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ACUTE-LPS MEDIATED MYELIN INJURY

By

GIDEON STONE

A thesis submitted to the University of Plymouth

In partial fulfilment for the degree of

RESEARCH MASTERS

School of Biomedical Sciences

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Author's Declaration:

At no time during the registration for the degree of Research Masters has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

This thesis has been proofread by a third party; no factual changes or additions or amendments to the argument were made as a result of this process. A copy of the thesis prior to proofreading will be made available to the examiners upon request.

I declare that the work contained in this thesis is original research carried out during the registration for this degree under the supervision of Prof. Robert Fern at Plymouth University. All the work was carried out by Gideon Stone, with the exception of LPS preparation which was performed by PhD Verity Mitchener, and PLP-GFP ear tag collection performed by Mr. Waldemar Woznica.

A programme of advanced study was undertaken, which included the following taught modules: PSYC703 Cognition and Biological psychology & PSYC705 Health and Wellbeing.

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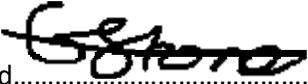
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Abstract:

Acute LPS-mediated myelin injury

Gideon Stone

Background: Multiple Sclerosis is a notorious neurological disease strongly linked to
auto-immune pathogenesis and involvement of inflammatory cytokines, resulting in
CNS white matter injury. To date, there is no known cure and a limited number of
successful treatments, with the exact pathogenic mechanisms of the disease being
unknown. Due to this there is a high demand for the development of effective
treatments. With the primary use of anti-inflammatory treatments, the symptoms can
be significantly reduced, and the occurrence of relapses and exacerbations can be
managed. The use of animal models such as EAE and cuprizone has helped drastically
with the understanding of the disease and have been used to formulate the current
treatments available to great success. This project will aim to speed up this process by
laying the groundwork for a novel in-vitro model of white matter disruption resulting
in speedy drug testing and reduced live animal experimentation.

Hypothesis: Acute LPS exposure to ex-vivo mice optic nerves will cause myelin
disruption to both myelinated axons and oligodendrocyte cell bodies.

Methods: Optic nerves from both CD1 mice and transgenic PLP-GFP mice were isolated
and stained using Fluoromyelin red, while being maintained in aCSF solution at 37°C.
This was followed by acute lipopolysaccharide (LPS) exposure at varying
concentrations for 120 minutes. After the insult period confocal microscopy was used
to capture images showing myelinated axons of the optic nerves, as well as the
oligodendrocyte cell bodies in the PLP positive mice which express the PLP promoter

that drives the expression of a green fluorescent protein (PLP-GFP). Following the microscopy, the program image J was used to measure the fluorescence of the white matter of the varying structures and allow for statistical analyses of the structural integrity of the optic nerves.

Results - Five mice were used per experiment. 1 μg-mol LPS showed significant myelin disruption ($p < 0.04$) in comparison to insignificant results gathered from 0.1 μg-mol LPS application ($p < 0.666$), both using CD1 mice. PLP-GFP negative mice also showed significant myelin injury following LPS insult, solidifying the results found in the CD1 mice ($p < 0.01$). Damage was not restricted to the myelinated axons but also affected oligodendrocyte cell body structure. Significant damage was shown with the use of PLP-GFP positive stacked images showing decreased fluorescence ($p < 0.027$), decreased cell area ($p < 0.007$) and increased cell process damage ($p < 0.001$), with no change to oligodendrocyte cell count ($p < 0.77$). Application of Methylprednisolone (MP) at a concentration of 10 μM limited inflammation in CD1 optic nerves ($p < 0.608$).

Conclusion - Acute LPS exposure on CD1 and PLP-GFP optic nerves elicit an inflammatory response and subsequent myelin injury through the activation of the MyD88 pathway. This pathway is strongly implicated in the pathogenesis of MS through the upregulation of TLRs and produced cytokines. Our findings suggest that LPS can be used as a new in-vivo model of MS, allowing for rapid therapy testing and reduced animal experimentation.

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Chapter 1. Introduction:

Multiple sclerosis (MS) is a widely researched neurological disease which affects over 2.3 million people worldwide with a female preference (Harbo, Gold and Tintoré, 2013). Over 100,000 individuals in the UK suffer from MS, making it one of the more common CNS conditions that affect young adults. Although MS can be a chronic disabling disease, the severe symptoms associated are the direct cause of death for 50% of patients; with the rest being a result of cardiovascular and respiratory diseases as well as accidents and suicides (Scalfari et al., 2013).

The roots of multiple sclerosis are unknown; however, there is speculation that environmental and lifestyle factors significantly influence the risk. The most common risk factors known are the following: Adolescent obesity supported by randomised studies (Mokry et al., 2016)**.** Epstein-Barr virus (EBV) infection as MS patients had a higher titre of EBV antibodies compared to controls (Ascherio and Munger, 2007); with emerging evidence that infectious mononucleosis can significantly increase the risk of MS development (Dobson and Giovannoni, 2018). Smoke exposure through both active and passive smoking has shown a dose-dependent relation to the risk of MS (Hernan, 2001). The implication of vitamin D levels through lack of sun exposure and diet is also prevalent in MS risk, with the consumption of rich sources of vitamin D providing protection from MS (Kampman and Brustad, 2008); the most prominent study supporting this was through the identification of high serum levels of 25-hydroxycholecalcifeol which is a form of vitamin D in the liver, this provided protective effects against MS (Munger et al., 2006). Geographic Locations that lie on the equator, such as regions of Africa, America, and Asia have a low number of cases of MS in comparison to those further away, such as the UK (Sharpe, 1986). This is most likely

due to lack of exposure to UV light which has shown to suppress EAE in mice lacking 7-dehydroxycholestrol (7-DHC), which is the precursor of vitamin D (Irving et al., 2019). Thereby supporting the role of vitamin D as a strong factor of MS.

Genetics are also thought to play a role in the onset of MS, with the risk of reoccurrence within families increasing with the higher amount of genetic sharing; for example, risk in monozygotic twins is 35%, dizygotic twins 6%, and siblings 3% (Filippi et al., 2018). The most prominent genetic effects are located in the human leukocyte antigen (HLA) class I and II region, although around 200 gene variants can affect the risk of MS, with different combinations further affecting the risk (Gourraud et al., 2012). Many of these variants encode immune system molecules such as immune modulators/pro-inflammatory cytokines. Single nucleotide polymorphisms (SNPs) in non-HLA-genes such as CD58 on chromosome 1p13 are also implicated in the disease. CD58 gene encodes for a costimulatory molecule LFA-3 responsible for interacting with the CD2 protein-ligand, which enhances CD4+ and Treg function (Jordan et al., 2014); therefore, playing a vital role in maintaining the immune response and tolerance towards self-antigens. SNPs in this gene may lead to a failure of immune tolerance, therefore, being associated with MS (Ching et al., 2019; Filippi et al., 2018).

The symptoms of MS:

The main clinical findings in MS are sensory disturbances such as paraesthesia, diplopia, ataxia, dysesthesias, and vertigo. On some occasions, these disturbances will resolve themselves; however, they can progress into chronic neuropathic pain (Toosy, Mason and Miller, 2014). Optic neuritis is also a common sign of MS, which is usually associated with impaired vision as mentioned above.

Symptoms In MS patients most commonly come to light between 20-40 years old, with no two patients having the same symptoms. The inflammatory pathology of MS can occur in many different CNS systems and involve both the adaptive and innate immune system.

Types of MS:

There are four different types of MS consisting of the following four major categories: Relapsing-Remitting MS (RRMS), Secondary-progressive MS (SPMS), primary-progressive MS (PPMS), and Progressive relapsing MS (PRMS) (Goldenberg, 2012).

RRMS: This is the most common form of MS, affecting around 80% of patients with the main characteristic being relapses of symptoms followed by periods of remission where the symptoms improve.

SPMS: This form of MS is usually a progression of those with relapsing-remitting MS. These patients will experience a disease progression along with its symptoms without and periods of relapse of less severity of symptoms.

PPMS: Affects almost 10% of MS patients and is much more resistant to treatments in comparison to RRMS and SPMS, with the characteristic of having symptoms that get worse slowly over time without any remissions or relapses.

PRMS: is the rarest form of MS, with less than 5% being affected. In this form, the disease is progressive from the beginning with no remissions and intermittent periods of worsening symptoms throughout its course (Goldenberg, 2012).

Studies have shown that more damage occurs in the first year of MS than in the five years after; therefore, it is imperative to get diagnosed and treated as early as possible. The Posner criteria had been used in MS diagnostics since 1980; this criterion

stated that the individual must show evidence of white matter lesions in two or more

sites in the CNS and suffered two relapses typical to MS; however, due to many

misdiagnoses, the McDonald criteria was implemented in 2010 (Thompson et al.,

2018). This new classification system was reviewed and published in 2017 by the

international panel on the diagnosis of MS. The main difference is the implementation

of clinical and laboratory elements (Murray, 2006); such as MRI and cerebrospinal fluid

analysis speeding up the diagnostic process, resulting in earlier diagnosis confirmation

that improves patient longevity through earlier application of disease-modifying

treatments (Omerhoca et al., 2018).

MS pathology:

The main hallmark of MS are lesions that can occur in both white and grey matter,

with white matter lesions being most harmful to the individual. White matter lesions

are most commonly found in the corpus callosum, cerebellar peduncles, spinal cord,

and optic nerves (Huang, Chen and Zhang, 2017). These lesions are the result of the

demyelination of myelinated structures in the brain through auto-immune

inflammation. In MS, the breakdown of the Blood-brain barrier (BBB) is well

established mainly due to pro-inflammatory cytokines and chemokines that are

produced by endothelial and other resident cells. In a healthy patient, the BBB will stop

specific molecules and cells from crossing into the CNS; however, in MS, the disruption

caused to the BBB will lead to an influx of activated cells such as B-cells and

macrophages. These activated cells will make their way into the CNS, leading to further

demyelination via inflammation. These are the most common characteristics of early-

onset MS, and although they still exist, demyelinated plaques are less common in

those with PPMS and SPMS (Filippi et al., 2018).

There are four main pathological features of MS 1) inflammation known to be the main trigger of events that lead to tissue damage in the CNS. 2) demyelination, which is the main hallmark of MS in terms of lesions, this refers to the process where the myelinated axons or oligodendrocyte cell body is severely damaged due to the inflammatory process, 3) axonal loss & damage and 4) gliosis which is the proliferation of glial cells in response to CNS insult (Huang, Chen and Zhang, 2017). Neuroinflammation is a key pathological event then occurs in MS causing demyelination. Although the exact mechanism of inflammation can't be reproduced in experiments LPS has been widely used in models as a gold standard of inflammation. Zhao and co (2019) had used LPS as a model of inflammation for MS, the LPS had activated the microglia and caused an upregulation of pro-inflammatory cytokines resulting in the cognitive impairment of mice (Zhao et al., 2019).

MS treatments:

As MS is an inflammatory disease, the most common anti-inflammatory treatments include interferon-beta (IFN-b), glatiramer acetate (GA), and corticosteroids. These treatments are mainly used to treat RRMS and SPMS; however, there have been no current effective treatments for PPMS and PRMS (Loma and Heyman, 2011).

The most popular out of the three mentioned is Interferon beta, an MS treatment and FDA approved from 1993; with sufficient evidence to show that it reduces the relapse rate and disability progression in MS sufferers. Interferon beta is effective in both RRMS and SPMS, the former being more effective. The exact mechanism is unknown; however, it is thought to reduce T cell activation and proliferation and have many other immunological effects, such as reducing the production of inflammatory

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cytokines while up-regulating anti-inflammatory cytokines via increased suppressor T cell activity (Dhib-Jalbut and Marks, 2009).

Glatiramer acetate, also known as Copaxone, is another standard anti-inflammatory treatment that has been approved for RRMS. Further studies into this treatment show that glatiramer acetate increases anti-inflammatory cytokines such as IL-10, TNF-alpha, and IL-4, which shows that it may switch the lymphocyte population from a Th1 to a Th2 state (pro-inflammatory – anti-inflammatory) (Tselis, Khan and Lisak, 2007).

This treatment is administered subcutaneously regularly and has had positive effects on MRI scan assessments. The possible symptoms include chest tightness, palpitations, dyspnea, and tachycardia (Loma and Heyman, 2011). These symptoms have been deemed safe and not threatening to the patient, with them occurring within the first 30 minutes of administration.

Corticosteroids also known as glucocorticoids such as methylprednisolone (MP) are heavily used to treat MS. They mimic cortisol which is a hormone produced by the adrenal glands and has anti-inflammatory effects leading to a reduction in relapses and management of exacerbations; through the inhibition of multiple inflammatory pathways responsible for producing cytokines, enzymes, and proteins (Krieger, Sorrells, Nickerson and Pace, 2014).

In MS, relapses are known as the manifestation of new inflammatory activity in the CNS with methylprednisolone being used since 1957 to counter this inflammation. The exact role of the anti-inflammatory mechanism of methylprednisolone is unknown; however, the best understanding is that the receptors it binds to is glucocorticoid receptors which can be found in the majority of the body and CNS. When activated via cortisol or MP, it will translocate to the nucleus and act as transcription factors binding

to glucocorticoid response element (GRE) inducing responses in immune function and metabolism (Krieger, Sorrells, Nickerson and Pace, 2014). Studies have proven that the transcription of these genes results in an upregulation of IL-10 and K-B inhibitor, which elicits anti-inflammatory effects which can reduce the demyelinating effects that inflammation has on the white matter (Krieger et al., 2014; Sloka and Stefanelli, 2005).

