and IL-1 receptor accessory protein-like 1 gene (IL-1RAPL1) (Estes and McAllister, 2015). These pathways are examined further in section I.iii.b on cytokines below.A major transcriptomic and network analysis of 19 brains from ASD individuals revealed several upregulated modules of genes including a neuronal module and an immuno-glial module (Voineagu et al., 2011). The immuno-glial module included astrocyte and activated-microglia markers as well as genes involved in innate immune/inflammatory response. Strikingly, the neuronal module correlated well with results from genome-wide association studies but the immuno-glial module did not, suggesting that the dysregulation of immuno-glial signalling is related to environmental factors.

Animal models of ASD have strengthened the hypothesis that neuro-immune interaction is causally related to onset of the disorder. The notion of maternal immune activation (MIA) taken from the epidemiological data has been applied experimentally to ascertain the effects of early immune challenge on behaviour and to delineate potential mechanisms. The pioneering contributions of the late Paul

Patterson (1943-2014) at Caltech should be particularly noted in this regard (see e.g. reviews by Patterson, 2011, 2009).

Experimental infection or inflammation has been induced in pregnant mice, rats and non-human primates (primarily rhesus macaques) to observe the effects on offspring. Typical approaches include intranasal administration of influenza virus and injection of synthetic viral RNA or bacterial lipopolysaccharide (LPS) to evoke antiviral or antibacterial innate immune responses respectively. Polyinosinic:polycytidylic acid (Poly I:C) is a synthetic, mismatched ribonucleic acid (RNA) which interacts with Toll-like Receptors and mimics viral infection. Despite differences in the immune responses elicited by these different means, the behavioural outcomes in the offspring have strong commonalities. These include deficits in communication, social interaction, elevated anxiety and reduced prepulse inhibition (Patterson, 2009). Structurally, localised reduction in Purkinje cell number are seen in the cerebellum after MIA in mouse, similar to deficits that have been reported in ASD individuals (Shi et al., 2009).

Prenatal exposure of mice to antibodies from human mothers of children with ASDs also elicits anxiety behaviour, reduced sociality, and repetitive-restricted behaviour such as marble-burying (Camacho et al., 2014; Singer et al., 2009).

In Rhesus macaques, prenatal administration of poly I:C resulted in offspring with deficits in social attention and reduced dendrite arborisation in layer III pyramidal neurons (Machado et al., 2015; Weir et al., 2015).

A highly intriguing study demonstrated that pathology in a mouse model of Rett syndrome, an X-linked form of ASD, could be arrested by bone-marrow transplantation (BMT) to repopulate the brain with wild-type myeloid cells (Derecki et al., 2012). Unfortunately a major, multi-centre attempt to replicate these findings was unsuccessful and clinical trials for BMT in Rett syndrome have since been suspended (Wang et al., 2015).

Attempts to delineate mechanisms of neuroimmune interaction have focused primarily on cytokines whose expression is first evoked in the mother and which cross the placenta to induce neuroimmune responses in the fetus (Hsiao and Patterson, 2012). MIA does not elicit behavioural deficits in an IL-6 knockout background and can be prevented by blockade of IL-6 or IL-1 pathways, or overexpression of the anti-inflammatory cytokine IL-10 (Estes and McAllister, 2015). In a very recent report, maternal IL-17a was necessary and sufficient to produce neurobehavioural deficits in mouse MIA (Choi et al., 2016).

The implication of multiple cytokines in the effects of MIA is likely a reflection of redundancy and complex regulation in the inflammatory cascade. In early inflammation for example, pro-inflammatory factors often induce their own

expression and expression of other pro-inflammatory factors in order to rapidly amplify responses. This might be considered a 'feed-forward' response in that further regulation of the inflammatory cascade is independent of the original stimulus. Conversely, negative feedback is required to terminate immune responses, where pro-inflammatory cytokines induce anti-inflammatory factors and also reduce their own expression (Schmitz et al., 2011).

In summary, multiple lines of evidence strongly implicate neuroimmune interaction in the aetiology of neuropsychiatric disorders including ASD. Although much of the evidence from human patients is correlational, successful attempts to reproduce behavioural effects in animal models point towards a causal role. A better understanding of the mechanisms and pathways involved may facilitate the development of interventional strategies to prevent or mitigate disease.

I.iii. Mechanisms of neuro-immune interaction

In this section I will briefly review some of the multiple interlinked cellular and molecular pathways implicated in developmental neuroimmune interaction.

a. Cellular

Microglia are the macrophages and primary immune modulators of the CNS. Their role in responding to injury and infection in the brain was first described in 1919 by the brilliant histologist Pio del Rio Hortega, who trained with Santiago Ramon y

Cajal. Hortega described microglia as constituting a 'third element' in the nervous system: neither neuronal nor typically glial. Hortega's description of the morphology and properties of these cells was astonishingly precise and insightful considering the relatively primitive tools available to him: primarily light microscopy and silver-staining. Most of his findings are unchallenged today. He noted that microglia are fundamentally distinct from neurons and other glia in that they are of mesodermal rather than ectodermal origin. He described their amoeboid morphology when they enter the CNS, that they then become highly branched or ramified with an apparently well-defined territory, and can reassume an amoeboid morphology when reacting to injury or threat. In these circumstances, they are capable of migration, proliferation and phagocytosis (Kettenmann et al., 2011; Rio-Hortega, 1939). So complete was Hortega's description that for almost a century, little was added to it. The last decade or so has finally seen a major resurgence of interest in microglia. A number of excellent recent reviews are available (Frost and Schafer, n.d.; Hong et al., 2016; Kettenmann et al., 2013, 2011; Michell-Robinson et al., 2015; Tay et al., 2016a, 2016b).

Fate-mapping studies have confirmed the mesodermal origin of microglia; specifically they arise from yolk-sac erythromyeloid progenitors which migrate into the CNS and colonise it early in development (Ginhoux et al., 2010; Tay et al., 2016a). Specification occurs by embryonic day (E) 7.5 in mouse and colonisation of the CNS begins from E9.5. In human embryos, colonisation begins by 4.5 weeks of

gestational age at the diencephalon and telencephalon and is at first restricted to white matter (Verney et al., 2010).

It had long been thought that microglia remained in an inert or 'resting' state unless responding to injury or infection. Advances in imaging, particularly multiphoton microscopy through skull windows in mice, permitted visualisation of microglia in the brains of live animals. Early seminal reports found that 'resting' microglia were in fact highly active, extending and retracting fine processes into their environment in apparent surveillance (Davalos et al., 2005; Nimmerjahn et al., 2005). These findings led to concerted efforts to re-evaluate the role of microglia in health and disease. Indeed, microglia have now been implicated as regulators of neurogenesis, circuit formation, refinement, maintenance and function in addition to neural death and disassembly.

A number of *in vitro* studies have suggested that microglia can positively influence proliferation or survival of neural progenitor cells (NPCs) (Chamak et al., 1994; Morgan et al., 2004). Freda Miller's group has demonstrated that cultures of cortical NPCs from microglia-null animals display reduced proliferation which can be rescued by addition of wild-type microglia (Antony et al., 2011). The authors propose that this is due to a loss of trophic factors such as BDNF, Fibroblast Growth Factor (FGF2) and IGF from microglial cells. *In vivo*, ablation of microglia appears to increase apoptosis of layer V cortical neurons by loss of trophic support, particularly

IGF-1 (Ueno et al., 2013), though the increased number of apoptotic cells seen in this study could simply reflect reduced phagocytic clearance.

It has also been proposed that microglia regulate neuronal activity and synaptic plasticity. This can occur by modulation of the NMDA receptor binding site, modulation of the chloride gradient through BDNF, purinergic signalling and by release of tumour necrosis factor alpha (TNF-α) (Kettenmann et al., 2013). The role of TNF-α in synaptic scaling has been particularly well described (Stellwagen and Malenka, 2006). Although the original reports implicated astrocytes as the main source, later studies have suggested that TNF-α is exclusively microglia-derived (Pascual et al., 2011). In zebrafish, contact between microglial processes and neuronal cell bodies apparently reduces neuronal activity by unclear mechanisms (Li et al., 2012); potentially implicating microglia in ramping down excito-toxicity.

As professional phagocytes, microglia are responsible for clearing cellular debris. It has long been known that after nerve injury, they can remove synapses from cell bodies, so-called 'synaptic stripping' (Blinzinger and Kreutzberg, 1968; Trapp et al., 2007). A number of studies have now suggested that microglia can employ this phagocytic ability in a more precise manner to 'prune' supernumary synapses in development and 'sculpt' circuits.

Contact of microglia with neuronal elements including synapses appears to be activity-regulated. Tremblay and colleagues used a combination of electron microscopy and *in vivo* two photon microscopy in mice to demonstrate that most microglial processes in visual cortex were in contact with synapse-associated elements including transient dendritic spines. When light levels were decreased, microglia were more likely to contact dendritic spines and those spines were more likely to disappear. Increasing light levels had the opposite effects (Tremblay et al., 2010).

Another group reduced activity in the visual system by enucleation, injecting tetrodotoxin or by lowering body temperature. In each case, there was a lower frequency of microglial-synapse contact. When the authors induced ischemia, microglia-synapse contact was prolonged and this contact was followed by disappearance of the terminal (Wake et al., 2009). In each of these studies, it remains unclear whether microglia are directly responsible for 'pruning' synaptic components but the data are consistent with this possibility.

In 2011, an exciting study made a further step in this direction. Using super-resolution microscopy, the authors demonstrated co-localisation of synaptic markers and microglial membranes, suggesting that microglia had in fact taken up synapses or synaptic components. In knockout mice not expressing Cx3cr1 (also known as fractalkine, a chemokine receptor expressed by microglia), fewer

microglia and more dendritic spines were seen than in controls. This may imply a deficit in synaptic pruning (Paolicelli et al., 2011).

The groups of Beth Stevens and Ben Barres have implicated the complement proteins c1q and c3 in synaptic elimination (Schafer et al., 2012; Stevens et al., 2007). The complement cascade forms part of the innate immune system; complement proteins are free-floating in plasma. C1q is the first protein in the cascade; it binds to pathogenic material and triggers binding of c3. Activated c3 can oposonize pathogens and debris (i.e. mark them for consumption by phagocytes) or destroy cells directly by formation of the membrane attack complex (Perry and O'Connor, 2008).

In a widely cited paper (Stevens et al., 2007), mice lacking c1q or c3 were shown to have deficient synapse elimination. In early development in mammals, retinal ganglion cells from both left and right eyes send projections to each lateral geniculate nucleus (LGN) of the thalamus. These overlapping retinogeniculate synapses refine and 'segregate' into eye-specific zones (Shatz, 1983). C1q and c3 null mice failed to develop this normal eye-specific segregation. Together with the previously described studies, these data may point towards an attractive hypothesis whereby inactive or inappropriate synapses are 'tagged' with complement proteins for subsequent elimination by microglia.

In an intriguing extension of this story, further work in Beth Stevens' group showed that microglia internalised synaptic material in both the monocular and binocular regions of the LGN during the peak period of retinogeniculate refinement (Schafer et al., 2012). The amount of synaptic material seen inside microglia could be modulated by changing the level of activity in the retinogeniculate projection. Specifically, blocking activity with tetrodotoxin (TTX) increased microglial phagocytosis while increasing activity with forskolin decreased it. Disrupting complement signalling by knocking out C3 or its receptor, CR3 (which is expressed largely in microglia) produced deficits in eye-specific segregation.

A few caveats should be noted. Firstly, most of the imaging data rely on co-localisation of synaptic components and microglia which can be difficult to reliably establish and interpret (Dunn et al., 2011). Secondly, even if co-localisation (of smoke and gun, as it were) is definitively demonstrated, it remains unclear whether microglial phagocytosis of neuronal components is a primary event or secondary to a more cell-autonomous process which causes synaptic disassembly. In many CNS areas, significant circuit refinement occurs in the absence of microglia. In mouse barrel cortex for example, microglia are excluded until around P5, which co-incides with the completion of the gross cytoarchitectonic structure and the closing of the critical period for activity-dependent plasticity (Arnoux et al., 2013; Crair and Malenka, 1995; Woolsey and Van der Loos, 1970). PU.1 null mice which lack microglia do not demonstrate gross deficits in synaptic pruning (personal

communication, Freda Miller, 2015). It remains difficult to reconcile the relatively small number of microglia with the vast number of synapses undergoing structural plasticity. A *single* Purkinje cell in the cerebellum forms an estimated 7.3×10^6 synapses with granule cells alone, for example (Huang et al., 2014).

In light of these considerations, contact-independent mechanisms of neural regulation by microglia may be particularly critical for their role in orchestrating neuroimmune interaction. Indeed, microglia are known to secrete and respond to a vast array of signalling molecules to orchestrate neuroimmune responses (Kettenmann et al., 2011). These include pro- and anti-inflammatory cytokines such as the interleukins IL1, IL2, IL6, IL10, TNFα, interferon gamma (IFNγ); growth factors such as transforming growth factor beta (TGFβ), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF); and many members of the matrix metalloproteinase (MMP) family.

Release of secreted factors from microglia is an attractive mechanism for neuroimmune interaction as it is one-to-many and can act at a distance, as opposed to contact-dependent mechanisms which are one-to-one or one-to-few. Some of these factors are discussed further in the next section.

In summary, current research suggests that microglia are central co-ordinators of neuroimmune interaction capable of regulating the birth, life and death of neural

circuits in both structure and function. We will now move on to consider other cell types that have been implicated in neuroimmune interaction.

T-lymphocytes are central components of the adaptive immune system. As previously noted, the presence of the blood-brain-barrier and the apparent absence of brain lymphatics had long led to the dogma that T and B cells had no role in the healthy brain. Intriguing recent work, particularly by Michal Schwarz and Jonathan Kipnis is challenging this opinion.

It has been shown that severe combined immunodeficiency (SCID) mice which lack T and B lymphocytes display impaired learning and memory (Filiano et al., 2015; Kipnis et al., 2004b). Cognitive training enhanced T-cell infiltration of the meninges and blockade of T-cells resulted in cognitive impairment. Replacing T-cells rescued cognitive skills (Derecki et al., 2010). It should perhaps be noted that this group's intriguing findings on Rett syndrome were not replicated in other labs (see section I.ii. animal models).

Astrocytes are macroglial cells which are amongst the most numerous in the adult CNS. They constitute another major group of cells which have been implicated in neuroimmune interaction. It was traditionally thought that the main functions of astrocytes were homeostatic and supportive, including buffering of electrolytes and pH, recycling of neurotransmitters including glutamate, and controlling blood flow

(Ransohoff and Brown, 2012). Recent work, particularly spear-headed by Ben Barres has demonstrated that astrocytes play much more active roles in regulating the formation, maintenance and function of synapses (reviewed in Clarke and Barres, 2013).

Developmentally, astrocytes are generated from neural precursor cells after neurons. The morphology of each astrocyte remains dynamic, with particular refinement during the period of synaptogensis. A single astrocyte can contact several tens of thousands of synapses. Astrocytes express a very wide range of proteins including ion channels and receptors and are capable of responding dynamically to their environment. They can use both secreted factors and contact-dependent mechanisms to modulate neuronal development and function.

Early evidence that astrocytes modulate synapse formation came from studies of cultured purified rat retinal ganglion cells (RGCs). In the absence of astrocytes, these cultured cells formed very few synapses. The addition of astrocytes or astrocyte-conditioned medium to the cultures strongly induced the number and strength of new synapses (Barres et al., 1988; Meyer-Franke et al., 1995).

Several astrocyte-derived factors have been implicated in excitatory synaptogenesis. Among these secreted factors are apolipoproteinE bound to cholesterol (Mauch et al., 2001); thrombospondins(Christopherson et al., 2005) and members of the

secreted-protein-acidic-and-rich-in-cysteine (SPARC) family of matricellular glycoproteins (Jones et al., 2011).

Astrocyte-derived factors may also play a role in maturing newly formed synapses. As synapses mature, there is increased expression and clustering of the receptors that mediate fast ionic responses to the neurotransmitter glutamate (known by the name of their specific agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors). Indeed, as glutamate is the principal excitatory neurotransmitter, AMPA receptors are the principal mediators of fast excitatory synaptic transmission in the mammalian brain (Anggono and Huganir, 2012). The glypicans are a family of heparan sulphate proteoglycans which are secreted by astrocytes. Applying glypicans to RGC cultures increased surface expression and clustering of AMPA receptor subunits and thus increased the frequency and amplitude of glutamatergic synaptic transmission (Allen et al., 2012).

Contact-dependent mechanisms have also been described. One study, for example, used time-lapse imaging in the hippocampus to show that astrocyte contact was associated with increased synaptic activity and the formation of more excitatory synapses. The mechanism appears to involve astrocytic modulation of integrin receptors on neurons with subsequent activation of the protein-kinase C system (Hama et al., 2004).

Astrocytes express Toll-like Receptors (TLRs) and complement proteins and thus participate in innate immune reactions. Reactive 'astrogliosis': a change in astrocyte morphology with branch retraction and an increase in their number has long been known to be a hallmark of CNS injury (Filous and Silver, 2016). Alongside microglia, astrocytes are the major sources of immune-molecules such as cytokines and chemokines in the CNS.

Again alongside microglia, astrocytes have strong phagocytic capability. Astrocytes in the optic nerve head express the gene galectin-3, also known as Mac-2 – a gene associated with phagocytic capability in microglia and other macrophages. These astrocytes upregulate Mac-2 in response to injury and are able to phagocytose axons and organelles in both pathological and normal conditions (Nguyen et al., 2011).

Several other phagocytic pathways are strongly expressed in astrocytes (Clarke and Barres, 2013). One example is a pathway involving the adaptor protein CRKII, the dedicator of cytokinesis 1 (DOCK1), the engulfment and cell motility protein (ELMO) and the Ras-related C3 botulinum toxin substrate 1 (RAC1). Together, these proteins are involved in controlling the complex modulation of the actin cytoskeleton that is required for engulfment and phagocytosis (Kinchen et al., 2005; Ziegenfuss et al., 2012). A second pathway hinges on the c-Mer tyrosine kinase receptor (MERTK), which interacts with integrins upstream of CRKII, DOCK1 and RAC1 signalling molecules (Wu et al., 2005). Finally, astrocytes express a RAC1-independent pathway that hinges on multiple epidermal growth factor-like domains

10 (MEGF10) (Lööv et al., 2012). As with microglia, such phagocytic capability may be employed in 'waste disposal' roles or more precisely to prune synaptic circuits. Evidence for the latter role remains somewhat circumstantial however the presence of motive, means and opportunity make such speculation highly tempting.