White matter:

The CNS consists of white matter and grey matter, with the former making up ~50% of the total brain volume in humans; containing axons, myelin-producing oligodendrocytes, astrocytes, and microglia, all of which play a significant role in the CNS allowing healthy brain function (Blumenfeld, 2010). The majority of axons present in white matter are myelinated, with the lipid-dense myelin sheath giving white matter its characteristic colour (Sampaio-Baptista and Johansen-Berg, 2017).

Axons surrounded by an insulating myelin sheath can be referred to as myelinated axons; the myelin sheath contains periodic unmyelinated gaps called nodes of Ranvier. In conjunction with the myelin sheath, these nodes increase the speed of an impulse sent through the neuron; this occurs by the pulse jumping from one node to another. This fast transmission is called saltatory conduction, which has a velocity of up to 150m/s compared to the velocity of an unmyelinated axon which is between 0.5-10ms (Purves and Williams, 2001). Axon diameter, internode length, ion channel density, and myelin thickness are all features that can further affect the speed of conduction. Myelination is essential for the correct functioning of the CNS as these finely calibrated conduction speeds are necessary for the coordination of signals across long distances (Sampaio-Baptista and Johansen-Berg, 2017; Moore et al., 2020).

Damage that may occur to the myelin sheath will slow down or even stop nerve impulses down myelinated axons. The short-circuiting of the impulses caused by myelin lesions will lead to various impairments such as sensory impairments (e.g.vision), difficulties in movement control and bodily functions, all of which are implicated in multiple demyelination disorders, especially MS (Susuki, 2013).

Oligodendrocyte precursor cells (OPC):

OPCs are a subtype of glial cell and are the fourth largest group of glia comprising 9% of cells found in the CNS (Dawson, 2003). These OPCs will migrate throughout the spinal cord, where they differentiate into fully functioning oligodendrocytes that specialise in myelin formation with any excess oligodendrocytes undergoing apoptosis (Bradl and Lassmann, 2009). In the brain, OPCs migrate from 3 main regions in the following order; Medial ganglionic eminence, lateral ganglionic eminence, and finally, the postnatal cortex. During development, the different lines of OPCs are in high competition with each other and will compete for the limited number of growth factors available. Regardless of which line of cells the OPCs initially belonged to, their migration is tightly regulated. There are many known regulatory signals involved in their migration, such as growth factors such as PDGF or FGF, chemotrophic netrins, and the chemokine CXCL 1 (Kettenmann and Verkhratsky, 2011); with the exact process in which these molecules play a role in OPC migration being unknown. Although most OPC will mature into oligodendrocytes, some remain in the mature brain and are therefore known as adult OPCs whose main function is remyelination which may occur due to various demyelinating diseases or traumatic brain injury (Bradl and Lassmann, 2009).

Although OPC have the capacity for remyelination through maturation into oligodendrocytes they also have a non-myelinating role through obtaining an immunomodulatory capacity. It is thought that OPCs express cytokine receptors and can respond to inflammatory cues. OPCs are also known to migrate to sites of injury which can result in cytotoxic death due to exposure to IFN-y . Interaction with pro-inflammatory cytokine IFN-y will cause the OPC to cross-present antigens to cytotoxic CD8+ T cells resulting in cytotoxic death. This pro-inflammatory phenotype has shown to promote tissue damage and block remyelination in-vitro and in-vivo (Kirby et al., 2019), and may be responsible for the fast acting in-vitro myelin disruption seen when CD1 optic nerves are exposed to LPS.

Studies have shown that the electrical activity of neurons is an important signal to initiate myelination. Gyllensten and Malmfors (1953) compared the myelinated neurons of mice reared naturally and mice reared in the dark; they concluded that light induces electrical activity in neurons that upregulates myelination (Gyllensten and Malmfors, 1963). Neuhoff et al. (1980) studied the effect of premature eye-opening in rabbits' optic nerves, which had shown to accelerate myelination, further supporting the use of light and neuronal electrical activity in remyelination (Tauber, Waehneldt and Neuhoff, 1980).

Oligodendrocytes:

Oligodendrocytes are a type of neuroglia and are the myelinating cells of the CNS (Bradl and Lassmann, 2009). In mice, myelin formation is initiated at birth and becomes complete around two months old; therefore, the mice used in this experiment will be adults in the range of 3-6 months old.   In the central nervous system these oligodendrocytes are responsible for the assembling of myelin proteins and production of the myelin sheath. The myelin sheath is an extension of the oligodendrocyte and acts to wrap around the nerve axon providing trophic support (Kuhn et al., 2019) .

When a mature oligodendrocyte connects to an axon the architecture of its plasma membrane changes to wrap the axons forming a compact myelin sheath. Studies using in-vivo imaging and 3D reconstructions have been used to explain and visualise the process of myelination (Snaidero et al., 2014). They had revealed that myelination of optic nerves occurs via plasma membrane extension laterally down the axon to form paranodal loops as shown in figure 1.
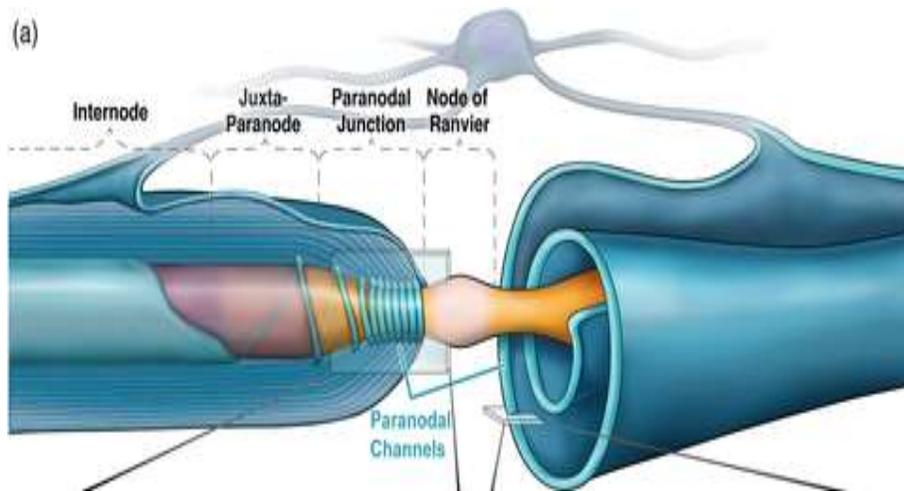
Figure 1: (a) Shows the organisation of the myelin sheath by oligodendrocyte myelination, which consist of compacted layers of plasma membrane that extend laterally down the axon and wrap around the axon (yellow). The edges of each sheath which end at the node of ranvier is characterised by a cytoplasm-filled channel (paranodal channel) that spirals around the axon, continuing along it. The image on the right conveys a partially unravelled sheath (Jarjour et al., 2020).

The main function of oligodendrocytes is the creation of myelin throughout development, adaptive myelination and remyelination after damage has occurred, with OPC being responsible for acting as a reserve ready to generate new oligodendrocytes.

Ca^{2+} activity is the one of the main factors that drives the myelin sheath formation in oligodendrocytes, which is why glutamate excitotoxicity that causes disturbances in Ca^{2+} regulation may greatly affect oligodendrocyte function. Ca^{2+} activity can also regulate neuronal activity which in turn regulates OPC proliferation, differentiation and survival.

Our knowledge of the oligodendrocyte biology of humans is limited but the main differences between mice and human brain/oligodendrocytes are thought to be as follows:

1) Regions of the human brain differ to mice, such as the neocortical regions being absent; and on the contrary, the olfactory bulbs are underdeveloped in humans but mature in mice (Jakovcevski, 2009).

2) Due to humans having a much longer life span and a more complex brain, the time scale for myelination is considerably different, with it taking decades in humans compared to a few weeks in rodents (Semple et al., 2013).

3) Humans have a much larger number of oligodendrocytes in the CNS (Valério-Gomes, Guimarães, Szczupak and Lent, 2018).

4) Cellular and molecular differences; for example, most rodent OPCs contain CXCR2 receptors. These receptors become activated via the chemokine CXCL1 contributing to inflammation, however, these receptors are rare in human OPCs (Bradl and Lassmann, 2009).

Optic nerve:

The optic nerve is a myelinated axon tract and mainly consists of retinal ganglion axons and glial cells. Optic nerves are considered part of the CNS and are responsible for transmitting visual information from the retina to the brain, including colour perception, brightness perception, and visual acuity (Vilensky, Robertson and Suárez-Quian, 2015). Oligodendrocytes can be found along the axis of the nerve with their processes extending orthogonally and radially to separate axons as shown in figure 2

(Butt et al., 2004), although oligodendrocytes play a vital role in the myelination process they also provide the nerve with structural support.
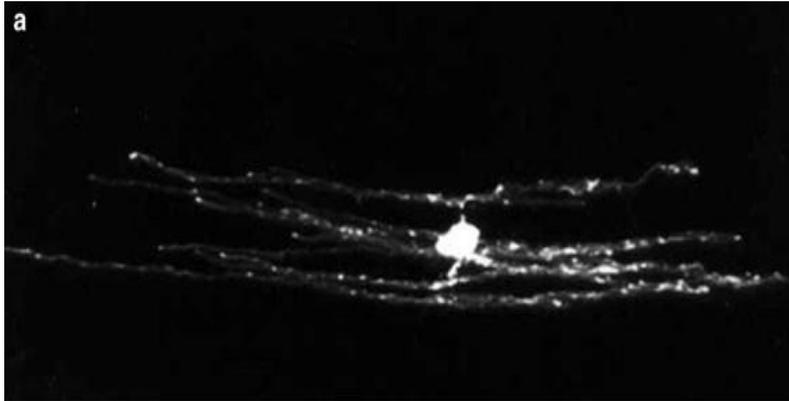
Figure 2: Confocal image obtained from a rat optic nerve oligodendrocyte. Shows the oligodendrocyte cell body with its processes which travel both down the axon and orthogonally to adjacent axons (Butt et al., 2004).

Damage to the optic nerves can cause permanent and severe loss of vision; with different portions of the optic nerve being related to different types of visual loss. Regarding multiple sclerosis, optic neuritis can be one of the initial signs of the disease. Optic neuritis is a demyelinating inflammatory disorder of the optic nerves which causes pain with eye movement and temporary or complete vision loss. Optic neuritis can be one of the first biomarkers of MS; with MRI scans showing that there is a significant risk of having MS if optic neuritis is present (Kale, 2016), although this is not the case 100% of the time.

The etiology of optic neuritis includes inflammation, infections, and genetic disorders, although idiopathic optic neuritis is common, referring to an unknown etiology. Once a patient has been diagnosed, both acute and long-term therapies are put in place. The most renowned treatment is the corticosteroid methyl-prednisolone (MP), which has

successfully treated optic neuritis relapses (kale, 2016). However, some patients are resistant to high-dose MP; therefore, plasma exchange is used as an alternative management strategy to significantly improve symptoms and vision loss (Roesner et al., 2011).

LPS:

Lipopolysaccharide (LPS) is the main component of gram-negative bacteria. It consists of a lipid, polysaccharide comprising an inner core, outer core and an O-antigen. The O-antigen is the part of the bacteria that gives protection against various dangers. The O-antigen not only protects the bacteria but is also responsible for stimulating our immune system through acting as an immunostimulatory molecule, eliciting an inflammatory response via multiple pathways (Triantafilou and Triantafilou, 2002).

LPS, once in the body, acts as a pathogen associated molecular pattern (PAMP) and can bind to the CD14/TLR4/MD2 receptor complex, which can be found in many immune cells such as dendritic cells, monocytes, b cells, and macrophages. Once the receptor is activated, it causes the secretion of pro-inflammatory cytokines, which have detrimental effects on the body (Bertani and Ruiz, 2018).

In the following experiments LPS was used as a model of inflammation on optic nerves eliciting similar demyelinating effects as MS. Cai et al., (2019) has shown that LPS application in mice produces CNS lesions in-vivo mice through the specific polarisation of microglia. These lesions are theorised to cause neuroinflammation and myelin damage through phagocytosis of apoptotic cells, pro-inflammatory cytokine, ROS and chemokine production, as well as the death of oligodendrocytes (Cai et al., 2019).

Microglial responses to LPS in rodents has also shown to significantly increase inflammatory cytokine production and Ca^{2+} related molecules, which would cause both inflammation and glutamate excitotoxicity, resulting in cell lysis and lesion formation, disrupting the myelin both directly and indirectly (Lively and Schlichter, 2018).

Therefore, LPS is responsible for the activation of microglia which is an important feature of neuroinflammation (Zhao et al., 2019). Although mild activation of microglia can play a protective role in response to damage, excessive activation of microglia can significantly contribute to neuroinflammation and myelin disruption through calcium and cytokine-mediated pathways , which is present in many neurodegenerative diseases (Ye et al., 2020).

Innate & adaptive immunity:

Innate immunity is known as the first line of defence and is responsible for initiating adaptive immunity. The main role of innate immunity is to remove pathogens and produce cytokines needed for T cell proliferation; this occurs in a non-antigen specific manner (Hemmer, Kerschensteiner and Korn, 2015). Dendritic cells, mast cells and natural killer cells are involved in innate immune reactions, with the most common immune cells found in MS lesions consisting of macrophages and microglia (Auffray, Sieweke and Geissmann, 2009). Microglia arise from primitive myeloid progenitors and migrate to the CNS during embryonic development, where they play essential roles in inflammation and CNS homeostasis.

Due to MS being a neuroinflammatory disease microglia are very important in both the protective and harmful effects that occur in the disease. The CNS and optic nerves do not contain many extrinsic immune cells and the most abundant immune cells that exist are the microglia (Yang et al., 2010).

Microglia are known to populate between 5-20% of the adult CNS and between 5-12% in mouse CNS and are essential for the immune regulation of the CNS by providing significant innate and adaptive immune responses. These are different from macrophages as they are thought to derive from myeloid precursors in comparison to macrophages which derive from monocytes (Guerrero and Sicotte, 2020) . The initial theory of proinflammatory (m1) and anti-inflammatory (m2) microglia is now considered invalid as there is evidence that shows that microglial phenotypes are transient and demonstrate temporal and spatial evolution (Guerrero and Sicotte, 2020).

Under healthy/normal conditions, the microglia number and function is strictly regulated by interactions with surrounding cells and the local microenvironment, with the main functions involving pruning of synaptic networks, apoptosis and the secretion of growth factors (Yang et al., 2010). When an insult occurs, for example in the progression of MS, activated microglia may shift into various functional states, in which functions such as proliferation, morphology, antigen presentation and phagocytic activity which dictate the release of inflammatory cytokines and chemokines (Bachiller et al., 2018). One way the microglia may become activated in-vitro methods is the application of LPS. Once the LPS binds to the microglia cell TLR4 receptor a chain of events occur which result in the translocation of Nf-kB to the nucleus where pro-inflammatory gene expression occurs producing cytokines such as TNF-a, COX-2 and IL-6.

The inflammatory responses and cytokine production caused by the innate immunity initiates the adaptive immunity, which acts in an antigen-specific manner. The adaptive immune response involves T and B cells with antigen-specific receptors on their cell

surfaces, and both play a role in autoimmunity. T cells can be further categorised into two main subsets: CD4+ and CD8+ T cells (Hemmer, Kerschensteiner and Korn, 2015).

MS is known mainly as an auto-immune disease implicating both T cells and B cells in the pathogenesis and progression. The exact mechanism of damage and role of these cells is unknown; however, many studies indicate that they are involved. CD4+ T cells have long been considered the basis of the disease as there have been substantial genetic risks involving the MHC class II locus. T cells are an essential part of the adaptive immune system, with each T cell expressing a specific T cell receptor (TCR). These cells mature in the thymus, where they become CD4+ or CD8+ dependent on what MHC complex they are restricted to (MHC class II and MHC class I, respectively) (Kaskow and Baecher-Allan, 2018). These T cells then undergo negative selection, which involves the deletion of the cells that can strongly recognize self MHCs; this process is key to our immune function as it halts the production and release of autoreactive T cells (Stritesky et al. 2012; Mingueneau et al. 2013).

The majority of self-reactive T cells are killed; however, there is one population of T cells with auto-reactive characteristics called Tregs, which play a fundamental role in maintaining immune homeostasis through the suppression of other immune cells (Sakaguchi et al. 2007).

The naive T cells will circulate in the blood and lymphatic system, waiting to come across their complement antigen. Once interaction between the specific antigen and MHC complex takes place, they differentiate into effector T cells. Of the population, some T cells will become memory cells, increasing the speed of effector T cell production the next time it comes across the same antigen. Both the interaction between the antigen and the antibody, along with the type of cytokine produced by

antigen presenting cells (APCs) such as dendritic cells, macrophages and B cells, will dictate the type of effector T cell produced, such as CD4+, CD8+ and Tregs. CD4+ are the main effector T cell-related to MS. These can be divided into three different subsets, Th1, Th2, and Th17, which regulate cytokine production (Arbour and Prat, 2015), along with cytotoxic T cells for CD8+, which primarily kill cells that show non-self-antigens on their MHC class I complexes (Kaskow and Baecher-Allan, 2018).

Effector T cells in the central nervous system (CNS):

CD4+ and CD8+ effector T cells are responsible for establishing and maintaining an inflammatory environment upon CNS entry, significantly contributing to demyelination and oligodendrocyte cell death. IL-17 and IFN-γ secreting cells activate antigen-presenting cells (APCs), resulting in an upregulation of major histocompatibility complex class I and II molecules. These are located on the APCs, which is detrimental to MS as this allows the restimulation of myelin-reactive T cells (Kaskow and Baecher-Allan, 2018). IL-17 also promotes pro-inflammatory cytokine expression such as IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor α (TNF- α). IL-17 secreting cells can also produce granzyme B, which can act to kill neurons through glutamate receptor (GluR3) interaction. IFN-γ can kill oligodendrocytes and inhibit CNS remyelination. CD8+ T cells contain cytolytic granules, consisting of perforin and granzyme, which are polarised towards demyelinated axons resulting in the death of oligodendrocytes and neurons (Kaskow and Baecher-Allan, 2018).

In relation to MS, it has been shown that MS lesions contain increased amounts of immune cells along with their counterpart cytokines and have become the main hallmark of the disease. These cells are responsible for the destruction of the BBB and

diffusion across it, thereby initiating many cascades and toxic effects such as inflammation, glutamate excitotoxicity and ROS production. This ultimately leads to loss of myelin, reduction of myelin-producing cells and neuronal cell death, thereby eliciting the neurologic dysfunction and the rise of symptoms found in MS (Frischer et al., 2009). Potent immune responses are detrimental to the CNS, which is why this is usually tightly regulated through astrocyte function and tight endothelial junctions, which both significantly contribute to the limited permeability of the BBB (Fletcher et al. 2010).

The critical difference betweenMS patients and a control group is that the auto-reactive cells in MS patients have already undergone an activation stage, as the majority of auto-reactive cells displayed a Th1 phenotype, compared to a control group that mainly contained naïve T cells, along with T cells from MS patients having a much higher affinity for MBP than the control (Kaskow and Baecher-Allan, 2018).

B cells:

The exact role that B cells may contribute to multiple sclerosis (MS) pathogenesis is unknown, but it is possible they do so in multiple ways. B cells are thought to be the source of differentiating plasma cells that may secrete autoreactive antibodies, possibly contributing to demyelination within the inflamed CNS (Häusser-Kinzel and Weber, 2019). Memory B cells can provide processed antigens to other APCs via its MHC class II complex, they are also thought to be important regulators of the immune system through the production of both anti and pro-inflammatory cytokines including IL-10 and IL-6, respectively (Lehmann-Horn, Kronsbein and Weber, 2013).

The EAE model & cuprizone model:

Due to a lack of understanding and the complexity of MS, we are unable to exactly replicate the pathogenesis of the disease. There are currently multiple experimental models which are commonly used that give an approximation of the main pathological features of MS, such as its inflammatory processes and demyelination. Many of the current treatments for RRMS have been developed and validated on the experimental auto-immune encephalomyelitis (EAE) model (Mix, Meyer-Rienecker, Hartung and Zettl, 2010).

The EAE is one of the leading models for studying MS and the testing/ development of drugs. The main difference between MS and the EAE model is that in the EAE model, the inflammation is artificially induced via external immunization, whereas the inflammation that occurs in humans is not. The external immunization that occurs in the EAE model is usually induced by known antigens, such as myelin protein (MBP) or spinal cord homogenate (SCH) or a passive transfer of myelin-specific T lymphocytes, which induces the development of myelin-specific T cells, immune cell influx into the CNS and direct inflammation and damage in the CNS (Torkildsen, Brunborg, Myhr and Bø, 2008). The onset of the disease replication usually occurs between 1-2 weeks (Constantinescu et al., 2011).

The EAE method was introduced in the 1930s by Rivers et al., (1993) but has since remained a prevalent model (Rivers, Sprunt and Berry, 1933). The EAE model is used in a wide range of species, especially rodents and monkeys, and is known to significantly vary in its experimental conditions such as species, strain, age, sex of animals, and specific induction methods. (Gold et al., 2006).

The best correlations between EAE and MS therapies are those using IFN-beta and GA.

The first correlation between the EAE model and MS was in 1982, which found that

IFN-beta was effective in 50% of MS patients (Jacobs et al., 1982). Around 25 trials

involving EAE with IFN-beta have been conducted, with the overall effect being

positive, although the effects had worsened in a small number of these studies.

(Farooqi, Gran and Constantinescu, 2010). Developed In 1971 by Teitelbaum, GA had

shown that it could effectively block the immunological effects of the EAE model. Its

most recent attention has been to its suppressive effects on Th17 development as well

as speculation of its roles in neuroprotective and remyelinating properties. (Arnon and

Aharoni, 2009).

The points stated above suggest a substantial body of evidence of concordance

between the EAE model and MS pathology and treatment. However, there have been

examples where EAE treatment success has not been followed through with accepted

MS treatments, for example, an approach to enhance anti-inflammatory cytokines

such as IL-10 and TGF-beta had shown to suppress the inflammation effects in the EAE

model, but when transferred to MS patients, were unsuccessful and caused adverse

side effects (Rott et al., 1994). Due to the abundance of evidence supporting the role

of pro-inflammatory cytokines in MS, cytokine therapy had been a popular therapy to

limit its pathogenesis, leading to early MS trials using an IL-1 receptor antagonist. This,

in theory, would compete with the pro-inflammatory cytokine IL-1; however, although

this trial was successful in the EAE model, when applied to MS patients there was

minimal success (Martin, McFarland and Boggs, 1995).

Another popular model is the cuprizone intoxication model, which mimics the acute

and chronic pathology of MS. Cuprizone is a copper chelator that inhibits the copper-

dependent mitochondrial enzyme cytochrome oxidase and monoamine oxidase

(Torkildsen, Brunborg, Myhr and Bø, 2008). The exact reason is unknown, but

cuprizone specifically targets the oligodendrocytes resulting in demyelination, with the

adequate dose being both strain and age-dependent. Slight damage to the BBB has

been shown to take effect in the cuprizone model; however, the T and B cells of the

adaptive immune system is thought to play the main role in the demyelination that

occurs; therefore, the cuprizone model reflects several essential characteristics of

progressive forms of MS as T and B cells (Zhan et al., 2020).

Fluoromyelin:

Fluoromyelin is a water-soluble fluorescent dye that stains lipids. Fluoromyelin red is

also photostable which indicates that it is a good reagent for confocal imaging. It is

widely used in-vitro for myelin staining, although this dye can't be applied in-vivo as

the BBB is impermeable (Wu et al., 2006). The myelin sheath has a very high lipid

density; therefore the dye will be able to stain the myelinated axons much more

intensely than any other membrane. This is shown by Brown and Monsma (2012),

who, when administering fluoromyelin red to medium containing cultures of Schwann

cells and neurons, lead to the staining of myelin sheaths with no adverse effects of the

myelinated cells or axons (Monsma and Brown, 2012). Other studies such as Fern et al

(2018) use fluoromyelin red along with other fluorescent biomarkers to measure the

myelin disruption that occurs when excess glutamate is released from central axons.

Similar to that of Choi et al (2018) who use the same fluoromyelin red stain to

determine demyelination and remyelination in a mouse model of MS (Sapkota, Park

and Choi, 2019). Proving that fluoromyelin red is a reliable measure of myelin integrity.

The use of the fluoromyelin lipid stain and confocal microscopy is very quick in comparison to electron microscopy techniques, with an optimum staining time of 1-2 hours, depending on the protocol. The live specimen can be imaged to a good standard of detail in colour, as EM is unable to analyse live specimens and can only produce black and white images, despite images being of a higher quality. Another advantage of Fluoromyelin is that it can be used in conjunction with other fluorescent markers such as GFP+ to give a multi-coloured image overlay, which can show multiple cell structures such as myelinated axons and oligodendrocytes. This creates a better visualisation of myelin and allows comparison of multiple cell organelles.

Hypothesis and aims:

Hypothesis: Acute LPS exposure to Ex-vivo mice optic nerves will cause myelin disruption to both myelinated axons and oligodendrocyte cell bodies.

To date, there are not many treatments that are effective for the progressive form of MS, and current strategies to slow down or even stop the progression lies within the improvement of innate immune responses and strengthening the neuroprotective pathways. Acute myelin pathology (lesions) is the main hallmark of this disease and current research has successfully produced models of acute myelin pathology. This project will explore the potential for using LPS-mediated injury of the myelin sheath and oligodendroglia as an effective model on par with models such as the EAE model and cuprizone model inducing white matter injury faster, with it being less time consuming and more practical for rapid drug screening.

Chapter 2. Methods:

The following work was carried out in a lab with the appropriate Personal protective equipment (PPE), including the use of lab coats, gloves, and masks to ensure no contamination or injuries occurred, thereby increasing safety and reducing the number of anomalies produced. All mice used were housed in humane pathogen-free conditions at Plymouth University. Mice were housed six per cage and selection ensured no mice were left alone for longer than 24 hours to limit any stress caused. The LPS used throughout this experiment was obtained from Sigma (batch #019M4009V).

Preparation of solutions:

Before the experiments could be conducted, multiple solutions were premade, including phosphate-buffered saline (PBS), 4% paraformaldehyde (PFA) solution, and artificial cerebrospinal fluid (aCSF). PBS is a water-based salt solution containing disodium hydrogen phosphate, sodium chloride and, in some cases, potassium chloride and potassium dihydrogen phosphate. This solution acts as a buffer solution as it maintains the pH, osmolarity, and ion concentration of that found in the human body, making it a suitable solution to store tissue overnight (Perchetti et al., 2020). PFA solution causes cross-linking of protein molecules and cell membranes, making it a renowned fixing agent (Kim, Kim, Okajima and Cho, 2017). aCSF is a buffer solution that is used to immerse isolated brain regions. In relation to this experiment, it keeps the structural integrity of the optic nerves throughout the transportation and duration of the experimentation by maintaining pH, osmolarity and oxygen supply to isolated tissue (Hájos and Mody, 2009).

1 x PBS was created using the following:

Distilled water	800 mL
Sodium Chloride	8 g
Potassium Chloride	200mg
Sodium phosphate	1.44 g
KH_2PO_4	245 mg
Desired pH	7.4
	Add distilled water until 1 L

Table 1: Reagents used for preparation of 1xPBS solution.