Oligodendrocytes are the myelinating cells of the CNS. Traditionally, they have been considered fairly inert or simply supportive. In inflammatory demyelinating disorders such as multiple sclerosis (MS), where they are attacked, it was thought they were 'passive victims', however immunomodulatory roles for oligodendrocytes have been proposed (reviewed in Zeis et al., 2016). Oligodendrocytes are capable of expressing a range of immune and immune-related molecules such as cytokines, chemokines, MHC molecules and complement proteins and receptors. In a cuprizone demyelination model, for example, adult oligodendrocyte precursor cells (OPCs) were found to strongly upregulate expression of IL-1β (Moyon et al., 2015). Oligodendrocytes and OPCs may be active participants in neuroimmune interaction.

b. Molecular

Pioneering work by Carla Shatz and colleagues revealed one of the first known instances of immune molecules playing roles in CNS development. Unbiased screens for factors involved in activity-dependent synaptic remodelling, surprisingly revealed that immune molecules including proteins of the **Major**

Histocompatibility Complex class 1 (MHC-1) family were strongly induced by changes in activity (Corriveau et al., 1998). MHC-1 receptors including the Paired immunoglobulin-like receptor B (PirB) have also been strongly implicated in CNS development. Mice lacking functional PirB demonstrated more robust cortical ocular dominance plasticity, including plasticity beyond the classical 'critical period' (Syken et al., 2006).

The massive genetic diversity of the immunoglobulin-superfamily and similar proteins has attracted interest as it immediately suggests attractive mechanisms (e.g. homophilic repulsion) for synaptic specification in development. Consistent with this, elegant experiments in drosophila have demonstrated that genetic diversity of the **Down syndrome cell adhesion molecules** (Dscam or DSCAM in vertebrates) is required for neuronal specification (Chen et al., 2006). Although massive molecular diversity at the DSCAM locus does not appear conserved in vertebrates, loss of mouse DSCAM causes arborisation and tiling defects in mouse retina (Kise and Schmucker, 2013). It is now thought proto-cadherins may subserve some of the roles of drosophila Dscam in vertebrates (Lefebvre et al., 2012).

The role of **complement proteins** has been noted above, particularly in work by the groups of Beth Stevens and Ben Barres (see section I.iii.a. microglia).

The inflammosome is "a cytosolic, multiprotein platform that enables the activation of pro-inflammatory caspsases, chiefly caspase-1" (Walsh et al., 2014). Caspase-1 is responsible for cleaving and thus activating pro-forms of inflammatory cytokines, notably Interleukin (IL)-1β and IL-18. First described by Tschopp and colleagues in 2002, this platform thus regulates and directs innate immune responses (Martinon et al., 2002).

The afferent wing of the inflammosome is formed by cytosolic pattern recognition receptors . These are usually members of the nucleotide-binding oligomerization domain-like receptors (NOD-like receptors; NLR) family or the pyrin and HIN-domain (PYHIN) family of proteins. Members of the NLRP family have both NLR and pyrin domains.

When activated by a pathogen, NLRPs oliogmerise and interact with adaptor proteins such as apoptosis-associated speck-like protein (ASC) containing an N-terminal caspase activation and recruitment domain (CARD). Caspases carry corresponding CARDs and can thus be activated downstream of NLRP activation. The precise mechanisms and pathways underlying inflammosome activation are still to be defined and remain an active area of investigation (Latz et al., 2013).

In the CNS, the highest expression of these inflammosome proteins (NLRPs, ASC, CARDs etc.) is in myeloid cells such as microglia and perivascular macrophages

(Guarda et al., 2011; Walsh et al., 2014). Notably however, neurons are also capable of expressing inflammosome components and of mounting IL-1β responses (Compan et al., 2012).

Several **cytokines** have been discussed above, particularly in the sections on ASD, microglia and inflammosomes. Cytokines are a loosely defined category of soluble signalling molecules that play roles in inflammatory and immune processes. They include chemokines (which regulate cell movement),interferons (named because they interfere with viral infection), interleukins (which were thought to involve signalling between leukocytes), lymphokines (between lymphocytes) and tumour necrosis factors.

Although cytokines are traditionally considered immune signals, neurons and glia are also capable of secreting and responding to them through cognate receptors. Cytokines may modulate neuronal survival, structure, function, and proliferation (Boulanger, 2009; Pribiag and Stellwagen, 2014; Vezzani and Viviani, 2015). The evidence for several individual cytokines will be considered below, particularly the three typical or canonical pro-inflammatory cytokines: IL-1β, IL-6 and TNF-α.

The **Interleukin-1 (IL-1) family** is an extensively studied group of cytokines including IL-1 receptor agonists (e.g. IL-1α , IL-1β, IL-18, IL-33), receptor antagonists (e.g. IL-1Ra, IL-36Ra, IL-38), and one anti-inflammatory cytokine (IL-37)

(for detailed reviews see e.g Dinarello, 2009; Garlanda et al., 2013). The role of IL-1β has been and is extensively studied in the CNS. As mentioned above, IL-1β is one of the products of the inflammosome cascade and so represents one of the first immune signals produced in the CNS following pathogenic insult.

The IL-1 receptor type 1 (IL-R1) mediates the action of IL-1β. Both ligand and receptor are expressed at low levels throughout the normal brain and are strongly enriched in post-synaptic fractions of rat hippocampus and cortex (Gardoni et al., 2011; Viviani et al., 2014). The signal transduction accessory protein (IL-1R AcPb) is specifically expressed in neurons and *in vivo* studies have demonstrated receptor expression in brain and spinal cord (Vezzani et al., 2011). Activation of the cytokine receptor can produce changes in neuronal excitability that are rapid-onset (seconds to minutes) and long-lasting (hours to days, weeks or longer) (Galic et al., 2012).

Exome sequencing studies have identified *de novo* synonymous SNPs in the IL-1β decoy receptor IL-1R2 (O'Roak et al., 2011; Sanders et al., 2012) in individuals with ASD. As synonymous SNPs do not alter the amino acid sequence of the encoded protein, they were long thought to be benign or 'silent' mutations. Mounting evidence suggests however that synonymous SNPs can contribute to pathology by a number of mechanisms including dysregulation of messenger RNA (mRNA) splicing, stability and the binding of micro-RNAs (Sauna and Kimchi-Sarfaty, 2011). The IL-

1β decoy receptor normally acts to inhibit IL-1β signalling; loss or impairment of function would therefore increase activity in this pathway .

A number of mutations and deletions in the the IL-1 receptor accessory protein-like 1 (IL1RAPL1) gene have been associated with X-linked intellectual disability and ASD (Estes and McAllister, 2015). The function of this protein in the canonical IL-1 signalling pathway remains to be established (Narayanan and Park, 2015). Interestingly, it appears to have highly pleiotropic effects in the nervous system. The Il1RAPL1 knockout mouse demonstrates deficits in associative learning and synaptic defects including a reduction in the number of dendritic spines (Houbaert et al., 2013; Ramos-Brossier et al., 2015). IL1RAPL1 interacts with PSD-95, a major post-synaptic scaffolding protein, and may regulate its localisation (Pavlowsky et al., 2010). It also interacts with regulators of Rho GTPase such as RhoGAP2 and Mcf2l, and so impinges on pathways for cytoskeletal remodelling (Hayashi et al., 2013). Finally, IL1RAPL1 may interact trans-synaptically with protein-tyrosine-phosphatase delta (PTPδ) to enhance synaptogenesi(Yoshida et al., 2011).

Interestingly, IL-1β may be released from neurons or glia following excitotoxic insult (Allan et al., 2005). Both inhibitory and excitatory effects of IL-1β have been described through actions on voltage- and receptor-gated channels and by modulation of neurotransmitter release properties (reviewed in Vezzani and Viviani, 2015). The precise effects are likely dependent on multiple factors including

the technical approach, the specific neural circuit, basal excitability, dose of IL-1β and the timecourse of application.

Conflicting evidence has also been presented for further roles of IL-1β. It has been reported that it reduces neuronal survival and growth, possibly by interfering with neurotrophin signaling pathways including neurotrophin-3 (NT-3) and BDNF (Soiampornkul et al., 2008; Tong et al., 2008). However, several *in vitro* studies have reported that it can enhance neurite outgrowth, possibly by induction of neurotrophins such as NGF, NT-3/4 or BDNF (Boato et al., 2011; Gölz et al., 2006; Gougeon et al., 2013; Temporin et al., 2008). In vivo studies have not borne these findings out: in a spinal cord injury model, IL-1β applied peri-lesionally reduced fibre outgrowth, while IL-1β knockout mice demonstrated significantly enhanced axonal sprouting and functional recovery (Boato et al., 2013). The authors suggest these contradictory findings may relate to differences in experimental approach or model, the lack of modulatory cells and signals in *ex-vivo* preparations, or may reflect the timecourse of IL-1β responses, with a hyperacute response of increased outgrowth followed by a lasting reduction in sprouting.

Interleukin (IL)-6 is another cytokine strongly implicated in modulating CNS structure and function (reviewed in Gruol, 2015). Exogenous application of IL-6 to acutely isolated nerve terminals from rat neocortex reduced subsequent responses to pharmacological stimulus, suggesting that IL-6 has an inhibitory effect on

synaptic transmission (D'Arcangelo et al., 2000). Conversely, chronic elevation in IL-6 was associated with enhanced NMDA currents and an increase in excitatory synapses (Qiu et al., 1998; Wei et al., 2011).

In studies of long-term potentiation (LTP) and memory, endogenous IL-6 expression was induced in the hippocampus by neural activity and appeared to interfere with or downregulate LTP. Administration of neutralising antibodies to IL-6 enhanced LTP (del Rey et al., 2013).

Tumour Necrosis Factor (TNF or TNF-α) was identified around 40 years ago as a substance released from macrophages in response to endotoxin that caused hemorrhagic necrosis of tumours (Carswell et al., 1975). Because of the marked wasting and shock it induces it was also known as cachectin. The cytokine has complex and pleiotropic signalling cascades based around cognate TNF-receptors that connect to necroptosis (regulated necrosis) pathways among others (reviewed in Berghe et al., 2014; Wajant et al., 2003).

TNF-α has important roles in the healthy and inflamed CNS (reviewed in Probert, 2015). Almost any pathological process in the CNS that involves cell death or degeneration has been associated with elevated levels of TNF-α. TNF-α can be produced endogenously by neurons, astrocytes and ependymal cells in the choroid plexus (Breder et al., 1993). Microglia are a particularly rich source (Kettenmann et

al., 2011; Sawada et al., 1989). In the mouse, a specific and saturable system has been identified which transports TNF-α across the blood-brain-barrier (Gutierrez et al., 1993).

Landmark studies by David Stellwagen, Robert Malenka and others implicated TNF-α as regulators of excitatory transmission in the CNS under normal conditions. Homeostatic synaptic scaling is a form of plasticity in which the strength of all synapses on a cell are uniformly adjusted in response to prolonged changes in activity levels. In mouse hippocampal slices, TNF-α was necessary and sufficient to induce scaling up of synaptic responses after prolonged activity blockade (Stellwagen and Malenka, 2006).

Constitutive TNF-α may be important for maintaining normal synaptic function . Blockade or deletion of TNF appears to reduce surface levels of AMPA receptors in cortex, hippocampus and motor neurons (Ferguson et al., 2008; He et al., 2012; Stellwagen et al., 2005). Conversely, TNF application reduced inhibitory surface GABA receptors on hippocampal neurons (Pribiag and Stellwagen, 2013; Stellwagen et al., 2005) and reduced surface AMPA receptors on inhibitory striatal neurons (Lewitus et al., 2014).

Interestingly, TNF-α may also regulate the activity of other cell types such as astrocytes. A provocative study demonstrated that vesicular release of glutamate

from astrocytes (gliotransmission) was dependent on TNF-α (Santello et al., 2011). The existence of gliotransmission by active vesicular release as a *bona fide* biological phenomenon remains controversial however (Sloan and Barres, 2014).

Neurotrophins including NGF and BDNF were discovered as factors which promote neuronal survival and growth, however evidence is accumulating that they can act as bridges between neuroinflammation and synaptic plasticity (reviewed in Calabrese et al., 2014; Linker et al., 2009).

BDNF is known to have pleiotropic roles in neuronal growth and activity. Expression of BDNF and its receptors is not limited to neurons or even to the CNS. Microglia strongly express BDNF, as do immune cells such as thymocytes, T-cells, B-cells, monocytes and eosinophils (Linker et al., 2009). The functions of BDNF in the immune system remains largely to be elucidated but may involve regulation of immune-cell survival and maturation.

The role of microglia-derived BDNF has attracted particular attention and appears to be critical in the development of neuropathic pain, likely by modulation of chloride flux (reviewed in Ferrini and De Koninck, 2013). Recent work demonstrates that microglial BDNF promotes learning-dependent synapse formation (Parkhurst et al., 2013).

Matrix metalloproteinases (MMPs) are a family of zinc proteases that are increasingly implicated in CNS function and plasticity. Particularly strong evidence has been accumulated for MMP-9, which I will focus on here (recently reviewed in detail by Vafadari et al., 2016). The original identification of MMP-9 was as an 'inflammatory' MMP released by activated leukocytes; its expression is strongly induced in inflammatory states and is necessary for the induction of experimental autoimmune encephalitis (EAE), an animal model of MS (Vandooren et al., 2014). In addition to leukocytes, MMP-9 is expressed by neurons and glial cells: notably microglia.

MMP-9 is typically released into the extracellular space where it has heterogenous substrates include matrix proteins, growth factors, cell-surface receptors and cell adhesion molecules (CAMs). As well as physically loosening peri-neuronal nets, such extracellular enzymatic activity may release bound signaling factors to further modulate synaptic function, plasticity and metaplasticity. Roles for MMP-9 have been described in dendritic remodelling, long-term potentiation, learning and memory and responses to enriched-environment conditioning (Tsilibary et al., 2014).

Nitric oxide (NO) is a gaseous signalling molecule that was originally described as a vascular endothelial-derived relaxation factor for smooth muscle. Much work has now demonstrated roles for NO as a modulator of neuronal structure and function

(reviewed in Calabrese et al., 2007; Hardingham et al., 2013). NO is produced from L-arginine by Nitric oxide synthase (NOS) which has three main isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). NOS isoforms are expressed by a wide array of cells including immune cells and microglia. Notably, iNOS is strongly induced by inflammatory signals such as pro-inflammatory cytokines.

NO has been demonstrated to act in both anterograde and retrograde directions to modulate synaptic formation and function. Pre-synaptically, it may modulate release of neurotransmitters including GABA, glutamate, acetylcholine and dopamine (Cossenza et al., 2014). Inhibition of NO signalling has been demonstrated to produce aberrant neural circuit formation in the retinotectal formation of the chick, mouse and ferret (Cramer and Sur, 1999; Wu et al., 1994).

NO is also capable of modulating the stress axis by altering release properties of or sensitivity to factors such as corticotropin-releasing hormone (CRH), adrenocorticotropin releasing hormone (ACTH) and anti-diuretic hormone (ADH) (Calabrese et al., 2007).

Finally, NO has been described as both a positive and negative regulator of adult neurogenesis (Gray and Cheung, 2014). The disparity may relate to the enzymatic

isoform: nNOS appears to inhibit proliferation while eNOS and iNOS enhance it (Chen et al., 2005; Sun et al., 2005).

I.iv. Objectives, hypotheses, experimental approach, rationale

As we have seen, neuro-immune interaction is a fascinating and clinically-relevant field with many outstanding questions. If MIA can perturb neural development, what specific effects does it induce on neural circuit formation, structure and function? Which key mechanisms are involved which might be amenable to clinical intervention? Would it be possible to arrest or even reverse such effects?

This thesis aims to answer some of these questions with in vivo experimental approaches.

Specifically, I will induce inflammation in the larval zebrafish (see next section for details of experimental model). Previous studies have demonstrated that it is straightforward to induce inflammation in this model, for example by adding lipopolysaccharide (LPS) to the fish water (Novoa et al., 2009). This is certainly a cruder technique than inducing a viral or bacterial infection but it can be justified. Firstly, the convenience of the approach facilitates relatively high throughput studies. Secondly, a direct and powerful inflammatory stimulus is more likely to produce significant observable changes in circuit structure and function. Thirdly, in a wide range of animal models, systemic inflammation (for example by LPS

administration) produces strong immune responses in the CNS including microglial activation (reviewed in Hoogland et al., 2015). Fourthly, as mentioned above, epidemiological studies have demonstrated that both bacterial and viral infections in pregnancy can be associated with an increased probability of ASD in the offspring (see section I.ii. epidemiology). Moreover, there are strong commonalities in effects produced experimentally with viral or bacterial stimuli, suggesting that the critical step may be activation of the innate immune system (section I.ii. animal models).

Much of the currently available data on the effect of immune activation on neural circuits uses electrophysiology, for good reason. This is an extremely powerful technique to examine the functional properties of cells and circuits. In order to approach the problem from a different angle and to maximise the advantage of the zebrafish system, this project will primarily use *in vivo* imaging. I will genetically target specific cell populations (see next section) and fluorescently label individual or sparse cells throughout their development. I can then observe these cells either at high temporal frequency (near video rate) for short periods or serially over days. I will then visualise and quantify aspects of axonal arborisation including rates of branch addition/retraction and overall arbor size, obtaining detailed metrics of neuronal development in the presence and absence of inflammation. To my knowledge, this will be the first attempt to visualise the effects of inflammation on a developing neural circuit *in vivo.*

If applying an inflammatory stimulus such as LPS to the larval zebrafish does dysregulate neural development, what are the mechanisms involved? As shown in the introduction, several cellular and molecular targets stand out as prime suspects. The role of microglia is likely crucial in regulating any form of neuroimmune interaction. I will examine their location during these experiments and observe them for morphological changes consistent with activation. If microglia do indeed physically 'prune' synapses in development and if this process occurs excessively in pathological situations, the zebrafish system would allow us to observe the whole process in real time. Further, we can use genetic or pharmacological approaches to disrupt microglial development or function and observe whether they are necessary to mediate effects of inflammation on neurons.

As described above, molecular candidates to mediate neuroimmune interaction include the canonical pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and the neurotrophic factor BDNF. I will perform quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiments to establish whether and how these factors are regulated in response to inflammatory stimulus in the zebrafish model. By establishing the time-course of their regulation, we will be in a better position to identify key regulators of the phenomena we observe. An ideal approach would be an unbiased screen (e.g. using whole-transcriptome next-generation sequencing) to identify *all* factors that are regulated by an inflammatory stimulus. This approach is however less suitable for exploratory experiments as it is limited

by the time and expense necessary to analyse multiple time-points. If exciting results are obtained and no candidate is identified from initial qRT-PCR, it would be a possible next step. Candidate molecules can be knocked down by morpholino or applied in recombinant form to assess their contribution to circuit development and pathology.

I hypothesise that inflammation will dysregulate neurite arborisation and that these effects are mediated primarily by microglial cells. (Their critical role was examined in section I.iii.a above). Further, I hypothesise that ablation of microglia or of specific pro-inflammatory cytokines (see section I.iii.b) that they release will prevent the deleterious effect of inflammation on circuit formation.

I.v. Experimental model

"Sometimes [tax] dollars go to projects that have little or nothing to do with the public good. Things like fruit fly research in Paris, France. I kid you not." - Sarah Palin (Enserink et al., 2008)

There is no 'right' or 'best' model to use to explore broad questions of neuro-immune interaction; complementary approaches shed light on different aspects of the problem. *In vitro* studies can be highly informative and permit the use of tissue from humans or other higher mammals, potentially closer to clinical scenarios and translation. Nonetheless, there is a necessary trade-off between experimental tractability and clinical relevance. Some extremely tractable and powerful systems, such as fruitfly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*) or

yeast (*Saccharomyces cerevisiae*) are often somewhat removed from clinical questions and may be more suited to fundamental research. The mouse model (*Mus musculus*) is highly tractable and has obvious mammalian relevance but mouse work is costly and low through-put; development occurs *in utero* which makes live-imaging very challenging. For these studies, the zebrafish (*Danio rerio*) model represents a 'sweet spot'.