4% PFA solution was made using the prepared 1x PBS solution as the solvent, mixed

with PFA powder at a ratio of 4g to 100mL PBS solution. The solution is then labelled

PFA and taken to a fume cupboard, where it is heated overnight at 60°C, ensuring the

flask is partially open to allow the heat to escape. Once heated for 24 hours, the 4%

PFA solution can be stored in a fridge until it is required.

The aCSF used throughout this research was created by adding the following

ingredients to 500ml of ddH2O:

Sodium Chloride	7.36 g
Potassium Chloride	0.22 g
Sodium phosphate monobasic	0.235 g
Magnesium Chloride	0.19 g
Sodium Bicarbonate	2.18 g
Calcium solution (1M)	2 ml
Glucose	1.8 g

Table 2: Reagents used in the preparation of aCSF.

All contents would be individually added using a weighing boat which would be rinsed

with ddH_2O between each use to ensure the weight of each substance was accurate

and to eliminate contamination. The contents would then be mixed thoroughly,

correctly labelled, and stored in a 4°C fridge.

Animal Selection & dissection:

Before commencing the experiment, the aCSF is placed on ice and bubbled with O_2 for 20-30 minutes. This oxygenates the solution allowing the optic nerve to survive longer after dissections, reducing any damage that may occur. The incubator would be preheated and have an oxygen pump inside to allow O_2 flow throughout the experiment.

A CD1 adult mouse would be randomly selected to initiate the experiment and undergo the schedule one procedure, in compliance with ethical regulations in place for the research animals. This involved suffocation for 5 minutes using CO_2, then confirming the kill via neck dislocation. This procedure was used for every animal throughout these experiments. Once confirmed dead, the mouse was transported to the fume cupboard and dissected, and the optic nerves extracted using appropriate equipment. This was done efficiently in a time-sensitive manner to ensure the optic nerves remain in the best condition. The longer it takes, the more damage that may occur to the nerves through lack of blood supply and essential nutrients. The optic nerves were then transferred to a small tube containing aCSF on ice to preserve the nerves while under transportation.

Fluoromyelin red staining:

aCSF is pre-heated to 37°C (+/- 0.5°C), then 3ml is pipetted into the two middle wells of a 12 well-plate (shown by 'B' in figure 3), with 2.5ml applied to the outer wells (shown by 'A' in figure 3). 25µl of fluoromyelin red stain is applied to the outer wells, and the optic nerves are then transferred over. The optic nerves were left in the FMR/aCSF solution for an hour staining period. Fluoromyelin red (FMR) is a water-soluble fluorescent dye that has a specific selectivity for myelin. It has a peak absorbance value

of around 560nm; therefore, this wavelength was used in the confocal methodology. The temperature of the solution inside the wells is regulated by a heat probe, ensuring it stays at 37°C +/-0.5°C. Due to the FMR being light-sensitive, a black sheet is placed across the front of the incubator to prevent light penetration.
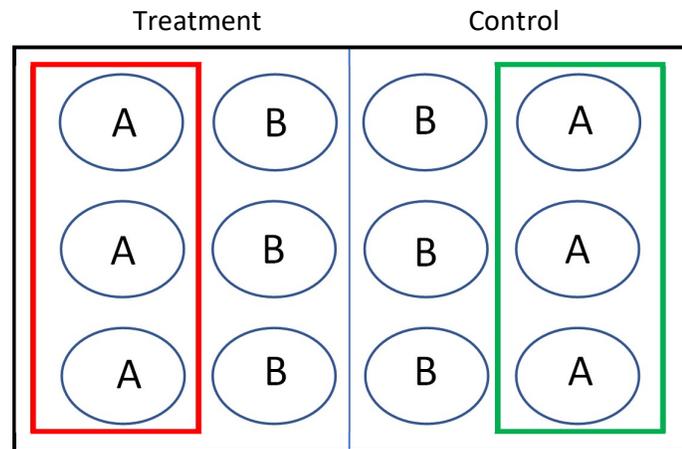
Figure 3: Contents of 12 well-plate throughout experiment. A- 2.5mL of aCSF with 25μL FMR where optic nerves are kept for 1-hour staining period and B- 3mL of aCSF. During treatment, the FMR and aCSF are replaced with A (red)- 3ml of LPS treatment, and A (green) 3ml of aCSF as a control. B wells used to suspend optic nerves between stages.

LPS application & PFA fixing:

During the staining period the LPS was defrosted and bubbled with O_2. After the staining period, the optic nerves are transferred to the middle wells, and the FMR solution is removed from the outer walls with a vacuum and replaced with 3ml of aCSF on the right side and 3ml of LPS into the left, as shown in figure 3 by the green and red box respectively. The concentration of LPS would vary depending on the experiment (0.1 μg-mol - 1 μg-mol). The use of LPS is to cause myelin disruption similar to that of

the myelin degradation found in all neurodegenerative diseases. LPS has proven to cause white matter disruptions in a rapid time frame as 180 minutes of LPS exposure has been used in rodent neonatal studies which resulted in the cell damage of oligodendroglia and myelin injury (Sherwin and fern, 2005).

The mesh holders would then be transferred to the outer wells, with the heat probe on the control side and returned to the incubator for a 2 hour insult period. After the 2 hours, the optic nerves were removed from the incubator and then transferred into micro-centrifuge tubes that contain 4% PFA then stored in a 4°C fridge for a 1-hour fixation period. This took place inside a fume cupboard as PFA is toxic.

Once the fixation period is over the optic nerves were transferred to a small petri dish containing PBS until required for confocal microscopy.

Confocal microscopy:

Confocal microscopy is an optical imaging technique that increases the resolution of images using a confocal pinhole that is efficient in blocking out of focus fluorescent light. Due to the confocal microscope (LEICA) being very light-sensitive, blackout curtains had to be used, ensuring the room was as dark as possible, removing any light variables.

To ensure the images are of the best quality, the laser must be set appropriately. If it were too low, the quality of the image would be inadequate, and if set too high, although the image would be of high quality, the tissue would be prone to bleaching if exposed to the laser for an extended period of time, which will not only damage the tissue but also affect the reliability of the images.

Initially, the optic nerves were imaged on the confocal while they were free-floating in solution with a few drops of aCSF being added to ensure the nerve did not dry out during imaging. This method was not effective as it was challenging to manage the amount of aCSF on the slide; therefore, a waterproof pen was used to ensure the aCSF would stay in a specified area. However, these images were still of very low quality; therefore, a mounting technique was acquired and used as follows:

Images of the optic nerve were taken along the surface of the nerve avoiding the ends which could have been damaged from the dissection process. Using a wide mouth pipette, the optic nerves were placed onto the middle of the microscope slide, then 2-3 drops of mounting solution were applied. Mounting grease was applied to the four corners of a coverslip and gently applied to a microscope slide, ensuring not to crush the nerve. A few attempts were needed to perfect this as if the nerve had been crushed, the images created would not be quantifiable; however, once perfected, the quality of the images was significantly better, and therefore this mounting method was used.

The Confocal was set up with the following settings and remained constant throughout all experiments to eliminate variables:

- bidirectional - on

- focus - 1

- Pinhole size - 1 AU

- laser strength - 15.7

- gain - 820

This protocol was used as a basis for five experiments with minor changes between each one as identified in the following:

Experiment 1: Used 0.1 μg-mol of LPS to see if any acute myelin disruption occurred in CD1 adult mice.

Experiment 2: 1μg-mol of LPS was used to identify myelin disruption in CD1 adult mice.

Experiment 3: 1μg-mol LPS treated with a final concentration of 10μm Methylprednisolone (obtained from Bio-techne) on CD1 adult mice.

Experiment 4: PLP-GFP negative adult mice were used for the 1μg-mol LPS.

Experiment 5: PLP-GFP positive adult mice were also used to look at myelin & oligodendrocyte damage, with a concentration of 1μg-mol LPS.

For creation of the stacked images in experiment 5. The following additional stack settings were required on the confocal microscope as follows:

- Beginning (μM) - 7.52
- End (μM) - 2.60
- Z position (μM) – 3.09
- Focus (μM) – 9.52
- Z size (μM) – 4.92

There were 12 steps for the processing of the stacked image with each step being 0.41μM in depth. Creating a stacked image consisting of 12 images with a total depth of 4.92μM. Due to the stacked images taking significantly longer than standard imaging, only three images were taken per optic nerve.

PLP-GFP genotyping:

To identify mice that were PLP-GFP positive, the PLP colony had to be genotyped for experiment 5. PLP-GFP positive mice express a green fluorescent protein in oligodendrocytes, Schwan cells and enteric glia. For this experiment, it was the expression of this gene in the oligodendrocytes which was identified, to allow us to monitor the potential damage a 120 min incubation period of LPS would have on the oligodendrocytes and their cell processes. This would allow for a detailed overlay of the FMR stained myelin and oligodendrocytes as shown in figure 8.

Initially, ear tags needed to be collected from each of the PLP-GFP mice in the animal house for DNA extraction. Following this, a lysis buffer A was made using the following:

- EDTA pH8 - 20µl

- 5M NaOH - 500µl

- Distilled water - 49.48ml

The ear notches are transferred to a microfuge tube, and 50µl of lysis buffer A was added to the tissue. Ensuring the lids are fully closed, then the tubes were placed into the hotshot machine. The DNA was isolated from the ear notches using a hotshot method. This form of DNA extraction isolates the DNA by heating the ear notch to 95°C for an hour. It was essential to ensure that there was no excess tissue in the tube as this could interfere with the extraction. Once the hot shot extraction was complete 50µl lysis buffer B was added to neutralise the extracted DNA. Lysis buffer B was made using the following:

- 1M tris HCl pH5 - 4ml

- Distilled water – 46ml

Following extraction, a PCR master mix was formulated. The following volumes are specific for one PCR; therefore, the amount was multiplied by however many PCRs are being run:

- DdH2O - 19µl

- Green buffer (GB) - 2.5µl

- Deoxynuceloside triphosphates (dNTPs) - 0.125µl

- Forward primer (FP) - 0.125µl

- Reverse primer (RP) - 0.125µl

- Taq polymerase - 0.125µl

Initially the following primers were used in the PCR MasterMix:

Forward primer: AAG GGC ATC GAC TTC AAG G

Reverse primer: TGC TTG TCG GCC ATG ATA TAG

These primers did not successfully bind the extracted DNA samples during the PCR. The exact reasons for the primers not working were unknown, but it was likely due to them being contaminated or expired as they had been in storage for a prolonged period of time. Therefore, the following primers were used:

Forward (Plp1): TGG TAA CAT GGG CTG CTT G

Reverse (EGFP): CTG AAC TTG TGG CCG TTT AC

Various concentrations of the primers were used to no success in the range of (0.1-1uM). Finally, a concentration of 0.5µM was selected as instructed by the genotyping support on the Jackson lab website.

Then 22μl of the PCR master mix was added to each microfuge tube along with 3ul of

extracted DNA sample. The pipette tip was changed each time the DNA is added to

prevent cross-contamination. This mix is then put into a PCR (G-storm) which will run a

specific programme sequence that will replicate the DNA. The following PCR sequence

was initially used:

- 95°C for 30s

- Denaturing Temp: 95°C for 30s
- Annealing Temp: 60°C for 30s   X40 cycles
- Extension Temp: 72°C for 30s

- 72°C for 10m

- 10°C for storage
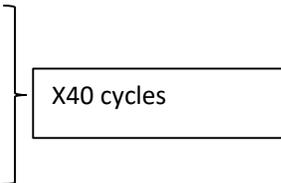
This programme was not successful as the result would show non-specific banding as

shown in figure 4.

Figure 4: An example of the non-specific banding acquired from the PCR for the PLP-positive gene, A) is the band needed to show positive results, B) is the non-specific banding acquired from the PCR.

The non-specific banding shown could have happened for multiple reasons, such as:

1) Due to a lengthened time scale, the primer could have dissociated and re-joined, causing primer dimer.

2) Due to contamination via human error by failing to change pipette tips between each sample in one of the stages of this protocol.

3) The wrong concentration of primers had been used.

4) Wrong annealing temperatures.

Thus far, the first three points had been addressed, which had still resulted in poor results; therefore, the heating and cooling programme of the PCR was altered to see if the annealing temperatures could be optimised, which resulted in the following programme being used:

- 95ºC for 30s

- 95ºC for 30s ⎤
- 70ºC for 30s ⎬ X20 cycles
- 72ºC for 30s ⎦

Then

- 95ºC for 30s ⎤
- 60ºC for 30s ⎬ X20 cycles
- 72ºC for 30s ⎦
- 72ºC for 10m
- 10ºC for storage

The results gathered from this programme were better than the original programme, however upon further refining this further, the optimum programme was found, as follows:

- 94ºC for 30s

- 94ºC for 30s ⎤
- 65ºC for 30s ⎬ X10 cycles
- 68ºC for 30s ⎦ Decreasing by 1.5ºC every cycle

Then

- 95ºC for 30s ⎤
- 50ºC for 30s ⎬ X28 cycles
- 72ºC for 30s ⎦
- 72ºC for 10m
- 10ºC for storage

Positive results were conducted with this programme, as shown in figure 5. Using a 0.5µM concentration of primers with the PCR master mix specified above.
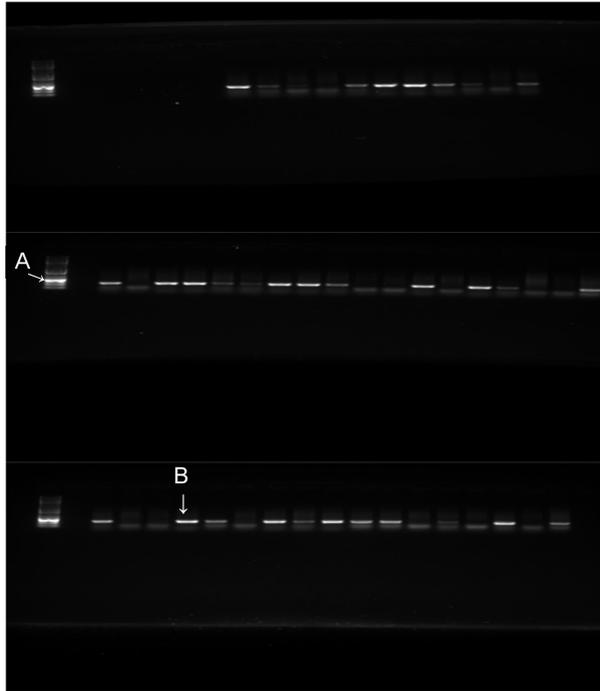
Figure 5: showing the successful results of the genotyping using the new PCR cycle mentioned in the methods. A) target banding B) PLP-positive mice result.