Use of the zebrafish as an experimental model was pioneered by George Streisinger in the 1960s who was particularly interested in genetics and neurodevelopment (Wyatt et al., 2015). As a vertebrate, the zebrafish shares many fundamental genetic and developmental pathways with humans. It is also highly experimentally tractable. In a well-established facility, fish are easy to breed and produce large numbers of eggs with frequent spawning (Lawrence, 2011). Zebrafish genetics facilitate knockdown or over-expressions studies; pharmacological agents or toxins can easily be added to rearing solution for *in vivo* exposure; the larvae develop rapidly and externally and their transparence at early stages allows excellent *in vivo* imaging (Haesemeyer and Schier, 2015; Kalueff et al., 2014).

Advances in imaging such as multi-photon microscopy now permit the visualisation of neuro-immune interaction in real time in live, intact organisms (Tang et al., 2013). This neatly sidesteps the dilemma of immune modulation which may be

induced by experimental procedures themselves, such as the creation of skull windows, acute brain slices or cellular dissociation and culturing.

In this section I will briefly introduce some relevant aspects of the zebrafish model: specifically, the retino-tectal system and the immune system.

Retino-tectal system. The question of how precise synaptic connections are formed in the brain is a fundamental one for modern neuroscience. Because of its experimental accessibility and its elegant topographic map, the zebrafish retino-tectal system has become a paradigmatic model in which to study neurogenesis, axonal pathfinding, synaptogenesis, synaptic refinement, activity-dependent remodelling and other aspects of structural-functional circuit formation and maturation (reviewed in Kita et al., 2015).

The output cells of the Zebrafish retina: the retinal ganglion cells (RGCs) differentiate between 29 and 34 hours post-fertilisation (hpf) and begin to project their axons out of the retina at around 36hpf (Stuermer, 1988). Most of these RGCs project towards the optic tectum, although they have at least nine other arborisation fields. In wildtype animals, all RGCs cross the midline and innervate the contralateral tectal neuropil, forming a retinotopic map. RGCs with their cell bodies on the nasal side project caudally and temporal RGCs project rostrally. The first functional synapses are formed by around 72 hpf. By 6-7 days post-fertilisation

(dpf) the visual circuit is essentially functional and growth of RGC arbors reaches a plateau (Ben Fredj et al., 2010; Meyer and Smith, 2006). Heroic experiments, largely in goldfish by Pamela Raymond, Claudia Stuermer and Stephen Easter demonstrated that continuing growth of the retina concentrically is parallelled by tectal expansion caudally and a continuous rearrangement of retinotectal connections to maintain topography (Cerveny et al., 2012; Easter and Stuermer, 1984; Raymond, 1986)

Work particularly by Herwig Baier's group has demonstrated that the tectum is arranged in an intricate stack of highly-organised laminae which likely subserve different functions and represent multiple superimposed topographic maps (Robles et al., 2013; Robles and Baier, 2012). RGC axons remain remarkably planar to permit laminal segregation.

It has been proposed that in mammals such as chicken and mouse, retinal projections and circuits form by overshoot and 'selective/biased branching', whereas in fish and frogs, the mechanism is 'directed growth' to the target area (McLaughlin and O'Leary, 2005). Indeed, initial studies suggested that zebrafish RGC axons grow directly to the target zone and subsequently arborise (Kaethner and Stuermer, 1992).

Although fish and frog RGC axons do not demonstrate the florid overgrowth and pruning seen in mouse and chick, time-lapse imaging suggests strong commonalities

in circuit formation in mammals and non-mammalian vertebrates. Zebrafish RGC axons continually extend and retract branches throughout pathfinding; some branches are tipped by growth cones which move primarily in straight trajectories without turning (Simpson et al., 2013). Branches which form in the direction of the target are maintained and those which do not are withdrawn. In fish and frogs, synaptogenesis and synaptic maturation play critical roles in the stabilisation of axon branch dynamics (Meyer and Smith, 2006; Ruthazer et al., 2006).

Christiane Nüsslein-Volhard and others harnessed the power of zebrafish genetics in the first major example of a vertebrate forward genetic screen: 'the Tübingen screen'. As part of this screen, hundreds of gene loci potentially involved in development of the retino-tectal circuit were identified (Baier et al., 1996). Work to delineate these effects continues.

Early stages of retinotectal circuit formation depend on chemogenetic cues, primarily gradients of ephrins and Eph receptors which help instruct medio-lateral and rostro-caudal mapping (reviewed in Kita et al., 2015; Klein and Kania, 2014).

Initial axon pathfinding and retinotopic organisation may appear grossly normal even in the complete absence of activity (Kaethner and Stuermer, 1992). A number of studies, not least by Edward Ruthazer and colleagues, have demonstrated that

neural activity is subsequently critical for circuit formation and refinement in fish and frogs (reviewed in Ackman and Crair, 2014; Okawa et al., 2014).

Zebrafish immunity. The last decade or so has seen a flood of laboratories using zebrafish to study the immune system, for many of the reasons noted above (reviewed in Oosterhof et al., 2015; Rauta et al., 2012; Renshaw and Trede, 2012). Fish immunity is a burgeoning school of research and can spawn vivid insights into evolution, human immunity and disease – and much dubious punnery in the headings of journal articles.

As vertebrates, zebrafish have many cellular and molecular immune pathways which are highly conserved through to mammals. These include pathways of the innate immune system such as TLRs, C-reactive protein, complement, macrophages and Natural-Killer-like cells. The adaptive immune system evolved around 450 million years ago and components such as T and B-lymphocytes and immunoglobulins are also present in zebrafish. Finally, zebrafish express a range of MHC molecules, chemokines and cytokines: TNF, interleukins, TGF, interferons and others (Rauta et al., 2012).

Bacterial lipopolysaccharide (LPS) can evoke strong immune responses in zebrafish with induction of pro-inflammatory cytokines, although high concentrations are

needed (Novoa et al., 2009; Yang et al., 2014). A high threshold may protect fish from mounting immune responses to debris in the water. LPS is detected by TLR-4 in mammals; interestingly signalling appears to be TLR-4 independent in zebrafish and may instead be mediated by TLR-1/2 (Zhang et al., 2014).

Rather than being orthologous genes (that is, genes which evolved from a common ancestral gene and maintained the same function), mammalian and zebrafish TLR-4 may be paralogous (evolved from a common ancestor but diverged in function) (Sullivan et al., 2009). Although the specific TLR implicated in LPS-response may be different to that in mammals, the strong similarities in the evoked immune response and the great degree of experimental tractability mean that the zebrafish remains a useful and powerful model of immunity (Renshaw and Trede, 2012).

Microglia have also been studied in zebrafish. As in mammals, they derive from yolk-cell progenitors which migrate into the CNS early in development. These macrophage-progenitors differentiate by around 18hpf and invade the head mesenchyme at 23-30hpf. Colonisation of the brain begins by 35hpf; from 48hpf onwards a particular concentration of amoeboid macrophages is noted at the optic tectum. At 60-72hpf these cells undergo a phenotypic transformation, becoming more ramified and upregulating expression of apoE (Herbomel et al., 2001, 1999). Francesca Peri and Christiane Nüsslein-Volhard generated a transgenic zebrafish line expressing green fluorescent protein (GFP) under the apoE promoter to visualise the behaviour of microglia *in vivo* (Peri and Nüsslein-Volhard, 2008). The

macrophage-expressed gene 1 (mpeg1) has also been used as a promoter to label microglia. The cells appear to 'morph' between more amoeboid and more ramified states between 3 and 10 days post-fertilisation (dpf), at which point they remain highly ramified, similar to mature microglia in mammals (Svahn et al., 2013).

The PU.1 gene has been shown to be necessary for specification of the myeloid lineage. Knockdown of PU.1 by injection of a morpholino oligonucleotide at the 1-cell stage effectively prevents differentiation of macrophages and their invasion of the CNS, at least for as long as the morpholino is effective (Rhodes et al., 2005). This provides a very useful approach to study the contribution of microglia to any inflammatory or developmental process.

In sum then, the zebrafish is an outstanding and well-characterised model with a wealth of experimental tools to study neuroimmune interaction in CNS development *in vivo.*

RESULTS – Contributions

Text was written by Nasr Farooqi with corrections and suggestions from Dr. Edward Ruthazer, Dr. Leanne Godinho and Dr. David Stellwagen. Experiments were conceived and designed by Nasr Farooqi and Dr. Edward Ruthazer with input from Dr. Jack Antel.

All experiments were performed and analysed by Nasr Farooqi with the following exceptions: Control morpholino experiments (Figure 9): some cellular reconstructions for these experiments were performed by Phil Kesner.

All figures were created by Nasr Farooqi with corrections and suggestions from Dr. Edward Ruthazer and Dr. Jack Antel. Dr. Edward Ruthazer and Dr. Jack Antel supervised the project.

II. RESULTS

II.i. Microglia are excluded from tectal neuropil during circuit refinement

Microglia are critical regulators of neuro-immune interaction (see introduction). We were interested in the exciting proposition that they contribute to circuit refinement by physically pruning synapses. Our first question was: do microglia physically interact with the retino-tectal projection under normal conditions?

The area in which retinal ganglion cell (RGC) axons synapse with dendrites of peri-ventricular neurons in the optic tectum is known as the tectal neuropil. Formation and refinement of the retino-tectal circuit occurs within the tectal neuropil during the period 3-6 days post-fertilisation (dpf). We examined the distribution of microglia in the zebrafish during this period. Previous reports using vital dye staining and genetic labelling of microglia have suggested that at this stage, microglia do not enter the tectal neuropil (Herbomel et al., 1999; Svahn et al., 2013).

Consistent with this, we performed static and time-lapse *in vivo* imaging of 4dpf zebrafish larvae from the ApoE:GFP transgenic line in which microglia express GFP (Peri and Nüsslein-Volhard, 2008).We found that microglia congregate in the tectal cell body area without entering the neuropil (**Fig. 1A**). This suggests that under normal conditions, physical microglia-synapse contact is not a major contributor to circuit refinement in this system.

Interestingly, analagous results have been demonstrated in mouse barrel cortex. The barrel cortex is a region of somatosensory cortex which receives projections from the thalamus. Each

whisker barrel receives thalamic input corresponding to a single whisker (vibrissa) (Woolsey and Van der Loos, 1970). The formation and refinement of barrels is a classic model of circuit formation, refinement and plasticity (Margolis et al., 2014). Hoshiko and colleagues found that microglia are excluded from the barrels until at least P5 at which point the basic formation of the circuit has occurred. Although further refinement in barrel cortex continues, particularly at around 2 weeks post-natally when active whisking begins, P3 is generally considered the closing of the 'critical period' for barrel plasticity(Vitali and Jabaudon, 2014). Microglial entry may be excluded in early development to prevent disturbance of fine structural refinement (Hoshiko et al., 2012).

As the neuropil is a densely packed area, it is possible that microglia are simply physically excluded by steric hindrance. Alternatively, they may be excluded by repulsive signalling molecules. To ascertain this, we induced laser injury in the tectal neuropil and observed microglial responses *in vivo*.

Interestingly, in this situation microglia are able to translocate into the injured area within minutes (**Fig. 1B**). They also appear to abandon their usual 'tiling' self-avoidance and tightly cluster at the site of injury. Our results demonstrate that during the major period of retino-tectal circuit formation and refinement, microglia are capable of entering the tectal neuropil but do not do so under normal conditions. These results are consistent with the report that PU.1 null mice which lack microglia do not display gross deficits in early neural circuit formation (personal communication, Freda Miller). It remains possible that their role is much more pronounced in pathological situations or that they act by contact-independent mechanisms.

Figure 1

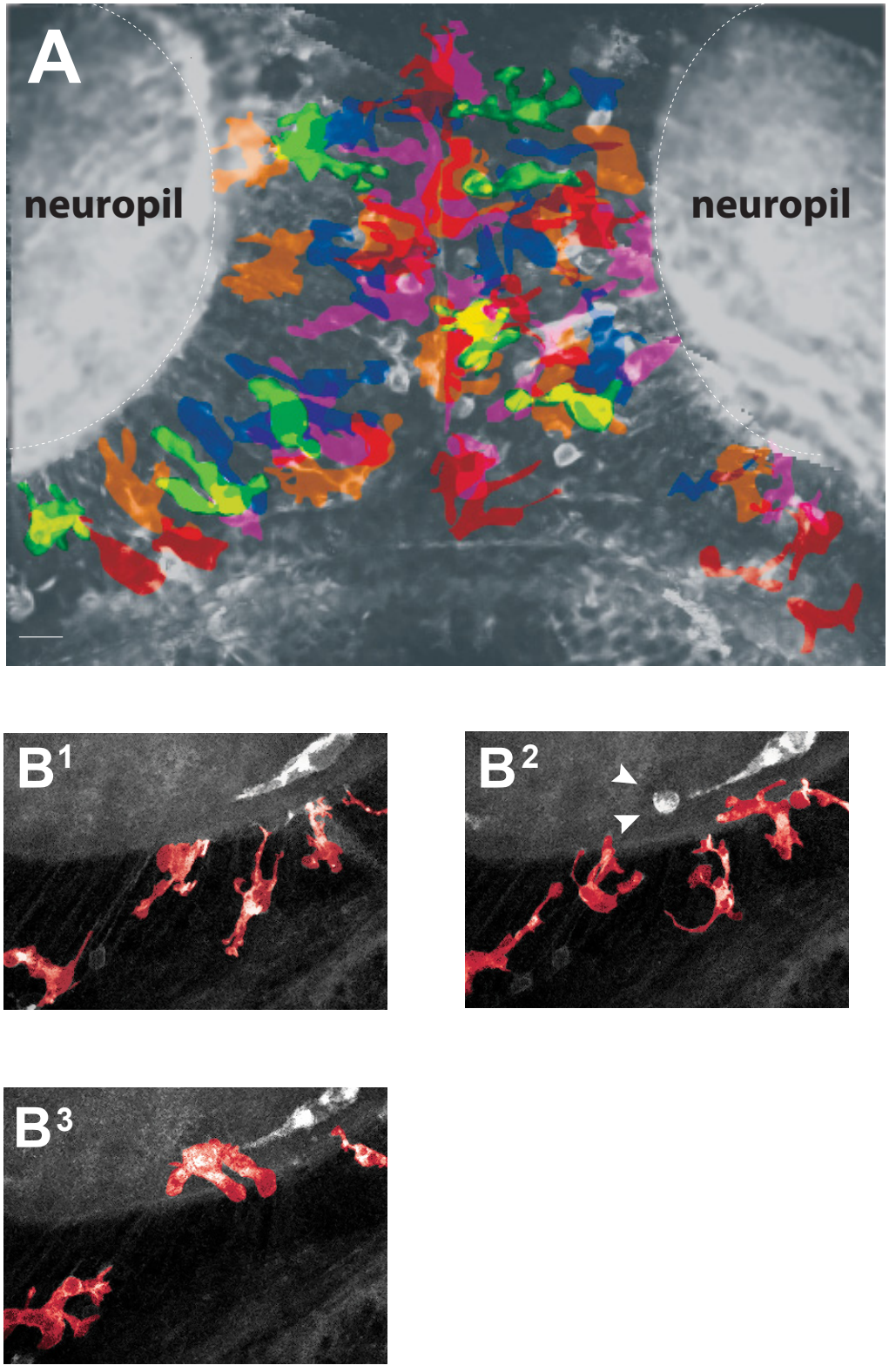


Figure 1: Microglia are excluded from the tectal neuropil

(**A**) Microglia do not enter the tectal neuropil under normal conditions. Representative superimposed images of tectal microglia from five ApoE:GFP zebrafish larvae at 4dpf. Each colour represents microglia from one animal. Note absence from the marked neuropil area

(**B**) Time-lapse *in vivo* imaging of microglia at tectal neuropil border before (B1) and immediately following laser injury (white arrows B2). Within 20 minutes, microglia have migrated to the injury site within the neuropil (B3). Microglia pseudocoloured.

II.ii Morphological activation of microglia by LPS

Although microglia are not present in the neuropil area during retino-tectal circuit formation and refinement, we wished to know whether they can be activated by an inflammatory stimulus. We bath-exposed larval zebrafish to bacterial lipopolysaccharide (LPS) diluted in E3 medium for two hours to trigger an innate inflammatory response (Novoa et al., 2009). Pilot experiments were performed to establish that an effective, sub-lethal dose was 25 μg/ml. Pilot experiments also demonstrated no difference with or without the use of dimethyl sulfoxide (DMSO) which is used to permeabilise the animals to chemical treatments. DMSO was therefore not subsequently used. Control animals were treated in exactly the same way, without LPS added to the E3 solution. The experimenter was blind to treatment condition.

We visualised microglia in the ApoE:GFP transgenic line generated by Francesca Peri and colleagues. Previous reports demonstrated that Apolipoprotein-E is a strong marker for zebrafish microglia (Herbomel et al., 2001). Expression of the green fluorescent protein (GFP) under the ApoE promoter labels a cell population in the CNS that is consistent in morphology and behaviour with known features of microglial cells (Peri and Nüsslein-Volhard, 2008). Notably transgenic lines that have been developed since using other promoters such as macrophage-expressed-gene-1 (MPEG-1) label a cell population of comparable number and features, raising confidence that the ApoE line represents the full complement of microglia (Svahn et al., 2013).

We performed *in vivo* two-photon microscopy and quantified number of processes on microglial cells before and after LPS treatment. This provides a metric of morphological complexity.

Microglia exhibit a spectrum of morphologies and activation states, ranging from ramified-surveillant to amoeboid-activated (Kettenmann et al., 2011; Svahn et al., 2013) (**Fig. 2A-B**). Two hours of LPS exposure induced microglia to adopt more amoeboid morphologies consistent with immune activation (**Fig. 2C**).