During the PCR process, the agarose gel is prepared using 2.5g of agarose mixed with 250ml of Tris-borate-EDTA (TBE), which is a commonly used buffer for DNA gel electrophoresis. This mixture was microwaved for three minutes, mixing periodically. Once heated, 8ul of gel red is added and further mixed. Gel red is a fluorescent nucleic acid stain used for agarose gel DNA electrophoresis. The contents would be poured into a mould containing a tip comb, producing wells for samples to be pipetted into. Once the gel sets after 30 minutes the combs were carefully removed and 12µl of the amplified DNA samples from the PCR would be pipetted into the wells. Between each pipetted sample, the pipette tip would be rinsed out, reducing the chance of

contamination. The gel would be ran for 60 minutes at 120V; once finished the gel would be carefully removed and placed into a gel imager (Syngene PXi) giving the images shown by figure 4 and 5.

Chapter 3. Results:

The central aspect of this research was to characterise rapid immune-related myelin injury using LPS. Currently, the primary model of immune activation with similar characteristics that occurs in MS such as inflammation and white matter injury is the EAE model. This model can imitate many of the features of the disease but is expensive, can cause the animals involved high levels of distress and it is also time-consuming.

Creating a model with the use of LPS in-vivo will not only take away the distress of the animals but will be rapid in comparison and therefore be a considerable advantage, giving a generation of dose-response relationships with potential drug targets, significantly faster drug testing cycle and reducing reliance on live animal experimentation.

The optic nerves used in the research were chosen as they are 100% myelinated structures, and these isolated brain preparations will retain all of the immune cascade elements, which is responsible for the myelin damage that occurs in the neurodegeneration of MS. The following results will prove that the LPS can be used as an effective inflammation mediated demyelination model to allow rapid drug testing, as mentioned above.

0.1 μg-mol LPS on CD1 mice:

Initially, a low concentration (0.1 μg-mol) of LPS was used to establish a concentration that would be effective in inducing an immunological cascade of inflammation and therefore damage the myelin of the CD1 optic nerves. Both images in Figure 6 show the myelinated axons, which can be identified by the ↓ shown in A and B portraying fluorescent red wave-like structures which travel throughout the nerve. FMR cannot penetrate the glial somata; therefore, black circular patches will indicate where cell bodies may be. The images are similar in fluorescence intensity and staining pattern which indicates that minimal damage has occurred. This can be verified by comparing the mean fluorescence measured using image J software, which was similar for the control optic nerves (24.68 +/- 5.00) and for the optic nerves treated with 0.1μg mol LPS (25.78 +/- 6.22), as shown in the graph in figure 6. The difference between control and treatment was not significant, which can be shown in the graph as the error bars do not overlap, and a paired t-test between the groups supports this with a p-value of 0.666. This was expected as the optic nerve being exposed to such a low concentration for such a short time period should not cause much inflammation. Due to this, a higher concentration of LPS was used with regard to previous literature (Zhang et al., 2013; Sherwin and Fern, 2005).
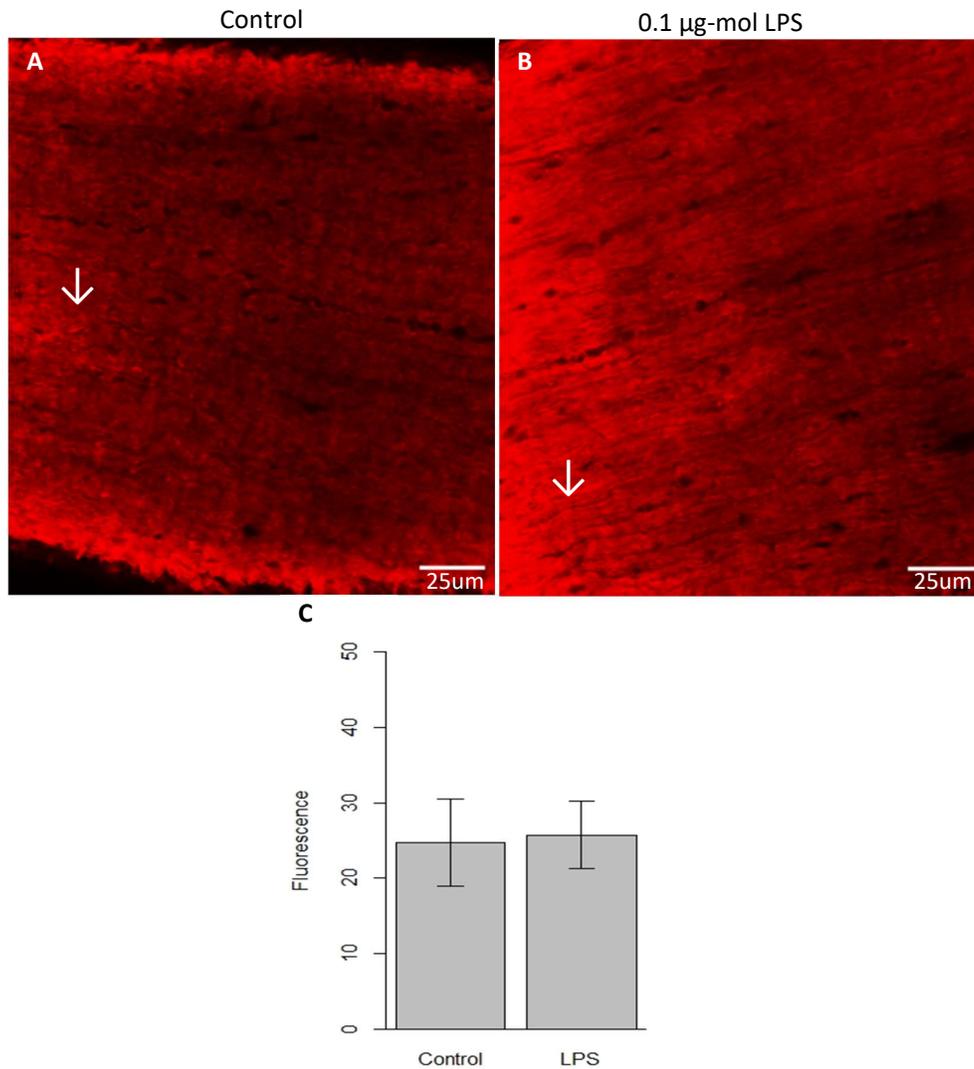
Figure 6: Live images from the confocal microscope Showing the successful uptake of fluoromyelin red into the optic nerve from a CD1 adult mouse. The control group (A) had been treated with 3ml of aCSF, with the test group treated with 0.1 μg-mol of LPS (B) for 120mins each. The myelinated axons can be identified by the red structures as shown by ↓ in both A and B. The graph (C) shows the mean fluorescence of FMR in the adult CD1 optic nerve when treated with 0.1 μg-mol LPS. Five mice were used (n=5) in this data set with 10 images being taken for each treated nerve, resulting in the analysis of 50 images per treatment group, with all fluorescence intensities being measured on ImageJ software.

1 µg-mol LPS on CD1 mice:

Due to the insignificance of the 0.1 µg-mol LPS, a higher concentration of 1 µg-mol LPS was used. The time period was kept the same as the less time needed to evoke an immune response the better, as it will open possibilities on a rapid inflammation and drug testing model. It was hypothesised that this concentration of LPS would cause a significant difference in damage compared to the control group; as such a high concentration of LPS should be significant in activating the immunological cascade. The same structures visible in figure 6, such as myelinated axons and oligodendrocyte cell bodies can be observed in figure 7. The main difference is that the image showing optic nerves treated with 1 µg-mol LPS is notably darker, and the myelinated axons are less apparent as shown by the ↓ in figure 7B. This would be due to inflammation resulting in demyelination, as shown by the FMR staining. The graph in figure 5 shows the mean fluorescence of the control optic nerves (29.55 +/-6.1) and optic nerves treated with 1.0 µg-mol LPS (15.56 +/-2.19). The error bars do not overlap, suggesting that the difference between the two treatment groups is significant, which is supported with a paired t-test giving a significance of $p < 0.04$(*).
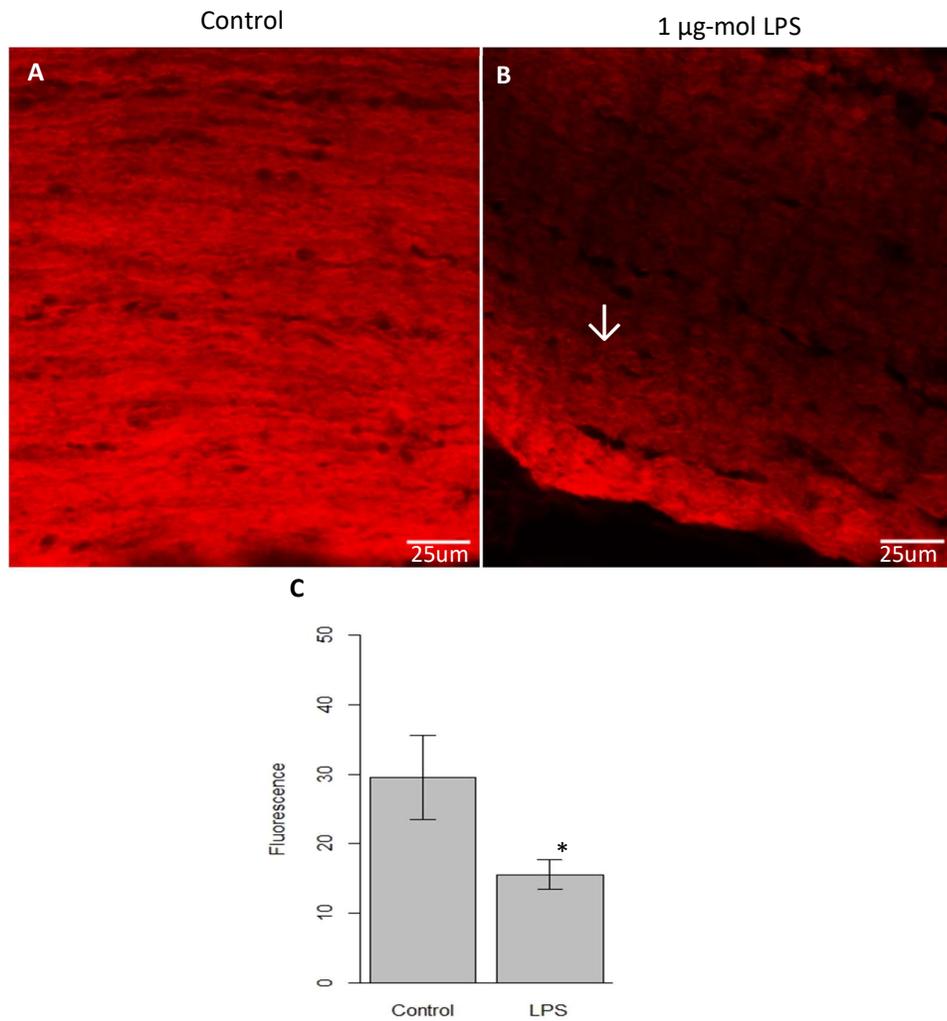
Figure 7: Live image from the confocal microscope showing the successful uptake of fluoromyelin red into the optic nerve of CD1 adult mice between the control group treated with 3ml of aCSF and the test group treated with 3ml of 1 µg-mol LPS for 120mins. ↓ represents the damaged myelin after LPS exposure. The graph (C) shows the mean fluorescence of FMR in the adult CD1 optic nerve when treated with 1 µg-mol LPS. Five mice were used (n=5) in this data set with 10 images being taken for each treated nerve, resulting in the analysis of 50 images per treatment group. With all fluorescence intensities being measured on ImageJ software.

1 µg-mol LPS on PLP-ve mice:

The basis of a Model of MS has been established with the 1 µg-mol LPS showing

significant results; a comparison between different strains of mice was conducted to

confirm the findings so far and prepare for PLP-GFP stacked imaging. Therefore, the 1

µg-mol LPS experiment was repeated with PLP-GFP negative mice as shown in figure 8.

The images reflect the same structures as mentioned previously, where the 1.0 µg mol

LPS treatment group contains a dark streak through the middle of the nerve, which

may be due to damage via acute LPS exposure. The graph in figure 8 compares the

mean fluorescence between treatment groups. The mean fluorescence of the control

group was higher than the group treated with 1.0 µg mol of LPS (39.39 +/- 5.75) vs.

(27.34+/-4.5, respectively). The paired t-test results show a significant difference

between the control and treated group with a p-value of 0.01(**), this significance can

be reinforced with the absence of overlapping error bars in the graph.