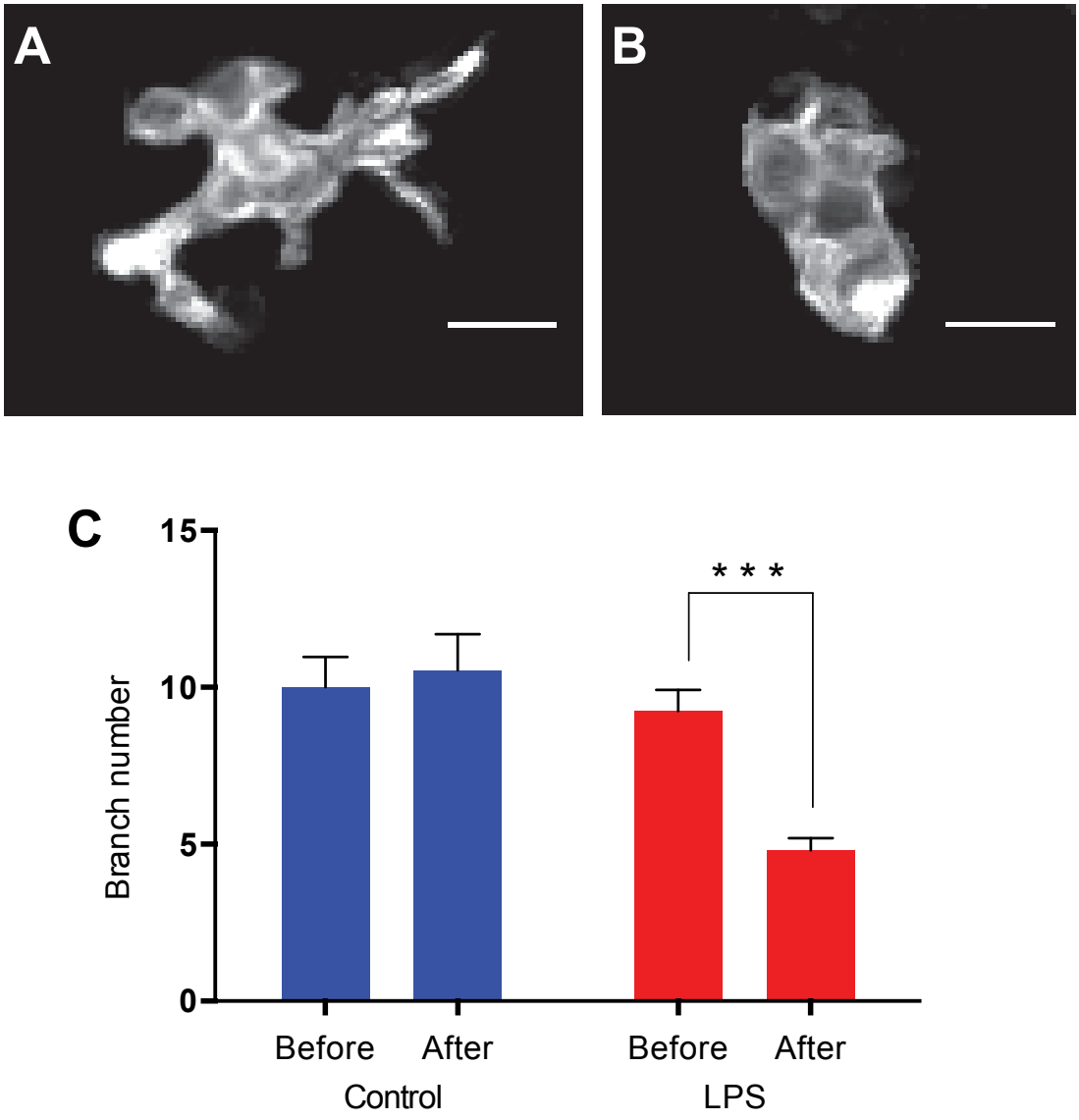
These data demonstrate that although microglia do not appear to directly contact developing retinal ganglion cell axons during development, they are closely apposed to the region in which refinement occurs. Microglia are also highly responsive to systemic immune activation, rapidly assuming an activated morphology. This raises the possibility that they are playing a contact-independent role to modulate developing circuits, for example by secreting soluble immune factors.

Figure 2 - Microglia assume an amoeboid morphology following LPS exposure, consistent with activation.

Microglia visualised in ApoE:GFP transgenic line at 3dpf. Representative images of typical morphology (**A**) before or (**B**) after 2 h bath exposure to LPS at 3 dpf. Scale bar 10um.

(**C**) Microglial branch number is significantly reduced following LPS exposure (n=53 cells from 4 animals (control), 91 cells from 7 animals (LPS)). Two-way ANOVA with Bonferroni correction. *** $p<0.001$.

Figure 2


A
B
C
15
10
5
0
Branch number
* * *
Before
After
Before
After
Control
LPS

II.iii LPS acutely upregulates axonal arborisation

To determine the effects of LPS on axonal arborisation in development, we performed one-cell stage plasmid injection to sparsely express fluorescent proteins under the RGC-specific promoter Brn3c identified by Herwig Baier and colleagues. Expression under the Brn3c promoter sequence labels a subset of retinal ganglion cells (RGCs) that project primarily to the *stratum fibrosum et griseum superficiale* (SFGS) layer of the tectum (Xiao et al., 2005). Distinct subsets of RGCs have distinct morphologies and projection zones and may subserve different functions in the visual system (Robles et al., 2013, 2011). By restricting our analysis to Brn3c RGCs, we aimed to minimise the degree of normal variation between RGCs in our different treatment conditions.
To obtain strong expression of our fluorescent constructs, we took advantage of the Gal4/UAS system which has been adapted for zebrafish (reviewed in Asakawa and Kawakami, 2008). Gal4 is a yeast transcriptional activator involved in galactose metabolism. When expressed, it binds to an upstream activating sequence (UAS) and strongly induces expression of genes downstream of the UAS. A major advantage of this system is the ability to generate 'driver' lines, in which expression of the Gal4 is linked to specific promoters. When crossed with expression lines containing the UAS and a reporter, specific expression is obtained. Here, although we did not create a driver line per se, we took advantage of the strong and specific transcription induced by the Gal4-UAS system.
We performed time lapse two-photon imaging of individual RGC axon arbors (every 6 min for 36 min at 3 dpf) and digitally reconstructed arbors to obtain 4-dimensional morphological data **(Fig. 3A-B)**.

The analysis was repeated after 2 h exposure to LPS or control solution. Comparison of the two epochs of imaging demonstrates that acute exposure to LPS increases rates of axonal branch addition and branch retraction (**Fig. 3C**).

Remarkably then, these data demonstrate that a brief exposure to an inflammatory stimulus has very rapid effects on axonal dynamics. Interestingly, *in vivo* imaging of dendritic spines in adult mice also demonstrated increased rates of turnover following LPS treatment (Kondo et al., 2011).

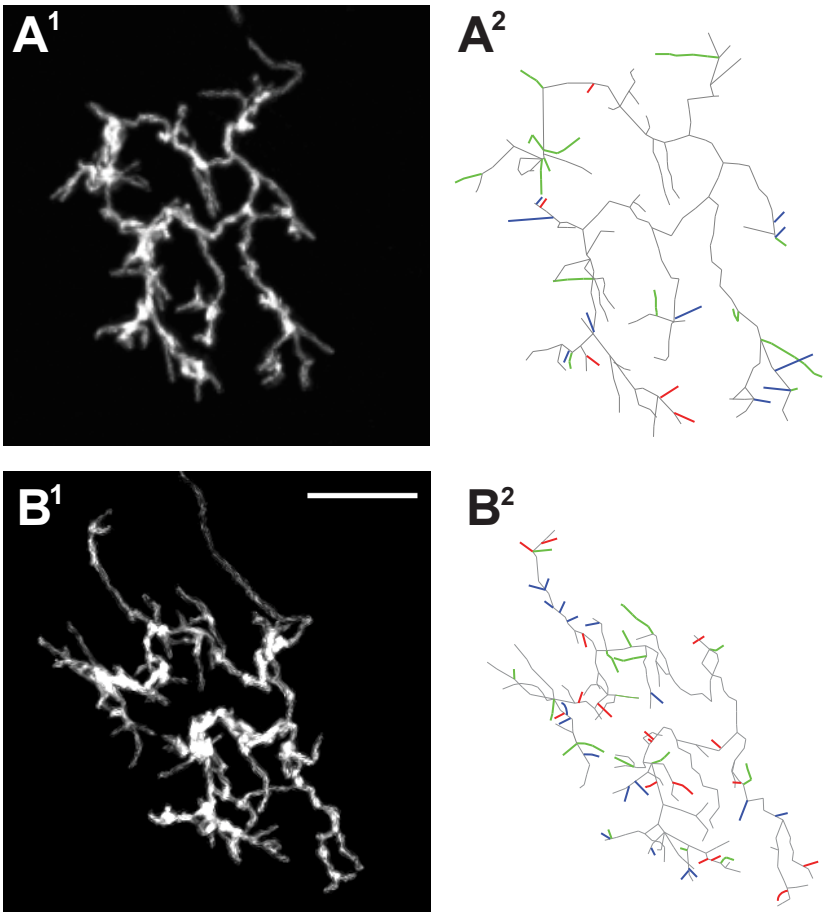
Figure 3 - **LPS acutely upregulates axonal arborisation dynamics**

(**A**) Time-lapse *in vivo* two-photon microscopy of individual RGC axon in 3 dpf zebrafish before and (**B**) after 2 h exposure to LPS.

Arbors are shown as projected z-stacks and as reconstructed skeletons. Branch additions during 36 min imaging period are marked in green, retractions in red and transient branches in blue.

(**C**) Rates of branch addition and branch retraction are significantly increased following LPS exposure (n=6 Control; 6 LPS).

Figure 3


A1
A2
B1
B2
C
Control vs LPS
**
*
Branch additions (% of baseline)
Branch retractions (% of baseline)
200
150
100
50
0
Control
LPS

II.iv Single LPS exposure reduces total arborisation over days

If LPS acutely increases axonal dynamics several questions immediately arise. Are these effects persistent or transient? Is overall growth increased or decreased? How is synaptogenesis affected? To examine the lasting consequences of LPS treatment we modified our experimental approach and used a different fluorescent reporter construct. The dsRed-synaptophysinGFP construct developed by Martin Meyer and colleagues is ideal for our purposes. The dsRed outlines overall arbor structure while the GFP-tagged synaptophysin enables visualisation of synaptic puncta. Both constructs are expressed on the same plasmid for convenience and efficiency (Meyer and Smith, 2006)(Meyer and Smith, 2006a)(Meyer and Smith, 2006).

We injected the plasmid construct along with the Brn3c:GAL4 driver at the one-cell stage and allowed animals to develop normally to 3dpf. At this point the RGCs were imaged once and the animals were gently dissected from their agarose mounting blocks. They were then treated with LPS or control solution as previously and re-imaged daily up until 6dpf.

Although acutely LPS had upregulated branch dynamics, surprisingly we found that a single 2 h LPS exposure at 3 dpf resulted in smaller arbors over days 4-6 post-fertilisation, corresponding to the main period of arbor elaboration (**Fig. 4, Fig. 5a**). These arbors also had fewer synaptophysin-positive pre-synaptic punctae than control-treated arbors (**Fig. 5b**). Finally, the number of branches was lower following LPS treatment, showing that the RGCs failed to arborise to the normal degree of complexity (**Fig. 5c**).

Our results initially appear paradoxical. Initial hyper-dynamic motility is followed by reduced arborisation. The data showing a reduced overall number of pre-synaptic punctae suggest a possible answer. High dynamics of RGCs: extending and retracting branches at an abnormally high rate may be a cause or consequence of reduced synaptic stability. In zebrafish larvae and Xenopus laevis tadpoles, synapse formation has been shown to be critical for branch stabilisation and elaboration. Fine branches which are not stabilised by synapse formation retract and disappear (Meyer and Smith, 2006; Ruthazer et al., 2006).

Inflammation induced by LPS may be interfering with normal synapse stabilisation and thus reducing overall arborisation.

Figure 4. RGC axon structure over days following LPS or Control treatment.

Representative images of single RGC axons expressing synatophysin:GFP and dsRed imaged daily for 4 days following control (**A1-4**) or 2 h LPS (**B1-4**) treatment. Treatment was administered at 3 dpf. Scale bar 10um.

Figure 5. LPS treatment results in smaller, simpler RGC arbors with fewer synapses

RGCs in LPS-treated animals have significantly smaller terminal arbors at 6dpf (**A**). These arbors have significantly fewer pre-synaptic punctae (**B**) and significantly reduced number of branches (**C**) (n= 5 control, 5 LPS). *$p<0.05$, two-way repeated measures ANOVA with Bonferroni correction.

Figure 4

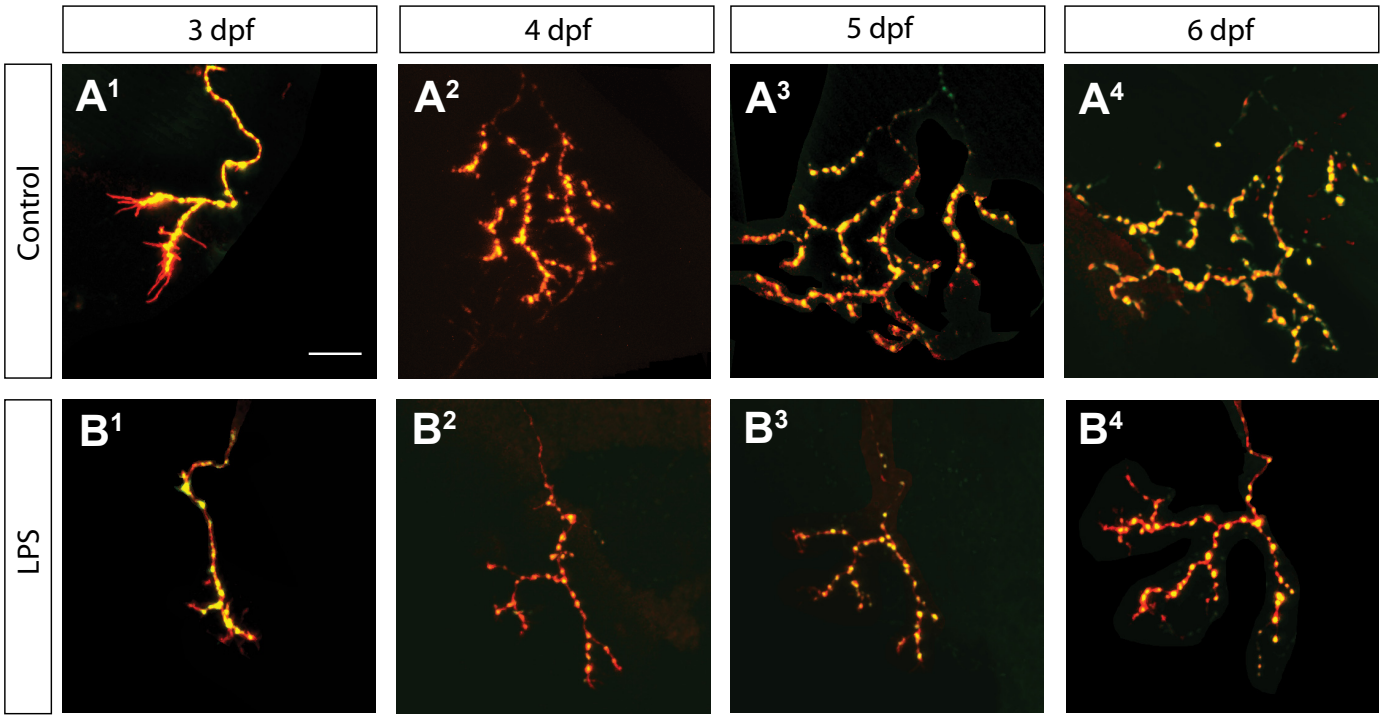
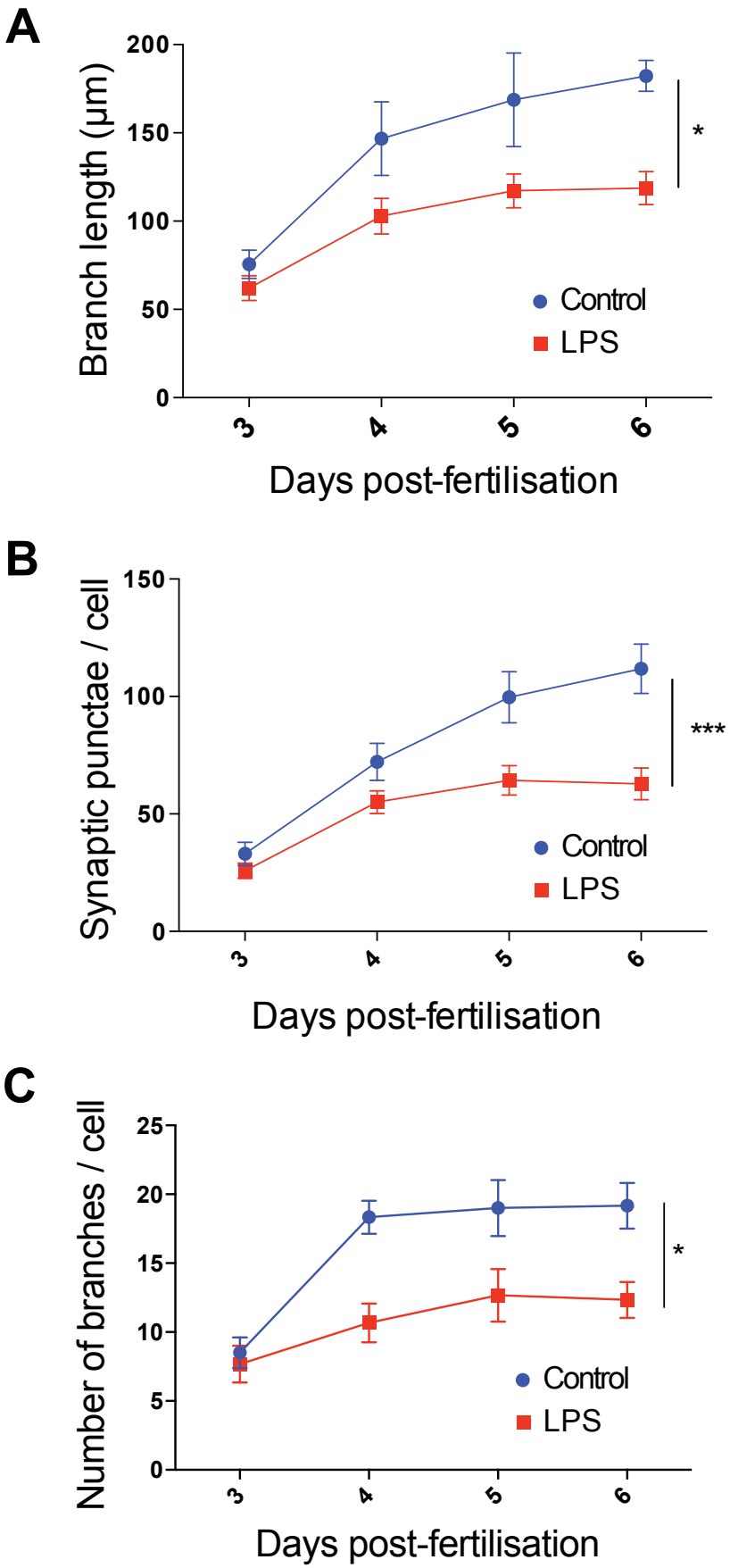


Figure 5 Control treatment vs. LPS (WT animals)



II.v. LPS effects are absent in PU.1 morphant animals lacking microglia

Having established acute and lasting effects of LPS administration, we began to examine some of the mechanisms that underlie these effects. As discussed, microglia are prime candidates as cellular regulators of neuro-immune interaction.

To understand how we manipulated microglia, a little background on their ontogeny is necessary (McGrath et al., 2015). Hematopoiesis is the formation of blood cells. In mammals, hematopoietic stem cells differentiate into common lymphoid and common myeloid progenitors. The lymphoid progenitor gives rise to lymphocytes and natural killer cells. The common myeloid progenitor gives rise to megakaryoblasts (leading to platelets), proerythroblasts (leading to erythrocytes – red blood cells) and myeloblasts.

Myeloblasts differentiate into myelocytes/granulocytes (including basophils, neutrophils and eosinophils) and monocytes (including macrophages and myeloid dendritic cells). Microglia are derived from primitive macrophage progenitors in the yolk sac (Ginhoux et al., 2013).

The PU.1 transcription factor has been identified as critical for hematopoietic specification. Mice lacking the PU.1 gene have multiple hematopoietic abnormalities. Most strikingly they lack monocytes and macrophages in the myeloid lineage and lack B-cells in the lymphoid lineage (McKercher et al., 1996). Erythroid development is spared. PU.1 was also studied in zebrafish. Morpholino knockdown prevents differentiation of monocytes/macrophages and markedly reduces expression of granulocyte markers (Rhodes et al., 2005). As in mouse, the development of other lineages including the erythroid appears to be spared.

We took advantage of the PU.1 morpholino effect to prevent macrophage/monocyte specification, including that of microglia. To confirm the effect, we injected antisense morpholino against PU.1 at the one-cell stage and subsequently imaged ApoE:GFP transgenic animals. Morphant larvae appear entirely deficient in microglia (**Figure 6**).

Figure 6 - Microglia are absent in PU.1 morphants.

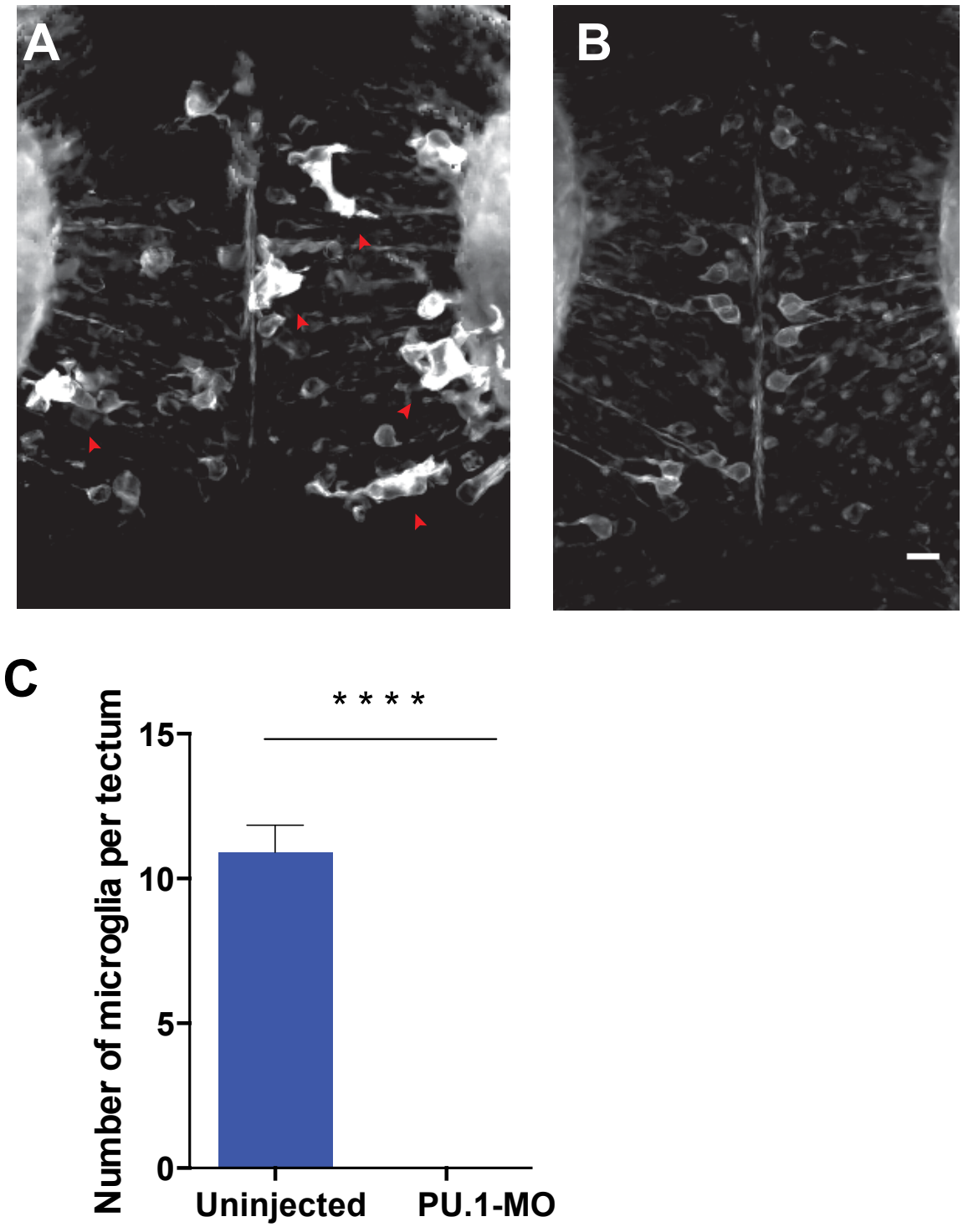
One-cell stage injection of morpholino against transcription factor PU.1 effectively prevents microglial differentiation and colonisation of the tectum.

(**A**) Control uninjected animal with microglia (red arrowheads).

(**B**) PU.1 morphant animals lack microglia. Imaged in ApoE:GFP transgenics. Scale bar 10μm.

(**C**) Uninjected controls have 10.9 ± 0.9 per tectum (n=11 animals), no microglia were seen in PU.1 morphant animals (n=13). Unpaired t-test, **** $p<0.0001$.

Figure 6


A
B
C
* * * *
Number of microglia per tectum
15
10
5
0
Uninjected
PU.1-MO

We then analysed RGC arborisation in PU.1 morphants and found that the effects of LPS were absent in animals lacking microglia.

In the first set of experiments, performing imaging acutely after LPS exposure, the previously observed upregulation of branch dynamics was absent in PU.1 morphants treated with LPS (**Fig. 7A-B**). Instead, arborisation dynamics (rates of branch addition and retraction) were not significantly different from controls. The LPS effect was preserved in animals injected with a scrambled control morpholino, excluding a non-specific morpholino effect **(Fig. 14)**.

These results suggest that the acute effects of LPS on axons require the presence of microglia or other PU.1-specified macrophages.

We went on to examine whether the lasting effects of LPS were also dependent on microglia/macrophages. In multi-day imaging, PU.1 morphants exposed to LPS again developed arbors resembling those of controls that were not exposed to LPS (**Fig. 8**). Total arbor size (**Fig. 8A**), number of synaptic puncta (**Fig. 8B**) and number of branches (**Fig. 8C**) were not significantly different between LPS or control-treated groups. The stunted arborisation previously observed in response to LPS was absent.

These results suggest that the lasting effects of LPS on axonal arborisation also depend on the presence of microglia or other myeloid cells that require PU.1 for their specification.

Figure 7

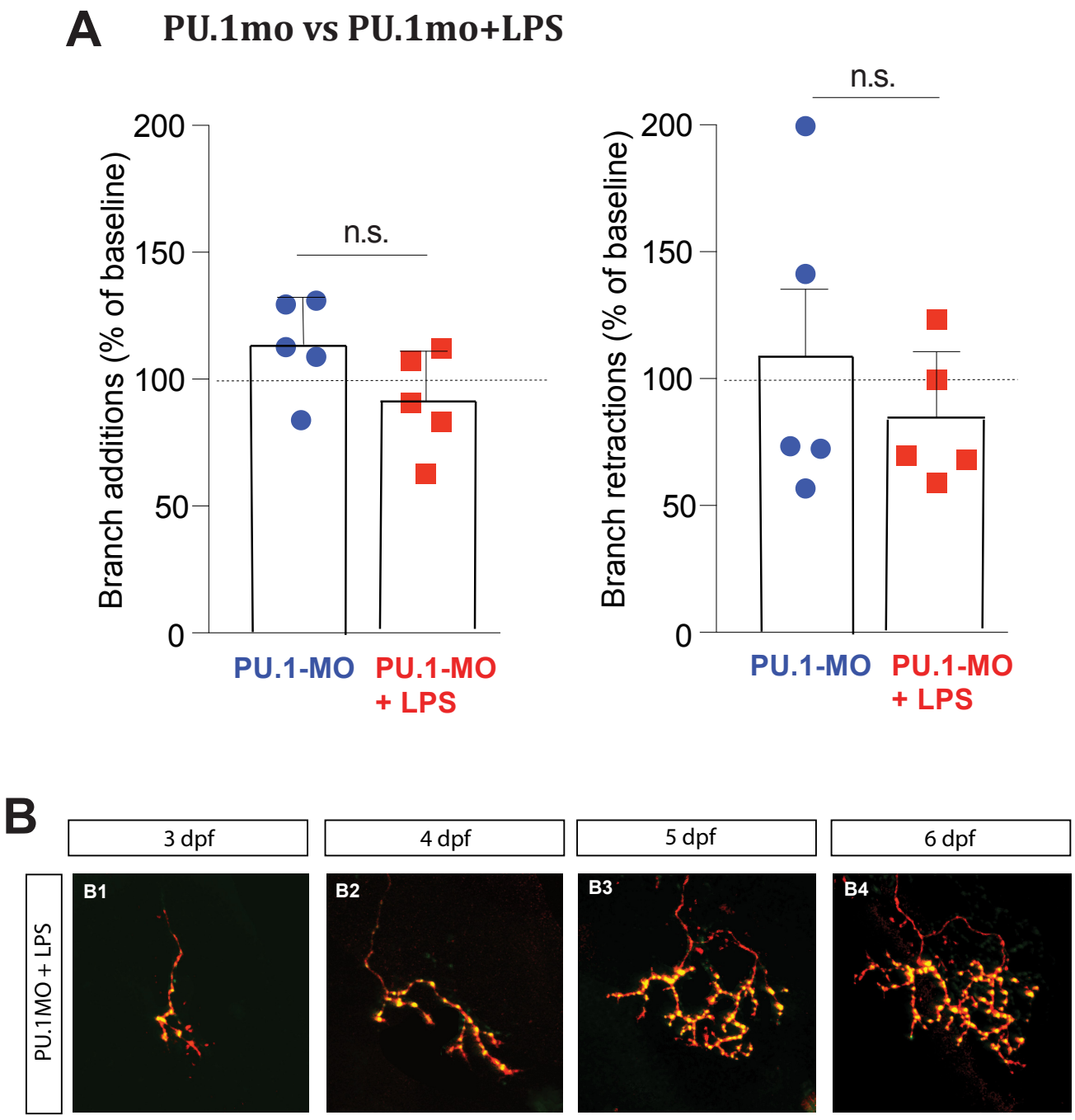

A
PU.1mo vs PU.1mo+LPS
Branch additions (% of baseline)
200
150
100
50
0
n.s.
PU.1-MO
PU.1-MO + LPS
Branch retractions (% of baseline)
200
150
100
50
0
n.s.
PU.1-MO
PU.1-MO + LPS
B
3 dpf
4 dpf
5 dpf
6 dpf
PU.1MO + LPS
B1
B2
B3
B4

Figure 8

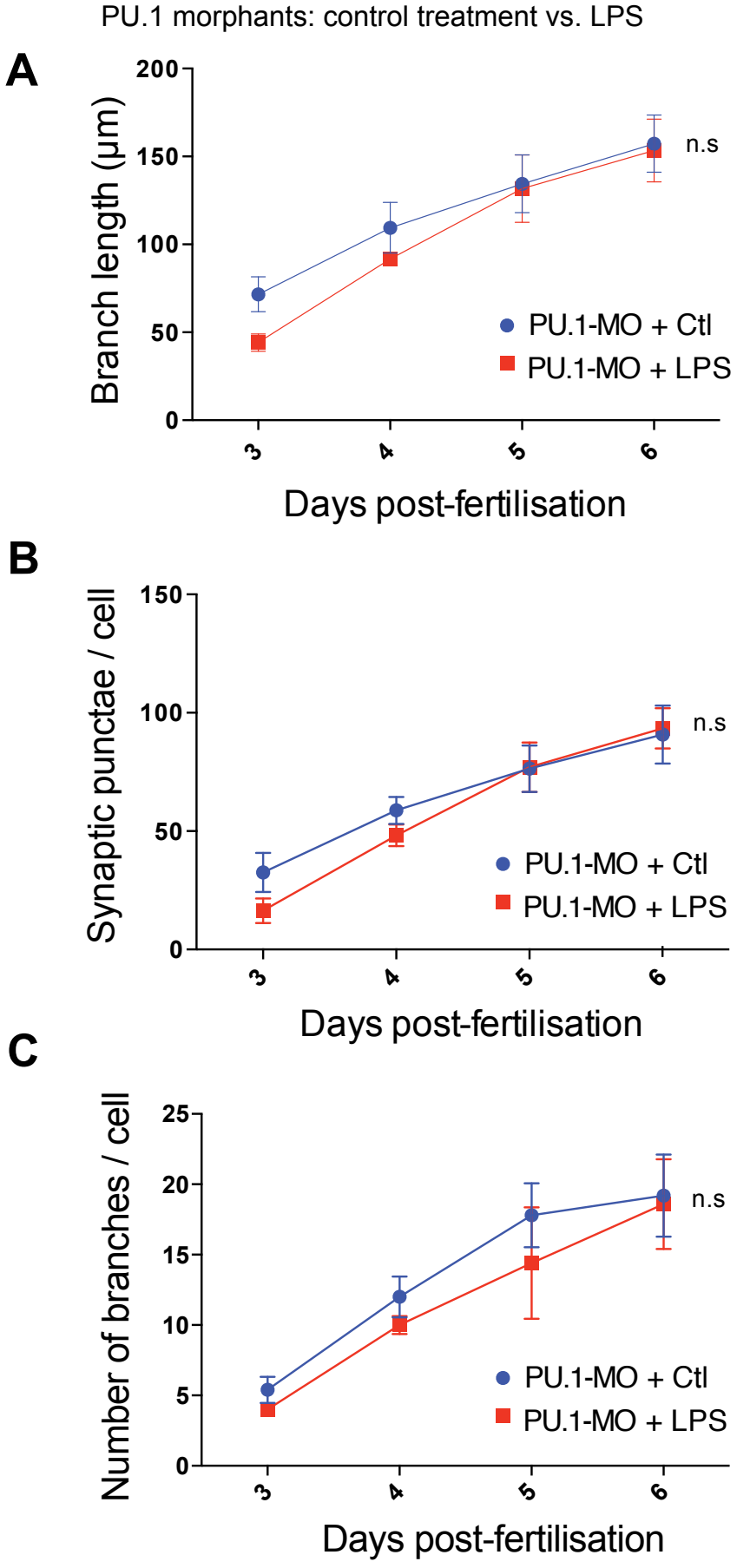

PU.1 morphants: control treatment vs. LPS
A
Branch length (µm)
200
150
100
50
0
n.s
PU.1-MO + Ctl
PU.1-MO + LPS
3
4
5
6
Days post-fertilisation
B
Synaptic punctae / cell
150
100
50
0
n.s
PU.1-MO + Ctl
PU.1-MO + LPS
3
4
5
6
Days post-fertilisation
C
Number of branches / cell
25
20
15
10
5
0
n.s
PU.1-MO + Ctl
PU.1-MO + LPS
3
4
5
6
Days post-fertilisation

Figure 7. In PU.1 morphants acute axon dynamics are unaffected by LPS

Time-lapse *in vivo* two-photon microscopy of individual RGC axon in 3 dpf PU.1 morphant zebrafish before and after 2 h exposure to LPS. Rates of branch addition and branch retraction (**A**) do not significantly change after LPS exposure. *p<0.05, **p<0.01, two-way repeated measures ANOVA with Bonferroni correction for multiple comparisons. Error bars = SEM. Scale bar 10 μm.

(B) Single RGC axons in PU.1 morphant fish expressing synatophysin:GFP and dsRed imaged daily for 4 days following control or 2 h LPS treatment. Representative serial imaging is shown. Scale bar 10um.

Figure 8. In PU.1 morphants arborisation and synaptogenesis are unaffected by LPS

Total arborisation length (**A**), number of synapses (**B**) and branch number (**C**) are unaffected in PU.1 morphants treated with LPS (n= 5 PU.1MO, 5 PU.1MO + LPS). *p<0.05, two-way repeated measures ANOVA with Bonferroni correction.

II.vi Timecourse of inflammatory cytokine induction by LPS

Given the paucity of normal physical interaction between microglia and RGC axons at these stages, we hypothesised that a secreted factor may mediate the effects. Pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNFα, IL-6 and IL-1β have been shown to be robustly upregulated in zebrafish in response to LPS (Novoa et al., 2009).

To ascertain the timecourse of this response, we exposed zebrafish larvae to LPS for 2h at 3dpf and performed qRT-PCR immediately, at 4dpf and at 6dpf (**Fig. 9A**). Our results indicate a rapid and robust induction of IL-1β and IL-6. A modest induction of TNFα was also observed. IL-1β induction was maintained for 24h and then returned to baseline. BDNF transcription was modestly decreased at 24h after LPS exposure.

PU.1 morphants demonstrated no significant changes in pro-inflammatory cytokine or BDNF expression in response to LPS exposure **(Fig. 9B**), suggesting that microglia or other myeloid lineage cells are the major *in vivo* source of these signaling factors.