PLP-ve Control   PLP-ve 1 µg-mol LPS
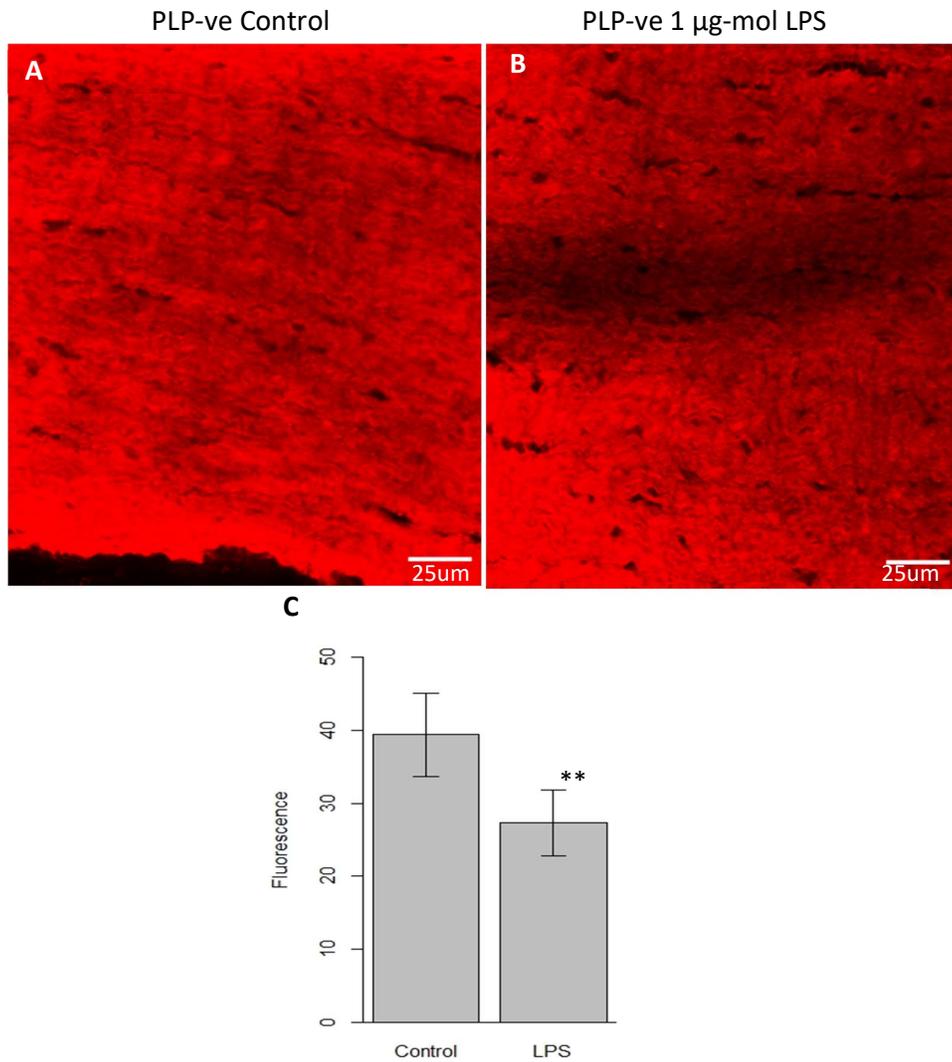
Figure 8: Live image from the confocal microscope showing the successful uptake of fluoromyelin red in the optic nerve of PLP-GFP negative mice. The control group (A) was treated with 3ml of aCSF, and the treatment group (B) was treated with 3ml of 1 µg-mol LPS. The Graph (C) shows the mean fluorescence of FMR in adult PLP-GFP negative optic nerve when treated with 1 µg-mol LPS. Five mice were used (n=5) in this data set with 10 images being taken for each treated nerve, resulting in the analysis of 50 images per treatment group.

1 μg-mol LPS with 10 μM methylprednisolone:

1 μg-mol LPS exposure for 120 minutes produced a significant reduction in live myelin staining; therefore, this experiment was repeated with 1 μg-mol of LPS; however, both the LPS and control group was treated with an anti-inflammatory treatment called methylprednisolone (10μm). This would test if the damage was caused by inflammation and also that this protocol can be used as a model for drug testing. Both groups were treated with the MP to negate bias. The image shows that slight damage may have occurred due to the slightly reduced fluorescence in figure 9(B); however, the mean fluorescence for the control group of mice was 29.80 +/- 7.11 and the mean fluorescence for the optic nerves treated with 1 μg-mol LPS was 26.99 +/- 7.24. This is not a significant difference, as shown by the overlapping error bars in the graph in figure 9. A paired t-test was performed on these results, which solidifies that the results were not significant with a p-value of 0.608. This was to be expected as LPS induces inflammation, therefore, an anti-inflammatory treatment should prevent the immunological cascade from occurring.
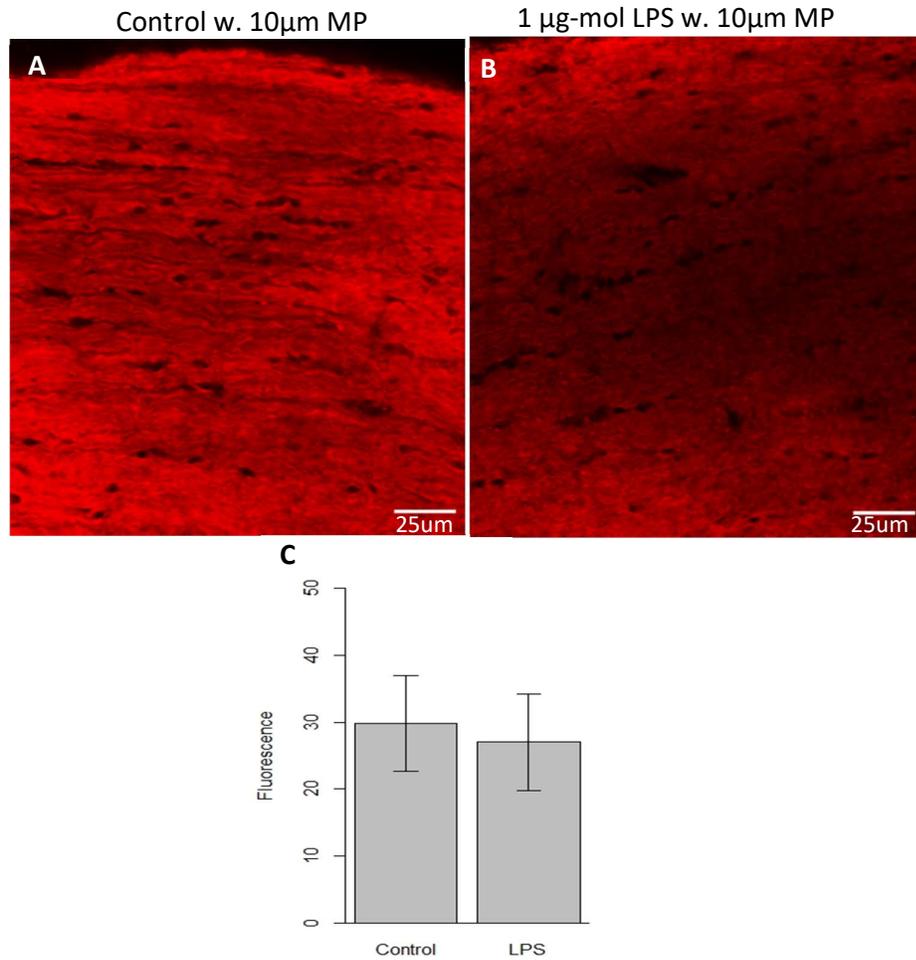
Figure 9: Live images from the confocal microscope showing the successful uptake of fluoromyelin red. The control group (A) was treated with 3ml of aCSF, and the test group (B) was treated with 3ml of 1 µg-mol LPS. Each nerve was treated with 30ul of 1M methylprednisolone to give a final MP concentration of 10µM. The Graph (C) shows the mean fluorescence of FMR in both the control and treated groups. Five mice were used (n=5) in this data set with 10 images being taken for each treated nerve, resulting in the analysis of 50 images per treatment group.

1 µg-mol LPS on PLP +ve mice:

Originally further experiments were to be conducted on various concentrations of MP and NMDA receptor antagonists; however, COVID-19 had severely impacted the original plan leaving minimal time left. Therefore, it was thought it would be best to consolidate further the results found so far, by researching the effect of acute LPS exposure on other aspects of myelination in the optic nerves, such as the oligodendrocyte morphologies and viability. This would provide an incite as to where and how LPS causes damage solidifying its use of an inflammation model for MS.

The genotyping results that had been successful in figure 5 enabled progression to the next set of experiments.

Figure 10 shows that many myelinated structures can be observed. Although the number of oligodendrocytes seems to stay constant, there is a notable decrease in overall fluorescence and the number of oligodendrocyte cell processes. Therefore, the following were measured and statistically analysed: Myelin fluorescence, oligodendrocyte cell size, cell count and a damage rating of the cell processes. Graph (G) shows the mean fluorescence of the PLP-GFP positive stacks for the control 66.22 (+/- 9.87) and 1 µg-mol LPS 38.62 (+/- 2.95). The error bars and paired t-test analysis with a p-value of 0.027* show a significant difference in the amount of myelin present. Graph (H) portrays the mean number of GFP(+) oligodendrocytes in the control 31.12 (+/- 3.6) and LPS 29.99 (+/- 3.79). Paired t-tests show there is no significant difference between the two groups (P= 0.077). Graph (I) shows the average cell area of GFP (+) oligodendrocytes between groups, with the control having a mean of 18.9 µm (+/- 2.09µm) and LPS 12.57µm (+/- 1.31µm). There was a significant difference between groups, as shown by the non-overlapping error bars and the p-value of 0.007**. Graph

(J) shows that the subjective damage rating of the oligodendrocyte cell processes is more significant in the optic nerves treated with 1 ug mol LPS (2.8 +/- 0.13) compared to the control optic nerves (1.53 +/- 0.17). The paired t-test results show that the difference is significant (P= 0.001), which can be supported through the error bars not overlapping. The high magnification images allow identification of cellular damage as in A the cell processes can clearly be seen extending from the oligodendrocyte to other myelinated axons in the nerve, in comparison to the high magnification image in B, in which there is significant loss of GFP+ processes this significant change is supported by the cell process data.

The data show that LPS exposure can cause significant damage to the myelin and oligodendrocyte processes via activating various immunological cascades, and that the exposure time period of 120 mins is too short to kill the oligodendrocytes but does cause cell shrinking and oligodendrocyte cell prosses damage.

Control | 1 μg-molLPS

25um
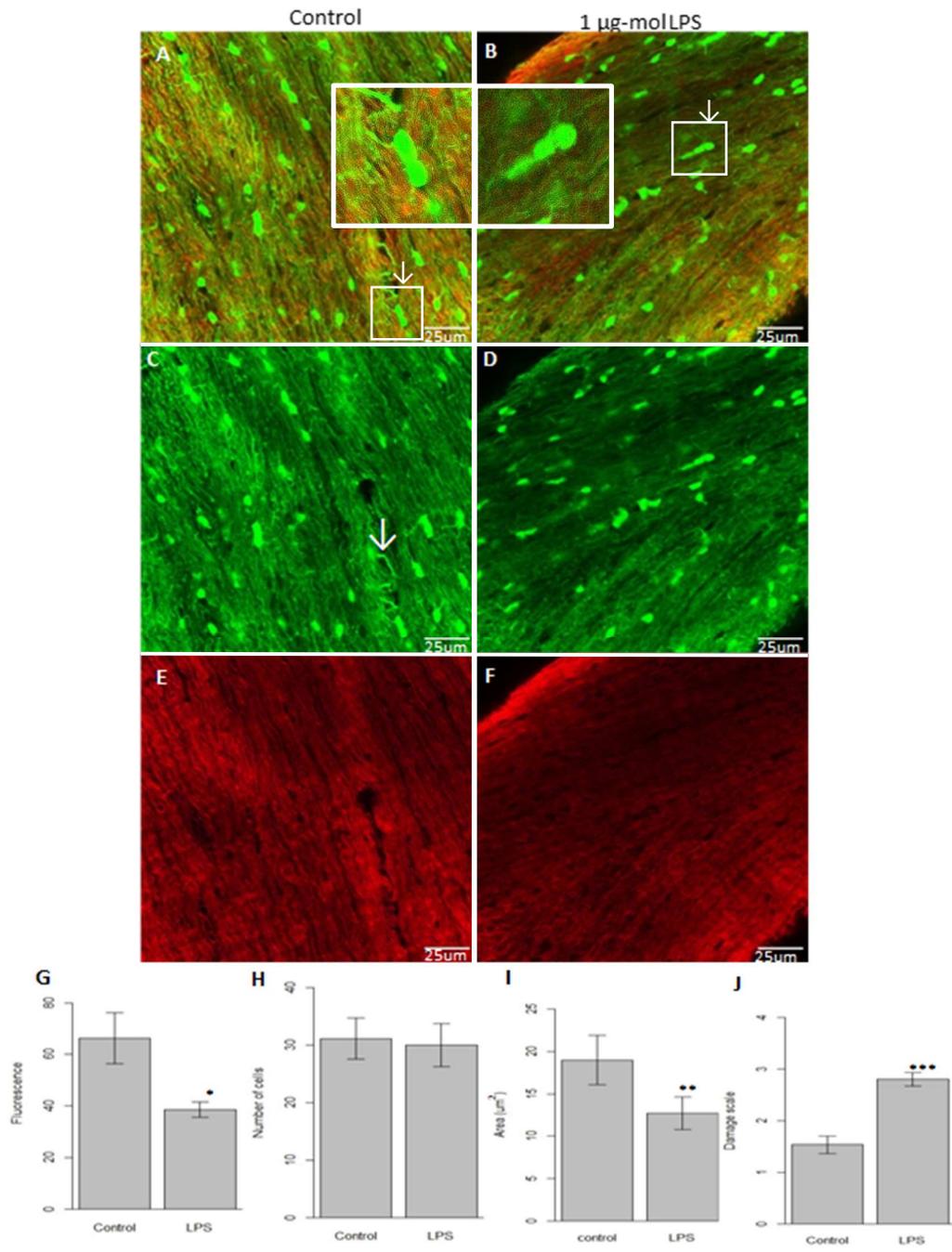
Figure 10: Shows the live confocal images from the optic nerves obtained in adult PLP-GFP positive mice showing oligodendrocyte morphology and structures of myelin. The left side is the control group, with the right being treated with 1 µg-mol LPS; the nerves were stained with Fluoromyelin red, and images were overlayed with the natural fluorescence from the PLP-GFP gene creating a stacked image. Image A and B represent the overlay of fluorescence of PLP and fluoromyelin with the ↓ in A and B representing the oligodendrocyte cell bodies which are shown in the magnified image. The high magnification images in A and B allows identification of oligodendrocyte cellular damage, through cell shrinking and loss of GFP+ cell processes. C and D show the PLP fluorescence with ↓ in C indicating the oligodendrocyte cell processes and the ↓ in D indicating significant cell process damage; finally, E and F representing the fluoromyelin fluorescence. Graphs G, H, I and J show the mean between Control and LPS treated on overall fluorescence (G), Total number of oligodendrocytes (H), Average oligodendrocyte cell area (I), and damage rating of cell processes (J), respectively. The damage scale is a subjective scale in which the oligodendrocyte cell process damage as shown by the ↓ in D was rated between 1-4, with 1 being no damage and 4 being significant damage. Five mice were used (n=5) in this data set with 3 image stacks being taken and analysed for each treated nerve.