Figure 9

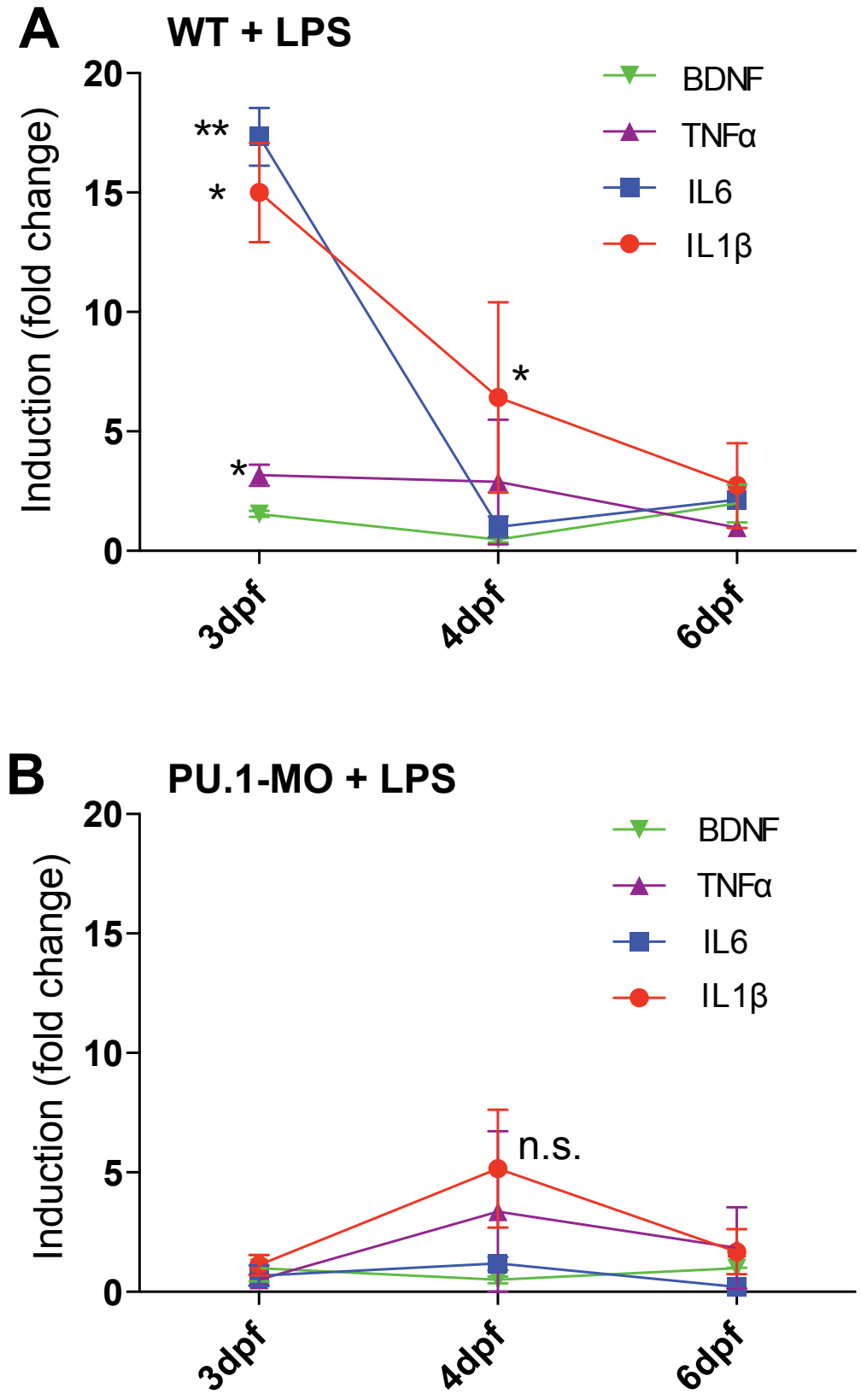

A
WT + LPS
Induction (fold change)
20
15
10
5
0
**
*
*
*
BDNF
TNFα
IL6
IL1β
3dpf
4dpf
6dpf
B
PU.1-MO + LPS
Induction (fold change)
20
15
10
5
0
n.s.
BDNF
TNFα
IL6
IL1β
3dpf
4dpf
6dpf

Figure 9. LPS treatment induces pro-inflammatory cytokine expression. qRT-PCR data illustrating relative fold change of gene expression induced by 2 h LPS treatment at 3 dpf. Change is shown relative to control-treated animals with expression normalised to housekeeping gene GAPDH. LPS treatment elevates pro-inflammatory cytokines IL-6, IL-1β and TNFα at the first time-point (immediately after 2h treatment) in wildtype (**A**) but not in PU.1 morphants (**B**). IL1β expression remains elevated 24 h later and then returns to baseline. (One-sample t-tests; experiments were performed in technical and biological triplicate. * $p<0.05$, ** $p<0.01$).

II.vii IL-1β is necessary for the effects of inflammation on circuit formation.

IL-1β has been demonstrated to play a role in axonal structural plasticity *in vitro* and in slice culture (Boato et al., 2013, 2011). We hypothesised that blocking IL-1β translation with an antisense morpholino might prevent the effects of LPS.

We first performed acute imaging. Fertilised zebrafish eggs were injected with the IL-1β morpholino and labelling plasmids at the one-cell stage and allowed to develop to 3dpf. Imaging at high temporal frequency demonstrated no significant difference in rates of branch addition (**Fig. 10A**) or branch retraction (**Fig. 10B**) after treatment with LPS compared with control.

Later effects were also absent. Arborisation following LPS treatment in IL-1β morphants was comparable to control-treated IL-1β morphants. No significant difference was observed in total arbor length (**Fig. 11A**), number of synaptic puncta (**Fig. 11B**) or number of branches (**Fig. 11C**).

Early and late effects of LPS were preserved in control morphants (**Fig. 15**), excluding non-specific morpholino effects. These results suggest that IL-1β is necessary for the effects of LPS on axonal development.

The similarity of the results for IL-1β and PU.1 morphants suggest the possibility that the two morphants have a common mechanistic pathway: IL-1β release from PU.1-specified cells such as microglia.

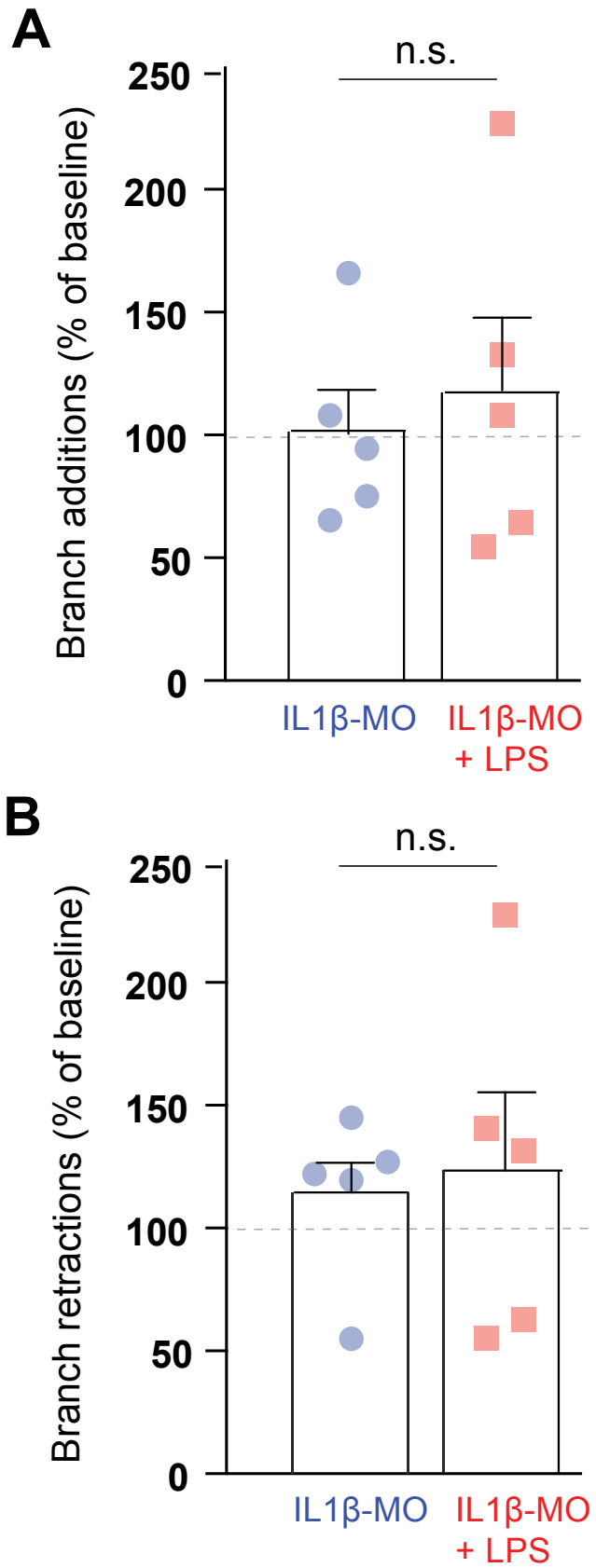

A
n.s.
Branch additions (% of baseline)
250
200
150
100
50
0
IL1β-MO
IL1β-MO
+ LPS
B
n.s.
Branch retractions (% of baseline)
250
200
150
100
50
0
IL1β-MO
IL1β-MO
+ LPS

Figure 11

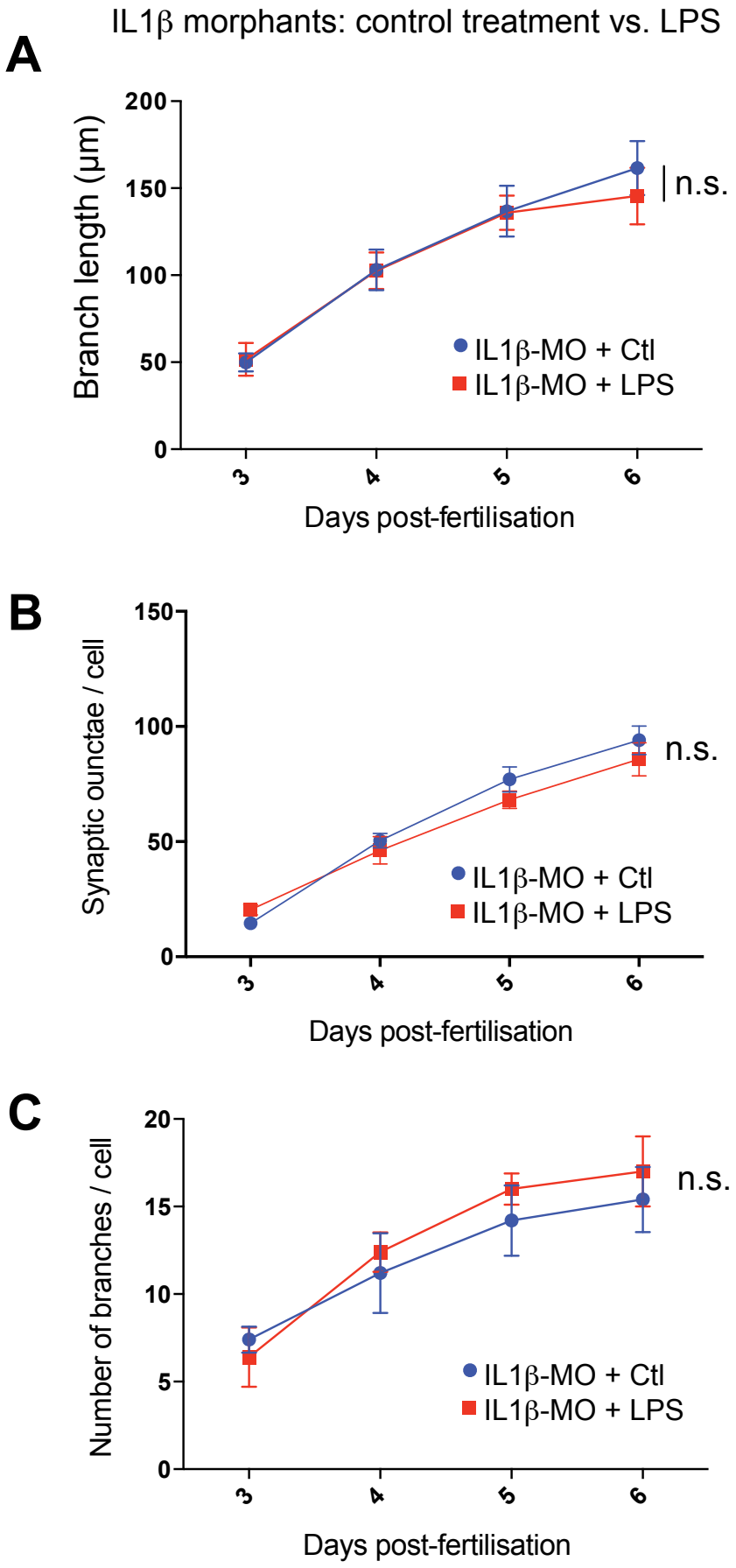

IL1β morphants: control treatment vs. LPS
A
Branch length (µm)
200
150
100
50
0
n.s.
IL1β-MO + Ctl
IL1β-MO + LPS
3
4
5
6
Days post-fertilisation
B
Synaptic ounctae / cell
150
100
50
0
n.s.
IL1β-MO + Ctl
IL1β-MO + LPS
3
4
5
6
Days post-fertilisation
C
Number of branches / cell
20
15
10
5
0
n.s.
IL1β-MO + Ctl
IL1β-MO + LPS
3
4
5
6
Days post-fertilisation

Figure 10. In IL-1β morphants acute axon dynamics are unaffected by LPS

Time-lapse *in vivo* two-photon microscopy of individual RGC axon in 3 dpf IL-1β morphant zebrafish before and after 2 h exposure to LPS. Rates of branch addition (**A**) and branch retraction (**B**) do not significantly change after LPS exposure. *p<0.05, **p<0.01, two-way repeated measures ANOVA with Bonferroni correction for multiple comparisons. Error bars = SEM.

Figure 11. In IL-1β morphants arborisation and synaptogenesis are unaffected by LPS

Multi-day *in vivo* two-photon microscopy of individual RGC axons in 3-6 dpf IL-1β morphant zebrafish in the days following a single 2-h exposure to LPS at 3dpf.

Total arborisation length (**A**), number of synapses (**B**) and branch number (**C**) are not significantly different in IL-1β morphants treated with LPS or control (n= 5 IL-1β-MO + control, 5 IL-1β-MO + LPS). *p<0.05, two-way repeated measures ANOVA with Bonferroni correction.

II. viii. IL-1β is sufficient for the effects of inflammation on circuit formation.

To ascertain whether IL-1β is sufficient to reproduce the effects of inflammation, we replaced LPS as our inflammatory stimulus with IL-1β.

Fertilised eggs were injected at the one-cell stage with Brn3c:GAL4 and the UAS:dsRED-synaptophysin:GFP expression construct. At 3dpf they were screened for expression.

Larvae with one or sparse clearly visible RGCs were imaged, then removed from the imaging chamber and lightly anaesthetised with Tricaine. Recombinant IL-1β at a concentration of 250 ng/μl or vehicle control was pressure injected into the venous circulation at the duct of Cuvier using established techniques (Benard et al., 2012). Larvae were allowed to recover and re-imaged daily as per previous experiments. The anaesthetic and injection process represent considerable invasive interventions and hence stress for the organism. As this would likely confound any imaging performed immediately afterwards, the acute imaging protocol was not performed in this set of experiments.

Animals injected with recombinant IL-1β demonstrated stunted arborisation in the days following injection, similar to that resulting from LPS exposure (**Fig. 12A**). They also demonstrated a reduced number of branches and synaptic puncta (**Fig 12B-C**) relative to vehicle-injected controls.

Figure 12

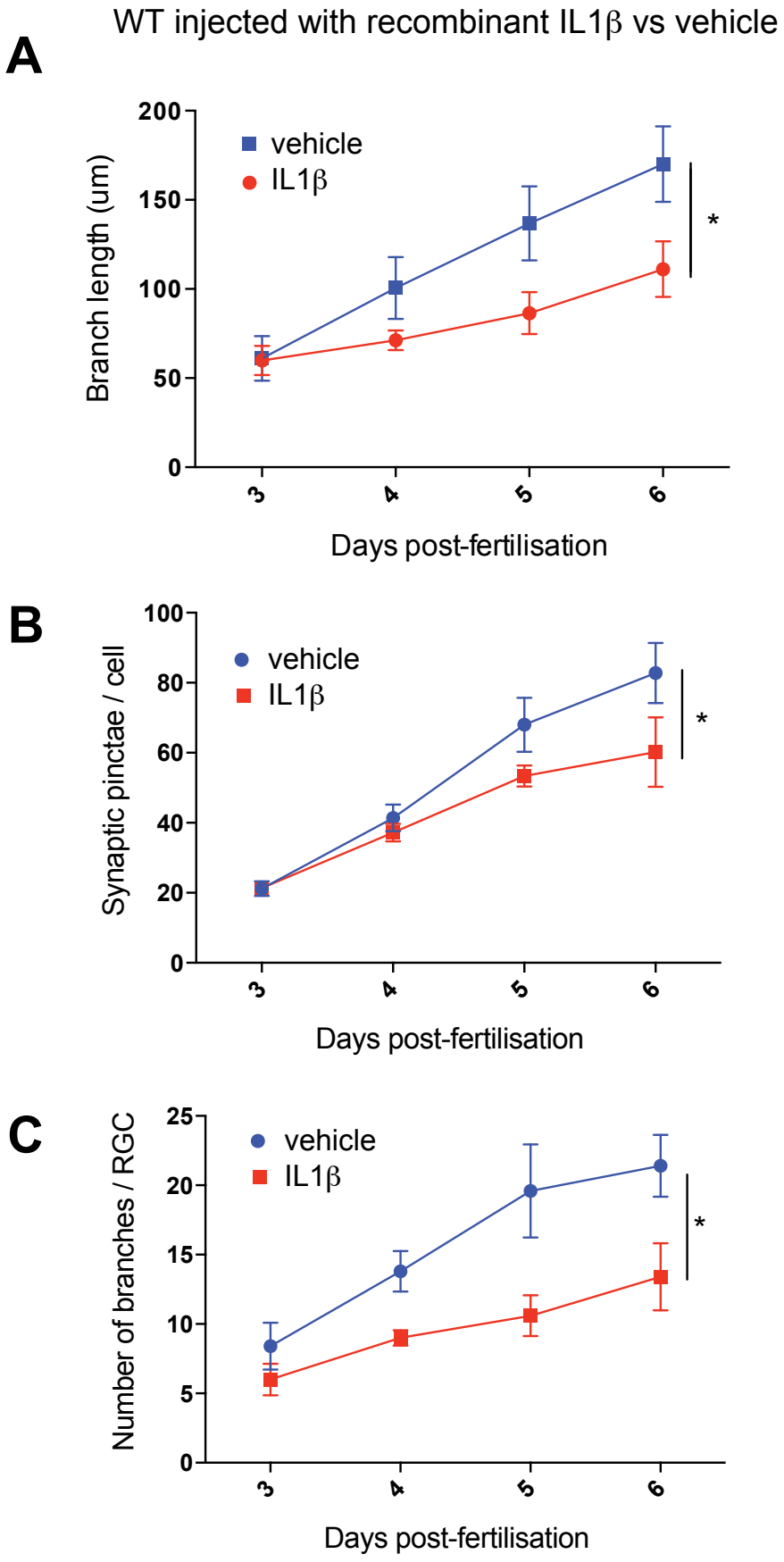

WT injected with recombinant IL1β vs vehicle
A
Branch length (um)
200
150
100
50
0
vehicle
IL1β
*
3
4
5
6
Days post-fertilisation
B
Synaptic pinctae / cell
100
80
60
40
20
0
vehicle
IL1β
*
3
4
5
6
Days post-fertilisation
C
Number of branches / RGC
25
20
15
10
5
0
vehicle
IL1β
*
3
4
5
6
Days post-fertilisation

Figure 12. Injection of recombinant IL-1β mimics the effect of LPS

Multi-day *in vivo* two-photon microscopy of individual RGC axons in 3-6 dpf WT zebrafish larvae in the days following a single injection of recombinant IL-1β at 3dpf.

Total arborisation length (**A**), number of synapses (**B**) and branch number (**C**) are significantly decreased in animals injected with recombinant IL-1β relative to vehicle-injected animals (n= 6 recombinant IL-1β, 5 vehicle). *$p<0.05$, two-way repeated measures ANOVA with Bonferroni correction.

II. ix. Recombinant IL-1β dysregulates arborisation even in the absence of microglia

We have so far established that IL-1β is necessary and sufficient for inflammatory dysregulation of axonal arborisation. Separately, we have established that microglia or other PU.1-specified macrophages are necessary for an inflammatory stimulus like LPS to impact axonal arborisation. We have also established using qRT-PCR that PU.1 morphants lacking microglia lack observable induction of pro-inflammatory cytokines including IL-1β after LPS challenge.
The remaining question is whether these two processes are indeed entirely separate or whether one is downstream of the other. That is: could IL-1β produced in the periphery act directly on neurons to dysregulate their development? Or as in the case of LPS, are microglia or other macrophages necessary to mediate the effect?