Summary of results:

The variability between the control groups of CD1 mice is minimal which further supports the damage that 1 µg-mol LPS had caused. Another secondary finding is that there is a slight variation in myelin fluorescence between CD1 strain and PLP negative when treated with 1 µg-mol LPS This could be down to genetic variability or the possibility that PLP contain fewer receptors responsible for the cause of damage such as TLRs.

From these results, we can summarize that 0.1 µg-mol LPS is not efficient in causing damage over 120 min in isolated mouse optic nerve; but 1 µg-mol is sufficient to invoke an immune response and damage the myelin. This had been tested on two mouse strains with the same conclusion, and further analyses of the effects were demonstrated in the results from the confocal stacked images. These showed that oligodendrocyte cell bodies and cell processes were significantly damaged when exposed to 1 µg-mol LPS. The exact mechanism at which this damage occurs is unknown; however, we can conclude that it is down to inflammation via inflammatory cytokine production, ROS and glutamate excitotoxicity through previous research. These results verify that LPS could be used as a rapid model of myelin damage that replicates the effects of MS. This was further tested through the experiments using methyl-prednisolone, which is a world-known anti-inflammatory that has been used for MS symptom relief. CD1 mice were used as an accurate comparison of data sets. This verifies that the damage that has occurred in the previous 1 µg-mol LPS data set is 100% due to inflammation as the results are not significant, which means the methyl-prednisolone treatment had prevented the damage that would have occurred. This

data set also proves to be an example of the effectiveness of methyl-prednisolone as an anti-inflammatory treatment.

Chapter 4. Discussion:

Overview:

The activation of immune cells such as T cells and microglia has shown to be the primary pathogenesis in many neuroinflammatory diseases, including multiple sclerosis. This inflammation is one of the critical characteristics in white matter injury in MS, which is why it has become an extensive research problem as, to date, there are few effective interventions. Although other working models of inflammation have been discovered, such as the EAE model and cuprizone, they lack rapid time frames and cause harm to the animals involved. The bacterial peptide LPS was used to induce inflammatory responses in the white matter of mice optic nerves, with fluoromyelin red being used as a stain to identify the significance of the myelin disruption. With GFP+ mice also being used to identify the changes to oligodendrocyte cell size, number and their GFP+ cell processes. The use of LPS in this project has revealed a rapid response for myelin disruption, supported by the aforementioned figures, in particular figure 10, showing significant myelin disruption to both myelinated axons and oligodendrocytes with evident loss of longitudinal GFP processes. The significance of these results gives a solid foundation for the use of LPS as a model of inflammation which could lead to speedy drug testing/ development to take place and reduced live animal experimentation. In this research the isolated optic nerves used in this project would only contain intrinsic immune cells for example microglia and therefore this project is unable to measure any damage through other extrinsic factors.

Low dose LPS:

Initially, a low concentration was used to find the concentration required to elicit an immune response and myelin disruption. As shown in figure 6, there was no significant difference between the control and LPS treated groups, which showed us that in order to invoke injury, a higher concentration would be needed. It is possible that slight damage did occur; however, this is hard to decipher from figure 4B. Acute LPS notoriously causes inflammation and damage in many models of neuro-diseases, however; few studies have shown that pro-inflammatory molecules associated with the immune response such as TNF- α, IL-1β can enhance remyelination. They are thought to enhance OPC recruitment, upregulating the expression of key transcription factors responsible for remyelination.

The TLR4 signalling involved may act to protect neurons in the presence of toxins by inducing the transcription of genes involved in OPC recruitment and myelin repair (Glezer, Lapointe and Rivest, 2006). Studies have found that a low-dose application of LPS increased platelet-derived growth factor receptor (PDGFR-γ) transcript levels; which are important in regulating cellular processes such as cell proliferation, cellular differentiation, and cell growth/development (Williams, 1989). The innate immune response has also shown to initiate the OPC recruitment through the stimulation of the TLR4 receptors in microglia, which leads to the production of inflammatory cytokines such as TNF-α, with the activation of TNF receptors also playing an essential role in the recruitment of OPCs and remyelination in models of demyelination (Church et al., 2017). The process of remyelination takes weeks, therefore, isn't relevant to our model making these findings of low relevance. Instead, it is widely known that LPS is a potent stimulator of inflammation and damage. When damage occurs to myelin, the

CNS usually undergoes remyelination through PDGFR-γ and other growth factors, increasing the proliferation and migration of OPCs to the lesion site (Chari, 2007). Here they undergo differentiation and mature into fully functioning oligodendrocytes which are responsible for wrapping the damaged axon with a new myelin sheath. Matured oligodendrocytes express proteins like myelin basic protein (MBP), which act to compact the membranes (Baaklini et al., 2019). Oligodendrocyte survival is crucial for a healthy CNS. The process of remyelination is usually disturbed in MS, through damage to oligodendrocytes and their cell processes or due to a shift in oligodendrocyte phenotype resulting in the impaired capability to undergo remyelination (Jäkel et al., 2019).

Due to the time-scale of remyelination taking weeks in comparison to the 120min in this study, we can conclude the results shown by figure 6 is due to the low concentration not having detrimental effects on oligodendrocytes.

TLRs in MS:

The Initial results led to a higher concentration of 1 μg-mol LPS being used to try and elicit white matter injury and formulate a new successful model of acute MS demyelination. As shown in figure 7, the damage that occurred was significant, and this can be identified by the darker appearance of the FMR stained myelin in figure 7B. The myelin structure had also become damaged as the myelinated axons, which can be identified clearly in figure 7A, are no longer apparent in figure 7B, with all visual analyses being supported with the significant results of a paired t-test. This indicates that acute LPS mediated injury has taken place through one of the various pathways of TLR4 activation resulting in inflammation and demyelination. This has been seen in other studies by Zhao et al., (2019); which concluded that LPS treatment in mice

caused an increase in pro-inflammatory cytokines and reduced levels of anti-inflammatory cytokines.

TLRs are a group of heavily involved receptors in pathogen recognition; they can be found on the cell surface of multiple immune-related cells such as dendritic cells, T cells, B cells, epithelial cells, and endothelial cells, with the specific TLR4 receptor not being expressed on oligodendrocytes and astrocytes. Therefore, the LPS receptor TLR4 involved in this study is only present on the microglia and axons of the optic nerves (Sherwin and Fern, 2005). The interaction between LPS and TLR4 expressing microglia and neurons, results in the direct demyelination on axons and indirectly on oligodendrocytes through pro-inflammatory cytokine production. The MyD88 dependent pathway shown by figure 11 has become the main topic of discussion for the pathogenesis of MS as both infiltrating and resident cells of the CNS express TLRs with TLR 2, 4, and 7 upregulation being found in patients with MS in comparison to controls (Hernandez and Baxter, 2013).
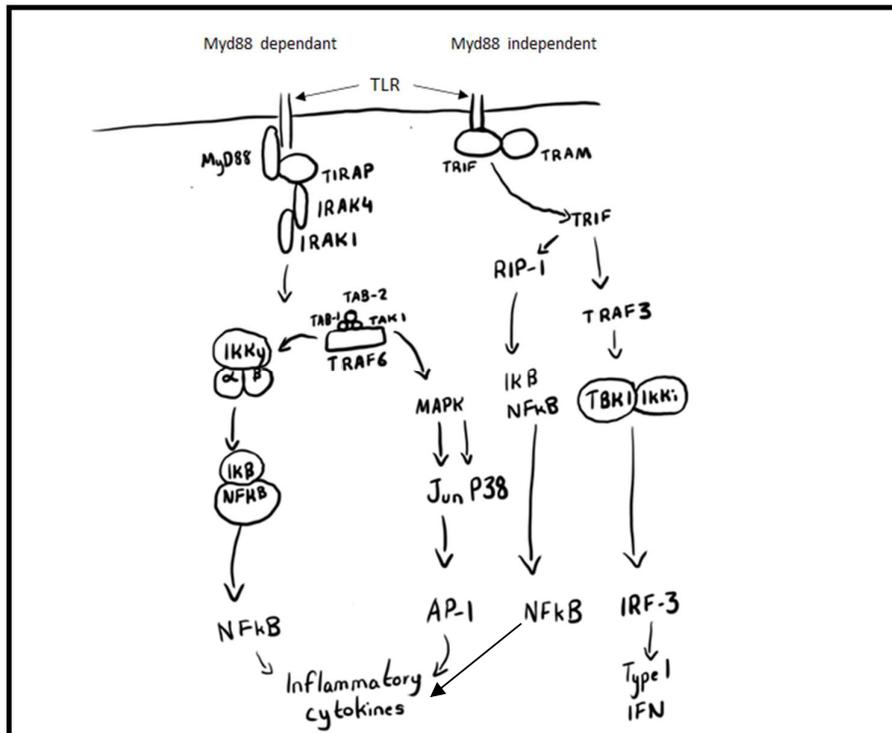
Figure 11: Showing the MyD88 dependent and independent pathways through TLR stimulation.

LPS is a significant component of gram-negative bacteria and acts as strong stimulators of the innate immune response; LPS will act as a PAMP and bind to TLR4 receptors, which will activate multiple pathways resulting in inflammation and cell death. TLR4 partakes in both MyD88 dependent and independent signaling pathways. Regarding the MyD88 pathway, once the LPS binds to its complement receptor on TLR4, MyD88 forms a complex with IRAK, which is part of the IRAK kinase family; this complex is known as the myddosome, during the formation of this complex IRAK4 activates IRAK1, which then undergoes multiple site autophosphorylation (Zheng et al., 2020). This phosphorylation then releases the MyD88. IRAk1 continues to associate with TRAF 6, which along with other subunits, promoting polyubiquitination of both itself and the TAK1 complex; this is a member of the MAPKK family and further forms a complex with

TAB1, TAB2 and TAB3, activating TAK1 through interaction with polyubiquitin chains that TRAF6 has created. Once TAK1 is has been activated, it activates the NF-kB pathway and the MAPKK pathway. TAK1 binds to the IKK complex, which consists of 3 subunits IKKα, IKKβ and IKKγ; this binding allows phosphorylation to activate IKKβ. This IKK complex then phosphorylates ikβ, which is an NF-kB inhibitory protein (Kawasaki and Kawai, 2014). Due to the phosphorylation of the inhibitory protein, the release of NF-kB is initiated, resulting in its translocation into the nucleus to induce pro-inflammatory gene expression. The activation of the MyD88 pathway is responsible for the expression of all inflammatory cytokines specifically IL-6 and IL-23 which are essential functions of Th17 & Th1 cell differentiation (Zheng et al., 2020).

The other pathway is referred to as the MyD88-independent pathway and it also involves the activation of TLR4 through LPS-TLR binding. This activates TRIF, which interacts with TRAF3 & 6 resulting in the recruitment of the kinase RIP-1. RIP-1 then interacts and activates the TAK1 complex resulting in the activation of NF-kB and the subsequent production of inflammatory cytokines. TRAF3 is also responsible for recruiting the IKK-related kinases TBK1 and Ikki for IRF3 phosphorylation. IRF3 then translocates from the cytoplasm to the nucleus in a dimeric form, where it induces type 1 IFN gene expression (Kawasaki and Kawai, 2014).

MyD88 -/- mice have been reported to not respond to acute LPS application, which would result in minimal inflammation occurring (Kawai et al., 1999). This shows that the MyD88 pathway is essential for damage via inflammation through LPS application, as shown in figure 7.

Regardless of the TLR receptor involved in the pathogenesis of MS, they all have one thing in common: the activation of the MyD88 dependent pathway (with the exception

of TLR 3), increasing pro-inflammatory cytokines production along with an increase of Th1 and Th17 regulation (Falck-Hansen, Kassiteridi and Monaco, 2013).

T cell pathogenesis:

Although the exact pathogenic mechanisms that occur in MS are currently unknown, it has been established that auto-antigen specific t cells are stimulated by antigen-presenting cells (APCs). These pathogenic cells can then migrate through the BBB after its disruption, allowing successful infiltration into the MS lesion to damage the myelinated axons via inflammation.