To establish this, we performed a 'rescue' experiment and injected recombinant IL-1β into PU.1 morphants lacking microglia.

Intriguingly, we found that IL-1β was able to reproduce the effects of LPS in dysregulating axonal arborisation, despite the absence of microglia (**Fig. 13**). Total length of terminal RGC arbors (**Fig. 13A**), number of synaptic puncta (**Fig. 13B**) and number of branches (**Fig. 13C**) were all significantly reduced in IL-1β vs. vehicle-injected PU.1 morphants.

Together, these data strongly suggest that IL-1β acts downstream of microglia to dysregulate neural development.

Figure 13

PU.1 morphants injected with recombinant IL1β vs vehicle

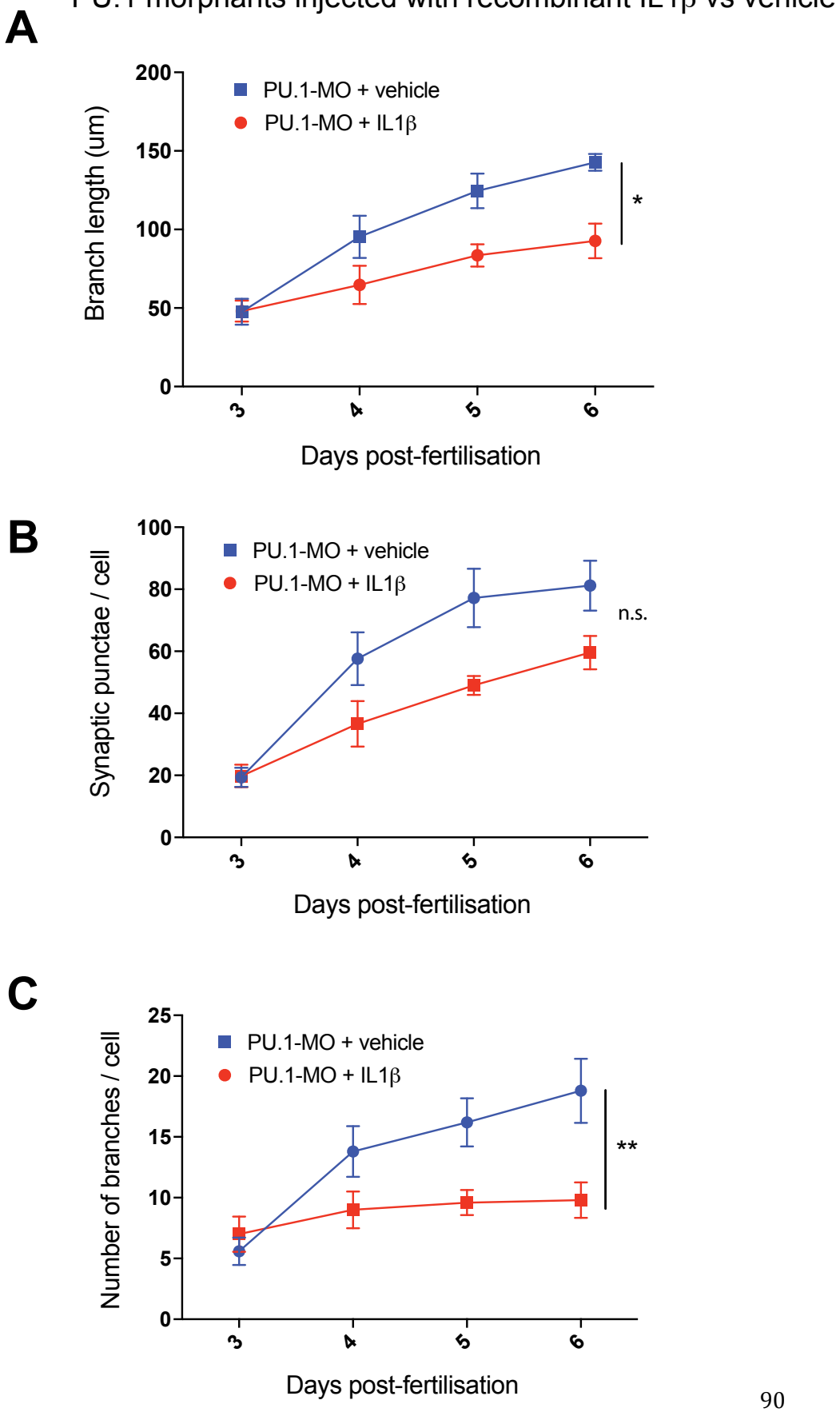


Figure 13.

Injection of recombinant IL-1β mimics the effect of LPS even in the absence of microglia

Multi-day *in vivo* two-photon microscopy of individual RGC axons in 3-6 dpf PU.1 morphant zebrafish larvae in the days following a single injection of recombinant IL-1β or vehicle at 3dpf.

Total arborisation length (**A**), number of synapses (**B**) and branch number (**C**) are significantly decreased in animals injected with recombinant IL-1β relative to vehicle-injected animals (n= 5 PU.1MO+recombinant IL-1β, 5 PU.1MO+vehicle). *$p<0.05$, two-way repeated measures ANOVA with Bonferroni correction.

[illegible]

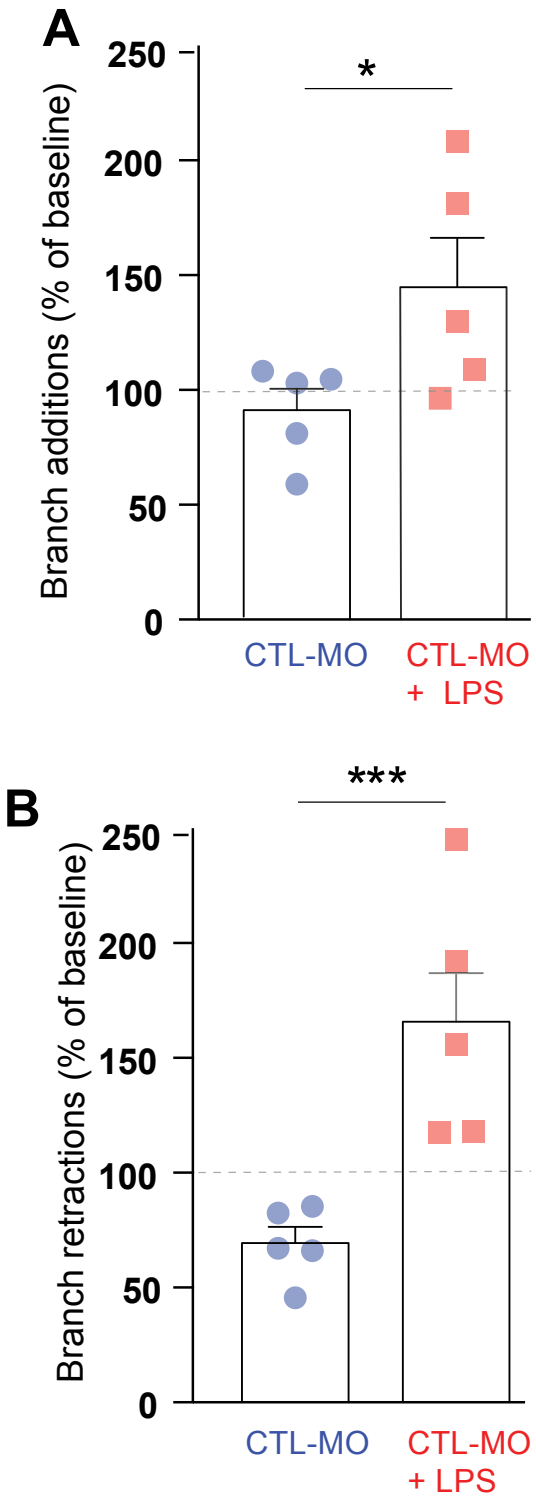

A
Branch additions (% of baseline)
250
200
150
100
50
0
*
CTL-MO
CTL-MO
+ LPS
B
Branch retractions (% of baseline)
250
200
150
100
50
0
***
CTL-MO
CTL-MO
+ LPS

Figure 15

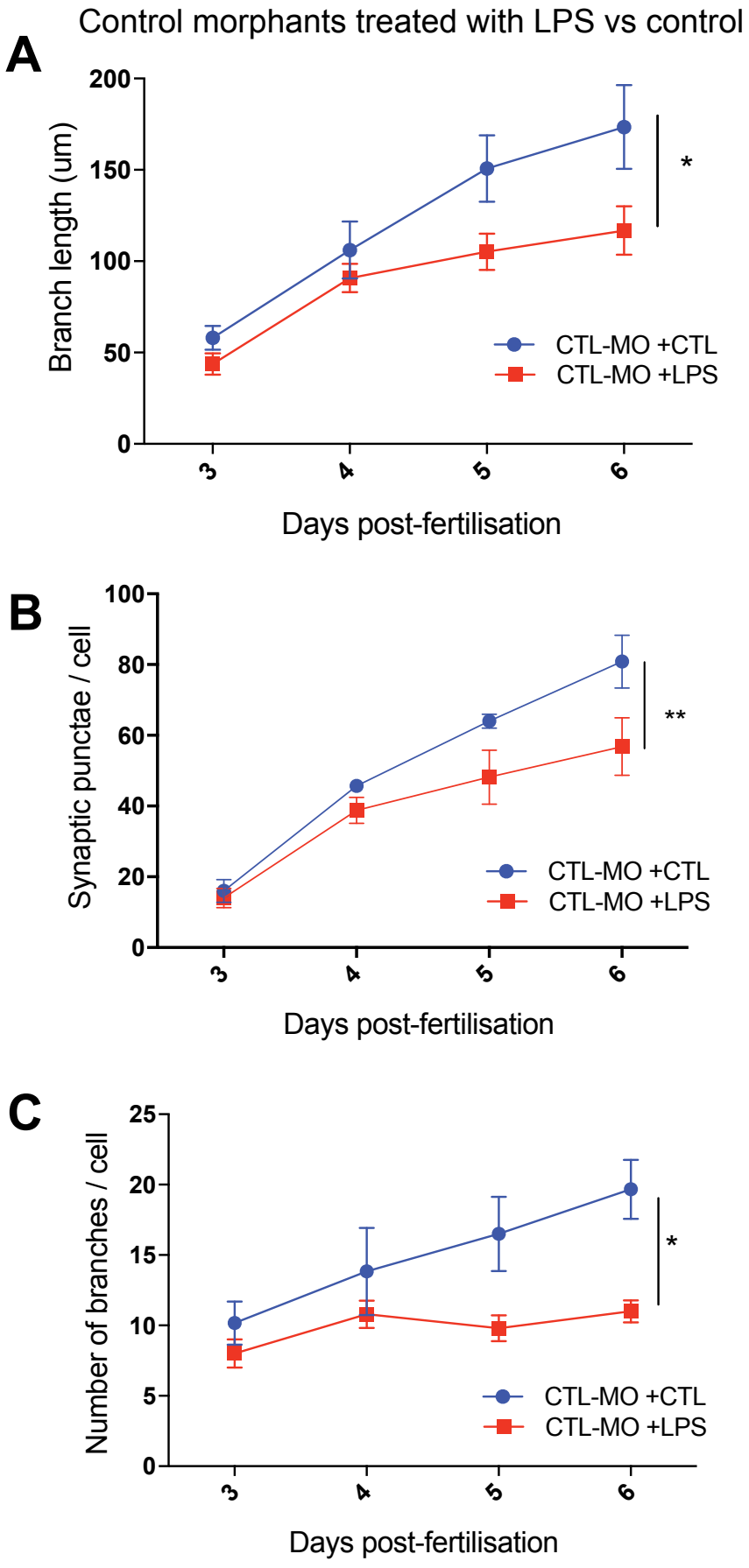

Control morphants treated with LPS vs control
A
Branch length (um)
200
150
100
50
0
3
4
5
6
Days post-fertilisation
*
CTL-MO +CTL
CTL-MO +LPS
B
Synaptic punctae / cell
100
80
60
40
20
0
3
4
5
6
Days post-fertilisation
**
CTL-MO +CTL
CTL-MO +LPS
C
Number of branches / cell
25
20
15
10
5
0
3
4
5
6
Days post-fertilisation
*
CTL-MO +CTL
CTL-MO +LPS

Figure 14 - LPS acutely upregulates axonal arborisation dynamics in control morphants

Time-lapse *in vivo* two-photon microscopy of individual RGC axon in 3 dpf zebrafish before and after 2 h exposure to LPS. Rates of branch addition (A) and branch retraction (B) are significantly increased following LPS exposure (n= 5 CTL.MO+control, 5 CTL.MO+LPS). *p<0.05, two-way repeated measures ANOVA with Bonferroni correction.

Figure 15. LPS effects are conserved in multi-day imaging in control morphants

RGCs in LPS-treated Control-morphants have significantly smaller terminal arbors at 6dpf (**A**). These arbors have significantly fewer pre-synaptic punctae (**B**) and significantly reduced number of branches (**C**) (n= 5 CTL.MO+control, 5 CTL.MO+LPS). *p<0.05, two-way repeated measures ANOVA with Bonferroni correction.

MATERIALS AND METHODS

Animal care

Zebrafish maintenance and breeding were performed according to standard techniques (Westerfield, 2000). Embryos were raised in E3 embryo medium at 28.5 degrees Celsius. 0.2 mM phenylthiourea was added to embryo water after 24 h of development to inhibit pigment formation. All protocols were reviewed and approved by the Animal Care Committee of the Montreal Neurological Institute, McGill University.

Fish lines

Microglia were imaged in the ApoE:GFP transgenic line, which was previously described (Peri and Nüsslein-Volhard, 2008) and generously provided by Dr. Francesca Peri. All other experiments were performed using wild-type embryos from *Tübingen–long fin* zebrafish (kind gift of Dr Pierre Drapeau).

RGC axon labelling and morpholino injection.

Linearised and purified plasmid DNA at a total concentration of 40 ng/µl was injected into the cytoplasm of 1-2 cell-stage fertilised zebrafish eggs. The following plasmids were used and have been previously described: Gal4:Brn3c (Xiao et al., 2005) (gift of Dr Herwig Baier); UAS:mYFP (Williams et al., 2010) (gift of Dr Leanne Godinho) and UAS:dsRed-UAS:synaptophysinGFP (Meyer and Smith, 2006) (single plasmid with double UAS cassette, gift of Dr Martin Meyer). Larvae were screened for expression at 3dpf. PU.1 (5′-GATATACTGATACTCCATTGGTGGT-3′) (Rhodes et al., 2005) and IL-1β (5'-TAACCAGCTCTGAAATGATGGCATG-3')(Banerjee and Leptin,

2014) morpholinos were previously described. Morpholinos were obtained from Gene Tools along with scrambled-control morpholino and were pressure-injected into cytoplasm or yolk of 1-2 cell stage fertilised eggs at a concentration of 0.5 mM (PU.1/control) or 0.25 mM (IL-1β).

Two-photon *in vivo* imaging

Unanaesthetised zebrafish larvae at 3-6dpf were mounted in 1.2% low-melting point agarose in custom-built imaging chambers. Imaging was performed on an upright confocal microscope (Olympus FV300) custom-converted for two-photon imaging, using a 60x water-immersion objective (NA 1.1, Olympus) and a Ti:sapphire femtosecond pulsed laser (Spectra-Physics). 1 µm z-steps were acquired with FluoView 5.0 software (Olympus). For multi-day imaging, larvae were dissected from agarose and returned to regular housing between imaging sessions.

Image processing

3 or 4-dimensional image stacks were denoised using CANDLE software (Coupé et al., 2012). Skin autofluorescence and cellular material other than the axon of interest were manually removed in ImageJ software (NIH). As zebrafish RGC axons are highly planar, XYZ-stacks were Z-projected for further analysis. Dynamo software (Dr Kurt Haas) was used to reconstruct axonal arbors and quantify total arbor size and rates of branch addition and elimination.

Microglial morphology was quantified using published techniques (Morrison and Filosa, 2013). Briefly, XYZ image stacks were Z-projected and imported into ImageJ. The image was binarized and skeletonized in ImageJ and quantitative data on branch number was extracted using the AnalyzeSkeleton plugin.

Treatments

Lipopolysaccharide (LPS) from Salmonella enterica serovar typhimurium (Sigma, L6511) was added to zebrafish E3 embryo medium at a final concentration of 25 μg/ml. After two hours, larvae were removed from LPS and rinsed in copious E3 embryo medium. Notably, we were unable to elicit inflammatory responses with LPS from E.coli.

For IL-1β experiments, 3 dpf larvae were briefly anaesthetised with 0.01% Tricaine (Sigma) in E3 medium and recombinant IL-1β (Sigma) or vehicle (ddH20) was pressure injected into the venous circulation at the Duct of Cuvier at a concentration of 250 ng/μl.

Quantitative reverse-transcription PCR

20-30 zebrafish larvae of the appropriate stage were collected in a microcentrifuge tube and euthanized by snap-freezing in an ethanol-dry ice bath. RNA was extracted with Tri Reagent (Sigma) and purified with RNEasy mini-kit (Qiagen) according to standard protocols (Lan et al., 2009), including a 30min DNAse digest. Reverse-transcription was performed on 1μg of total RNA with Superscript RT-II (Life technologies) according to manufacturer's instructions to obtain cDNA.

TaqMan quantitative real-time PCR (Life Technologies) was performed using the following assays: GAPDH (Dr03436842_m1), TNFα (Dr03126850_m1), IL-1β (Dr03114368_m1). Custom probes were designed against Danio rerio BDNF (Fwd: 5'-CTGCTGAATGGTCTCCTTTACGA-3'; Rev: 5'-GAACAGGATGGTCATCACTCTTCT-3') and Interleukin-6 (Fwd: 5'-TCAGACCGCTGCCTGTCTA-3'; Rev: 5'-CACGTCAGGACGCTGTAGAT-3'). No amplification was seen

in no-RT and no-template controls.

Data were normalised to the housekeeping gene GAPDH and relative gene expression was calculated according to the ΔΔCt method (Livak and Schmittgen, 2001).

Xenopus laevis experiments (see Discussion)

Xenopus laevis tadpoles (stage 45) were electroporated with farnesylated green fluorescent protein (fEGFP). Briefly, tadpoles were lightly anaesthetised in 0.02% Tricaine (Sigma). DNA was pressure-injected into the cerebral ventricle and platinum electrodes were applied to the surface of the head bilaterally. 2-3 pulses were delivered in each polarity. As the radial glial cell bodies are peri-ventricular, they constitute the bulk of the labeled cells. Two days later, tadpoles were again lightly anaesthetised and placed in a custom-built PDMS imaging chamber covered with a glass cover slip for imaging.

Statistical Analysis

Measurements were analysed for statistical significance at the $P < 0.05$ level using Prism 6.0 software.

IV. DISCUSSION

IV.i. Summary of main findings

We intially examined the distribution of microglia (the macrophages and immune regulators of the CNS) during the period of initial retinotectal circuit formation and refinement (3-6dpf). We found that under normal conditions, microglia remain outside of the tectal neuropil during this period. Interestingly, they are able to enter the neuropil in response to a laser lesion.

We then examined the response of microglia to an application of bacterial lipopolysaccharide (LPS). Consistent with induction of a strong innate immune response, we found that LPS induced an amoeboid shift in microglial morphology and induced transcription of the canonical pro-inflammatory cytokines TNF, IL-1β and IL-6.

As we were interested in how developing neurons respond to inflammation, we performed *in vivo* two photon imaging of retinal ganglion cell axons before and after application of LPS. We found that LPS exposure acutely upregulated retinal ganglion cell (RGC) axonal dynamics within 2 hours. Despite this initial increase in axonal dynamics, we found that total arbor extent, complexity and number of pre-synaptic puncta were decreased in the days following a single LPS exposure, relative to control-treated animals.

To examine the contribution of microglia to this effect, we took advantage of thePU.1 transcription factor, which is critical for myeloid specification. PU.1 morphants lack microglia and macrophages. The effects of LPS were occluded in PU.1 morphants. PU.1 knockdown also prevented upregulation of pro-inflammatory cytokines in response to LPS.

As our studies suggested that contact-independent mechanisms may predominate over contact-dependent ones for neuroimmune interaction in our system, we decided to examine specific factors secreted from microglia/macrophages which might underlie the effects we observed. We chose to study the pro-inflammatory cytokine IL-1β, as mounting data suggest a role in the regulation of neuronal structure and function (see Introduction, section I.iii.b). Similar to the results in PU.1 morphants lacking microglia, the effects of LPS were also occluded by morpholino knockdown of IL-1β. This result suggests IL-1β is necessary for the effects of LPS to manifest. To examine whether it is sufficient, we injected IL-1β directly into the bloodstream of developing zebrafish larvae. IL-1β reproduced LPS-like effects in RGCs (reduction in overall arbor length, complexity and number of pre-synaptic puncta).

Finally, we wanted to establish whether the effects of IL-1β were dependent on the presence of microglia/macrophages. To test this, we injected IL-1β into PU.1 morphants lacking microglia. Even in the absence of microglia/macrophages, IL-1β was sufficient to reproduce LPS-like effects.

IV.ii. Significance of findings

Effects of inflammation. Our results demonstrate that inflammation can have rapid effects on neural development; changes in arborisation dynamics were evident within 2h of exposure to LPS. This suggests that even a brief inflammatory or infectious insult during development can have detrimental effects on subsequent circuit formation. Moreover, a single 2h exposure to LPS had longstanding consequences, evident for several days after the insult – throughout the early period of circuit maturation.

Synaptic formation vs. maintenance/destruction. Reduced synapse density has been found in schizophrenia patients as well as in models of maternal immune activation (Elmer et al., 2013; Giovanoli et al., 2016; Roberts et al., 2015). It remains unclear to what extent this is due to failure of synapse formation or maintenance or increased synaptic destruction (Boksa, 2012).

Neuroimaging studies in ASD have demonstrated both increases and decreases in functional connectivity. It has recently been proposed that hyper-connectivity appears in younger cohorts while hypo-connectivity predominates in older cohorts (Nomi and Uddin, 2015). Intriguingly, mouse models of ASD that utilise genetic mutations associated with human ASD demonstrate increased rates of synaptic remodelling (Isshiki et al., 2014).

Our findings of an early increase in structural plasticity followed by reduced axonal arborisation suggest failure of synaptic formation or stabilisation, rather than increased loss as a possible mechanism for a reduction in synaptic density.

Paracrine function of microglia. A number of high profile studies have implicated microglia in physical pruning of synapses in development, raising the intriguing possibility that dysregulation of this process plays a role in the pathogenesis of neuropsychiatric disease (Paolicelli et al., 2011; Stevens et al., 2007; Tremblay et al., 2010).

Our data complement these studies by highlighting mechanisms independent of synaptic contact. Indeed, in the larval zebrafish system, physical contact by microglia can be essentially excluded as a contributor to normal or pathological brain development at least in the retinotectal system. Given the enormous number of synapses undergoing modification in the developing brain, paracrine mechanisms may allow a relatively small population of microglia to exert effects on a large number of neurons simultaneously.

. In the context of maternal immune activation, the primary immune response occurs in the mother. Translocation of inflammatory factors across the placenta and across the blood-brain-barrier follows (Estes and McAllister, 2016). As CNS-resident immune cells, microglia are well-placed to receive and amplify such signals.

IV.iii Caveats and limitations

Microglial distribution and depletion

Our findings of microglial absence from the tectal neuropil should be confirmed at later stages and with additional labelling techniques, although other investigators have used simple dyes or

other transgenic lines and similarly reported absence until at least around 11dpf, well past the developmental period in which these experiments were performed (Herbomel et al., 1999; Svahn et al., 2013).

Further, although we did not observe microglia translocation into the tectal neuropil with LPS treatment, this should be systematically examined throughout the developmental period. Our experimental induction of laser injury demonstrated that microglia were able to enter the neuropil under certain conditions. It is possible however that physical disruption to the cellular environment and extra-cellular matrix induced by the laser opens a new physical space which allows microglial entry. It would be of interest to induce laser lesion only at the deepest points of the neuropil to observe whether microglia are still able to penetrate.

Induction of inflammation

Our model of inflammation relies on high doses of bacterial LPS. Our preliminary experiments established that the dose was non-lethal and that it appeared to induce clear effects on RGC arborisation. For the purposes of these experiments – establishing the effects of immune activation on early circuit formation – we felt that this initial approach was legitimate. It would be important to assess whether there is a dose-response effect with LPS.

Further work would also be needed to assess how far this approach is 'physiological' in the sense of accurately modelling a typical *in vivo* inflammatory response. Studies on immunity in zebrafish have for example induced bacterial infection of early zebrafish embryos using immersion in a solution containing bacterial species such as *Edwardsiella tarda and Flavobacterium columnare:* Gram-negative bacteria which are typical fish pathogens (Chang and Nie, 2008; Phelan et al.,

2005). Another approach, which produces more consistent inflammatory responses than immersion with bacteria, is yolk injection with Mycobacterium marinum (Meijer and Spaink, 2011). Another formof immune stimulation would be administration of Polyinosinic:polycytidylic acid (Poly I:C), a structural mimic of double-stranded viral RNA which induces viral-type inflammatory responses via TLR3 in zebrafish (van der Vaart et al., 2013).

Timecourse of effects

The effects of LPS remained apparent at 6dpf, at which time the basic establishment of the retinotectal circuit has been completed. We cannot exclude however, that there is a gradual renormalisation of arbor size in the days and weeks following. Analysis of late stage RGCs following inflammation would be valuable.

Microglia vs. macrophages

As discussed, the PU.1 promoter is critical for myeloid specification. Although microglia seem the most likely source, we cannot exclude that the effects we observe are mediated or initiated by non-microglial cells including peripheral macrophages. *In vivo*, such effects are likely mediated by multiple parallel cellular and molecular pathways. Clinically, microglia are unlikely to be the first cells responding to systemic infection, nonetheless they are the immune cells best-placed to mediate or modulate effects within the CNS. As of yet, there are no microglia-specific promoters in zebrafish that do not also label peripheral macrophages.

Work in the mouse model is more promising in this regard. The fractalkine receptor CX3CR1 is often described as microglia-specific (e.g. Paolicelli et al., 2011; Schafer et al., 2012), however it is also expressed by circulating monocytes, natural-killer cells, dendritic cells and other tissue-

resident macrophages (Jung et al., 2000).

A 2014 study from Howard Weiner, Jack Antel and colleagues has recently proposed a promising molecular signature for microglia based on TGF-β signalling (Butovsky et al., 2014).
Very recently, an intriguing study from Ben Barres has described a protein of unknown function, transmembrane protein 119 (Tmem119) as a specific marker of microglia (Bennett et al., 2016). These studies suggest that more microglia-specific tools for research will soon be available.

Neuronal cell-type specificity

The Brn3c promoter that we used to label RGC axons labels a subset of RGCs which project predominantly to the *stratum fibrosum et griseum superficiale* (SFGS), the second-most superficial layer of the retino-recipient optic tectum(Xiao et al., 2005). We chose to use this promoter to limit one major source of variability: the RGC sub-type. It would be of interest to see whether these findings apply to all types of RGCs, to tectal cell dendrites and to developing axons and dendrites outside of the visual system.

IV.iv. Future directions

Further establishing the role of myeloid cell-derived factors. Our study implicates IL-1β as a key regulator of neuroimmune interaction, however the action of IL-1β remains to be clearly delineated. For example, does IL-1β act directly on RGCs or indirectly via another cell type? To establish this, we are currently working on techniques to establish IL-1β receptor expression

pattern and to knockdown the IL-1β receptor specifically in RGCs.

In our qRT-PCT experiments, we noted that IL-6 was strongly upregulated alongside IL-1β. It would be of interest to establish whether knockdown of IL-6 also impacts the neurodevelopmental effects that we observed, and whether IL-6 is upstream or downstream of IL-1β in this pathway.

We specifically looked at three pro-inflammatory cytokines; a gene array could be used to establish all the factors specifically regulated by LPS and the relevant timecourse (Stockhammer et al., 2009); further candidates for investigation could be selected in this way.

Further, it would be interesting to explore the mechanisms by which the effects of inflammation last significantly beyond the apparent renormalisation of cytokine induction. Recent studies have proposed that inflammation can cause lasting epigenetic change in macrophages which may polarise their baseline state and alter their responses to subsequent immune challenge (Patel et al., 2016). In the basal state, without active TLR signaling, transcription of inflammatory cytokines is specifically repressed by factors such as B-cell-leukemia 6 (BCL-6) and repressor complexes including histone deacetylases and demethylases (Barish et al., 2010). Chromatin accessibility of critical immune modulators such as members of the interleukin-1 family is limited by the structural configuration of the nucleosome. TLR signaling induces a cascade which releases these epigenetic 'brakes', recruits master activators such as NF-κB and leads to a full inflammatory response (Ivashkiv, 2013). The precise molecular mechanisms and timecourse of such effects remains to be established, however it is known that epigenetic modifications can be extremely long-lasting and even transmitted across generations. These effects may in effect

'prime' the immune system to remain in a more activated state and to respond more vigorously to future immune stimuli (Patel et al., 2016).

Prevention/treatment. Ultimately, our clinical aim is to mitigate detrimental neuroimmune effects by prevention or treatment. In this model, we can apply pharmacological or genetic neuroimmune modulators before, during or after inducing inflammation to attempt this. Examples might include corticosteroids, function-blocking antibodies or agents such as fingolimod and dimethyl fumarate which can traverse the blood-brain-barrier and impact neural, glial and immune function (Healy et al., 2015).

Specifically, our studies implicate the canonical pro-inflammatory cytokine IL-1β. A number of clinical studies have been performed and are in progress to examine the effect of IL-1β-blockade on inflammatory disorders (Dinarello et al., 2012). Although not all of these agents cross the blood-brain-barrier, maternal immune activation likely begins peripherally. One might hypothesise that blockade of circulatory IL-1β is sufficient to mitigate some of its effects in the CNS. Future experiments might aim to antagonise IL-1β at different timepoints (immediately after LPS exposure or later) and see whether the negative effects on circuit development can be mitigated or reversed.

Effects on neural activity. Activity is a key regulator of structural-functional circuit refinement and activity itself can be modulated by microglia and by inflammatory signals (Li et al., 2012; Pribiag and Stellwagen, 2014). In future work, we would like to establish the effect of LPS on spontaneous and induced activity in the visual system. The zebrafish model is excellent for calcium imaging which can be used to address these questions. If LPS has dramatic effects on

circuit activity, it would be interesting to see whether normalising the changes in activity affects the structural remodelling that we have observed. Modulation of activity may be an indirect way to mitigate detrimental neuroimmune effects.

Effects on cell number and proliferation. We did not directly observe microglial proliferation in response to inflammation, though this has been reported in the literature. Further, we would like to establish whether and how neural proliferation is affected by inflammation. Mounting evidence implicates both innate and adaptive immune responses in the modulation of neurogenesis, with both positive and negative effects described depending on the model, context and dose (reviewed in Kokaia et al., 2012). As we observe reduction in overall RGC arborisation following immune insult, it would be interesting to see whether this is accompanied by a compensatory increase in RGC number. Powerful *in vivo* imaging tools to assess cellular proliferation have been created in the zebrafish (Sugiyama et al., 2009).and could be exploited to address these questions.

Effects on circuit properties and behaviour. We noted reduced final arbor size of RGCs, in future work we would like to establish how this effects circuit properties such as the size of receptive fields of the tectal neurons which receive input from RGCs. Does the smaller arbor translate to a smaller receptive field? How does this affect behavioural responses such as the opto-kinetic reflex, predator escape or prey capture? We might hypothesise that the smaller area of RGC input would result in a homeostatic shift in the receptive field properties of tectal cell neurons to increase the activity of the remaining inputs. Studies on the effect of inflammatory factors such as TNF-α in the developing *Xenopus laevis* tadpole suggest that it may increase

dendritic growth of tectal neurons as well as synapse maturation and strength (Lee et al., 2010). The initial increase in dynamics that we observe however, suggest that the picture may be more complex.

Further, 'thigmotaxis' assays have been employed in the larval and adult zebrafish (Schnörr et al., 2012). Thigmotaxis ('wall-hugging') is used to assay 'anxiety'. In mammals such as rodents and humans as well as in non-mammalian vertebrates like fish, novel or stressful situations lead organisms to stick to the edges of their environment and to avoid traversing an 'open field' where, presumably they would be more vulnerable to attack (Crawley, 1985). Early immune activation in rodents produces anxiety-like behaviours such as impaired open-field test performance (increased thigmotaxis) (Claypoole et al., 2016; Roy-Lacroix et al., 2013). Analagous effects may be found in zebrafish exposed to LPS.

Heritability. An intriguing possibility is that effects of neuroinflammation can be transmitted across generations by epigenetic change (Garden, 2013). We would like to establish whether offspring of larvae exposed to inflammation also display defects in neural circuit formation and function.

Inflammation and other glial cells. Pilot experiments were performed on the response of radial glia to injury (see below). We would like to take this work forward and examine neuroimmune/neuroinflammatory properties of this fascinating cell type. Oligodendrocytes are another group of cells whose responses could readily be studied in the zebrafish or Xenopus laevis tadpole.

Radial glia are multifunctional glial cells that disappear early in mammalian development but persist until adulthood in fish and frogs. They serve as neural progenitor cells and as scaffolds to assist in cellular migration early in development. They also perform structural and functional roles to support and modulate neural activity and may subsume some of the functions ascribed to astrocytes in higher organisms (Chung and Barres, 2011; Sild et al., 2016; Sild and Ruthazer, 2011). Recent studies have demonstrated that inflammation can cause lasting phenotypic 'polarisation' of astrocytes, suggesting modification of glial cell responses as a mechanism for the long-lasting effects of a single, acute inflammatory exposure (Jang et al., 2013).

As experimental protocols to label and visualise radial glia were already available in the Xenopus tadpole in the laboratory (Sild et al., 2013; Tremblay et al., 2009), we performed experiments using the Xenopus model.

Early electroporation of eGFP into the tectum labelled a mixed population of cells including a number of radial glia, easily identifiable by their bottle-brush morphology (Golgi, 1886). Time-lapse imaging of radial glia after the induction of a laser lesion demonstrated apparent detachment of the radial glial endfoot from its position and rapid lamellar transformation of its surrounding membrane. The lamellar structure extends over the area of laser injury, apparently engulfing cellular debris (Figure 16; supplemental video 3). This would be consistent with recently reported roles for astrocytes in phagocytosis (Chung et al., 2013; Chung and Barres, 2011).

Figure 16

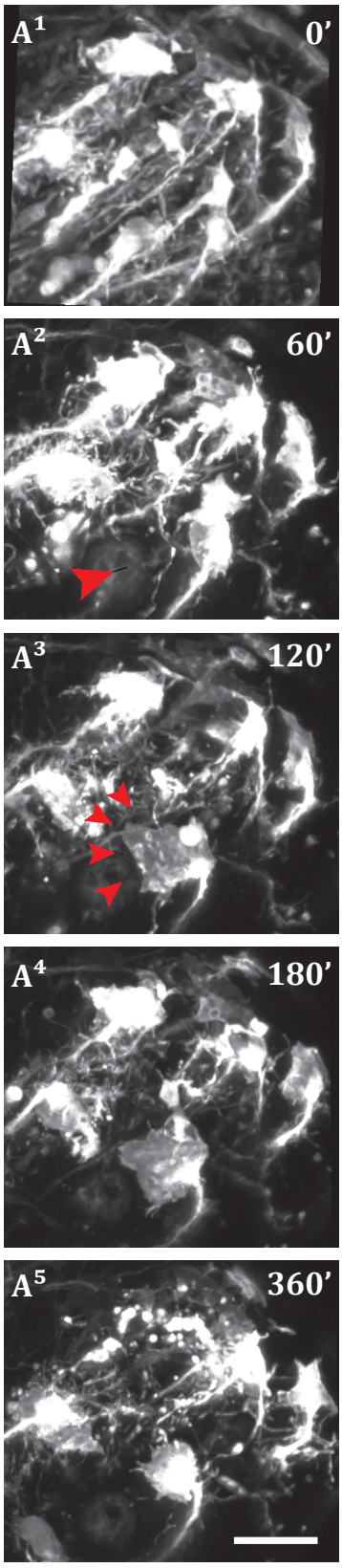


Figure 16. Radial glial cells respond with morphological transformation to laser-induced injury. Serial *in vivo* two photon imaging performed in *Xenopus laevis* tadpoles. Radial glial morphology demonstrated before lesion (**A1**), foot-process region at pial surface is demonstrated. Laser lesion performed (red arrow, **A2**) ablating one radial glial cell. Within one hour, dramatic structural remodelling of adjacent radial glia occurs with detachment of foot process and lamellar transformation (red arrows **A3**). Lamellar structure appears to engulf cellular debris and gradually resorbs into radial glial foot process (**A4-5**). Representative image series; time shown in minutes; scale bar 20μm.

IV.v. Concluding remarks

The process of development and specifically of brain development has often been considered as though it occurs in a vacuum. The German philosopher Martin Heidegger (1889-1976) claimed that Being (*Sein*) can only be understood as always-already Being-in-the-world (*In-der-Welt-sein*) (Heidegger, 1927). Increasingly, the environmental context of development is being recognised as critical – what we might call 'Becoming-in-the-world'. This environmental 'world' includes hostile pathogens and toxins – and the cellular and molecular programs of the innate immune system that respond to them, hardwired through millions of years of evolution.
Indeed, the parallel evolution of the nervous and immune systems and their shared cellular and molecular mechanisms illustrate the diversity of function that can be achieved with a parsimonious gene set. These shared mechanisms may come with the price of pathological 'cross-talk'. Through the hypothalamic-pituitary axis, stress hormones and epigenetics, factors such as socio-cultural history and economics (long considered outside the scope of scientific investigation) may impact neuroimmune function.

To our knowledge this is the first study that takes advantage of *in vivo* imaging tools to visualise early developmental neuro-immune interaction in real time. Our findings implicate secreted factors from myeloid cells including microglia.

Future studies on neuro-immune interaction will doubtless continue to shed light on normal and pathological development and hopefully give rise to new interventions for neuropsychiatric disorders.

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