Th17 cells are one of the leading causes of inflammation, as studies have shown that the amount of Th17 cells in the cerebrospinal fluid of MS patients is significantly higher than the control. The discovery of the implications of th17 in MS was with the EAE model renowned for identifying many immunological mechanisms of MS (Slavin et al., 2010).

Th17 cells can secrete many pro-inflammatory cytokines such as IL-17, IL-22, and TNF-α, which induce cell-mediated inflammation (Becher and Segal, 2011). Studies by Jadidi-Niaragh and Mirshafiey, (2011); indicate that IL-17 is also responsible for inducing the secretion of IL-1β, IL-6 while also mediating the dysfunction of the BBB. This can be supported by identifying these cytokines in demyelinating plaques found in the CNS and CSF of MS patients (Zheng et al., 2020). Furthermore, it is known that the TLR-MyD88 pathway results in the translocation of NF-kB into the nucleus, which mediates the secretion of IL-6, which in turn can initiate the differentiation of Th17 (jadidi, 2011). This is supported through the reduction of IL-17 production and Th17 cell differentiation of MyD88 -/- mice when stimulated with a TLR antagonist.

Th1 is the other type of T cell that is essential for the damage caused in MS; they are responsible for secreting a wide array of cytokines. The primary function of Th1 cells is in cell-mediated immunity and inflammation. The cell-mediated immunity is initiated by the interaction between the CD40L and CD40 receptor, expressed on B cells, dendritic cells, and macrophages, resulting in inflammation and opsonisation of the target antibody expressed by MHC II complexes which in MS can be MBP. This leads to the destruction and damage of the myelin seen in MS. Th1 cells also secrete large amounts of IFN-γ, TNF-α, and CCL2. IFN-γ facilitates the expansion of Th1 cells and up-regulation of MHC II complexes. TNF-α initiates inflammation by recruiting macrophages to the site of lesion and acts upon endothelium damaging the BBB, allowing a toxic influx of immune cells and auto-reactive T cells with CCL2 partaking in the recruitment of macrophages to the site of interest (Sekiya and Yoshimura, 2016).

Th17 and Th1 Phenotypes have been shown to be upregulated in the blood mononuclear cells of patients with MS, proving that they are essential contributors to the pathogenesis of MS (Arellano et al., 2017).

The previous roles of Th1 and Th17 discussed above can be related to the model of LPS we are trying to uphold; the same cytokines found to be elevated in MS will be produced through the TLR4-MyD88 pathway in our experiments, therefore, leading to the same detrimental effects such as the myelin disruption observed in figure 7, 8 and 9. The limitation of our model is that it has not been used on live mice; therefore, the involvement of the BBB and the auto-reactive t cell pathogenesis will not be exactly replicated. However, due to the same MyD88 pathway being activated followed by the expected immune response of inflammation, this model would effectively replicate many of the pathogenesis involved in MS.

B cell pathogensis:

B cells are a type of APC that presents antigens to CD4+ T cells and promote Th1 and Th17 responses. A portion of B cells will proliferate and develop into plasma cells which will secrete myelin-specific antibodies, which results in functional impairments and damage to myelin (Zheng et al., 2020). B cells are also responsible for secreting cytokines such as TNF-α, which act as pro-inflammatory. Abnormal levels of TNF-α secretion from B cells have been associated with MS (Bar-Or et al, 2010). The progression of MS can be linked closely with B cells as the TLR-my88 signalling is essential for B cell proliferation and differentiation, concluding that TLRs are a critical factor in the pathogenesis of MS (Häusser-Kinzel and Weber, 2019).

Dendritic cells in MS:

The presentation of antigens via APCs is an important event in the activation of auto-reactive T cells. Both the innate and adaptive immune system is heavily controlled by Dendritic cells (DCs). Data has shown that these DCs are responsible for providing the signals necessary for T cell polarisation, thereby significantly contributing to the MS pathogenesis. Once the auto-reactive T cells successfully cross the BBB, the resident DCs in the CNS provides the stimulating signals required to re-activate these auto-reactive T cells thereby, promoting the progress of MS (Zheng et al., 2020; Tai et al., 2018).

Studies show that when DCs become activated by pro-inflammatory signals, the DCs become more aggressive, which causes hyperfunction of the immune response resulting in inflammation and potential damage. An example of one of these inflammatory signals is the pro-inflammatory cytokines produced by the MyD88 pathway (Sweeney et al., 2011). LPS, a TLR-4 antagonist, has been shown to cause DCs

to secrete high levels of IL-6 and IL-12, which can both act as pro-inflammatory. This indicates that the activation of this pathway can promote the evolution of DCs into a pathogenic status (Zheng et al., 2020; Tai et al., 2018).

The Blood Brain barrier (BBB) in MS:

The BBB consists of endothelial cells, which are attached via tight junctions. The role of the BBB is to ensure the brain can function properly and act as a permeable barrier preventing potentially harmful molecules from entering the CNS. During MS, auto-reactive T cells and lymphocytes can cross the BBB, migrating into the CNS. This can result from the TLR-MyD88 pathway as TLR -/- mice in rodent studies resulted in a reduction of BBB disruption (Ortiz et al., 2014).

TLR2 + 4 can initiate the expression of adhesion molecules ICAM-1 via activation of the MyD88 dependent pathway and promoting lymphocyte adhesion on the vessel wall, as the reduced TLR signalling results in a lower expression of ICAM-1 (Zheng et al., 2020). Therefore, the disruption of the BBB is essential for MS development, and the TLR signalling pathway can directly destroy the integrity of the BBB (Ortiz et al., 2014). However, as mentioned previously the integrity of the BBB cannot be measured in our LPS model.

Oligodendrocyte pathogenesis:

Until recently it was thought that only a few cells of the CNS contained TLR receptors including B cells, NK cells, Treg Cells, macrophages dendritic cells as well as epithelial and endothelial cells however, recent finding has shown that they present in other CNS structures such as microglia, astrocytes, and oligodendrocytes (Bsibsi et al., 2002). Over the last decade, the evidence supporting the neuronal and oligodendrocyte

expression of TLRs has also significantly increased. Human NT-2 cells that have a neuronal-like phenotype express mRNA for TLR 1-4 (Préhaud et al., 2005), and TLR 1-9 protein expression in the mRNA of rat primary neuronal cultures (Tang et al., 2007). Wadachi and co found that both rat and human sensory nociceptive neurons co-express TLR4 and CD14, which support our hypothesis of the experiment shown by figure 7, 8, and 10 in which acute 1 μg-mol LPS exposure had caused significant damage to the myelinated structures of optic nerves of CD1 and PLP mice (Wadachi and Hargreaves, 2006).

As shown in figure 10, acute LPS application to PLP-GFP positive mice had caused significant damage to both the myelinated structures and the oligodendrocyte cell bodies, which are the key myelinating cells of the CNS. Various evidence supports the existence of TLR2 being expressed on oligodendrocytes (Nagyőszi et al., 2010). Its role is controversial as some evidence points towards it having an inflammation-mediated remyelination effect as TLR2 & TLR4 knock-out mice had shown reduced remyelination following spinal cord injury. However, there is ample contrasting evidence that supports the results in figure 10; as following LPS (TLR4 ligand) and zymosan (TLR2 ligand) application OPC proliferation was significantly inhibited with zymosan inducing a complete loss of oligodendrocyte (Hernandez and Baxter, 2013). Although some TLRs have been found to be expressed on oligodendrocytes which suggest the MyD88 does play a role in oligodendrocyte pathogensis; there has been minimal evidence of TLR4 expression therefore, the damage observed in figure 8 may not be due to the direct interaction between TLR4 activation via LPS on oligodendrocytes. Implicating other ways of observed damage.

There are multiple other ways in which the damage seen in figure 10 can have occurred. Firstly, the stimulation of various cells which express TLR4 in the CNS with an acute 1 μg-mol of LPS exposure could have activated the MyD88 pathway resulting in the production of IL-1, IL-6, IL-1B, TNFα and IL-12, these are pro-inflammatory cytokines and are responsible for inducing the differentiation of naïve T cells into Th1 and Th17, which secrete IFN-γ and IL-17 respectively. The production of all these pro-inflammatory cytokines may stimulate the production of ROS and excess glutamate thereby damaging the oligodendrocytes. In regard to MS, IL17/INFγ are key mediators of the facilitation of leukocytes migration across the BBB; this results in the migration of a higher concentration of auto-reactive T cells into the CNS, which in turn cause damage in the PLP optic nerves. IL-1 and IL-6 produced by both transcribed NfKB and activated CD4+ T cells inhibit Treg differentiation, thereby reducing IL-10 production, a key anti-inflammatory cytokine (Okun et al., 2009; Hernandez and Baxter, 2013).

Reactive Oxygen Species (ROS) in MS:

Another mechanism of pathogenesis involves the mitochondria, which are very important in the synthesis of ATP, which provides energy for cells. The majority of ATP is created through oxidative phosphorylation, which also results in a high concentration of harmful ROS and reactive nitrogen species (RNS) (Zagon and Mclaughlin., 2017).

Free radicals such as ROS are unstable molecules that consist of an unpaired electron in their outer shell, which causes them to react with many other molecules. ROS are naturally created through ATP production in mitochondria as a by-product during the conversion of O_2. The primary by-products are superoxide ($O_2^{-\bullet}$) and hydroperoxyl radicals (Lewen, Matz and Chan, 2000). $O_2^{-\bullet}$ detoxification by superoxide dismutase

(SOD) results in the production of H_2O_2. Under normal conditions H_2O_2 is metabolised into H_2O and O_2 by catalase; however, when this metabolism capacity is in inadequate the H_2O_2 is then converted into the most reactive oxygen radical $O_2^{-\bullet}$ that can be harmful (Ortiz et al., 2013). On the contrary, free radicals such as hydrogen peroxide can be created by immune cells as they are used to kill pathogens (Birben et al., 2012).

When mitochondria become disturbed through a rise of calcium ions via glutamate excitotoxicity, they produce fewer antioxidants which counter the harmful ROS. This leads to reduced levels of ATP being produced, cytochrome c secretion through the opening of the pores, and increased levels of ROS leading to apoptosis and oxidative stress (Zagon and Mclaughlin., 2017). This shows that mitochondrial dysfunction plays a vital role in the inflammatory process. The results of this mitochondrial dysfunction will disrupt the production and transport of ATP along the axons starving the CNS of energy needed to function. Recent finding using the EAE have found that mitochondrial dysfunction occurs in the early stages of MS and develop before the notorious inflammation occurs (Zagon and Mclaughlin., 2017).

Therefore, oxidative stress can be caused by 1) the production of high levels of ROS by activated glial cells such as microglia, astrocytes as well as 2) The activation of the lipoxygenase pathways. Although these free radical species are harmful, our body has developed small molecules such as uric acid, α-tocopherol, ascorbate, carotenoids, and glutathione which can scavenge the free radicals preventing any cell damage from occurring (Ortiz et al., 2013). Oxidative stress occurs when the number of ROS is higher than the number of antioxidant molecules. The transcription of these antioxidant molecules is driven by the transcription factor nuclear factor-E2-related factor (Nrf2). When oxidative stress occurs, Nrf2 translocates to the nucleus to activate antioxidant

response element (ARE) mediated gene transcription (Nguyen, Nioi and Pickett, 2009). The CNS specifically the brain is the organ that is most liable to oxidative stress due to the following reasons; 1) there is high consumption of oxygen through metabolic pathways 2) there is a higher concentration of easily peroxidizable fatty acids 3) there is a lower quantity of antioxidant enzymes compared to other tissues and 4) the brain has a has higher levels of iron (Fe) and ascorbate which if released through tissue disruption can lead to the two substances mixing which is a very potent pro-oxidant for the brain membranes (Ortiz et al., 2013).

Oxidative stress has been regularly implicated in the development of brain damage as ROS contributes to many mechanisms of MS pathogenesis. ROS that are produced on the interaction between monocytes and brain endothelium lead to tight-junction alterations and loss of BBB integrity resulting in the infiltration of leukocytes into the CNS. The leukocytes which enter the CNS produce even more ROS, which induces the myelin phagocytosis and breakdown by macrophages, oligodendroglia damage, and neuronal injury (Giovannoni et al., 1998; Hendriks et al., 2005).

Due to an inflammatory environment being responsible for creating both pro-inflammatory cytokines and ROS, which can be caused by acute LPS exposure, Lipid peroxidation is also another critical mechanism of damage caused in MS, this can be shown in figure 7 and 8 In which significant damage had occurred in both CD1 and PLP -ve mice. Lipid peroxidation (LPO) is caused by free radicals, which cause oxidative modifications of lipids initiating the LPO chain reaction resulting in the destruction of lipid affluent areas such as myelin which consists of roughly 30% protein and 70% lipids (Ortiz et al., 2013). Lipid peroxidation may also alter cell membranes' structure, affecting their physical and chemical properties and permeability, leading to increased

leukocyte infiltration across the BBB. Concerning our results, the seen loss of

oligodendrocyte size and cell processes in figure 8 may also be due to the ROS causing

oxidative stress in the optic nerve, initiating damage and inflammation. One of the

major sources of ROS production stems from NADPH oxidase. This complex consists of

4 proteins, including membrane bound cytochrome b, p47, p67, and Rac2 for humans

and Rac1 for rodents (Hsu and Wen, 2002). The exact mechanisms of ROS production

are unknown; however, Hsu and Wen (2002) showed that LPS causes the production of

pro-inflammatory cytokines, which induces phagocytosis; the opsonised particles

produced stimulate the NADPH oxidase complex resulting in the production of

superoxide radicals. This occurs through NADPH oxidase transferring one electron from

NADPH to oxygen (Hsu and Wen, 2002). ROS are capable of inducing death signals via

the activation of stress-related kinases such as JNK, Ca^{2+} mobilisation, up-regulation of

death proteins (Bax, bad), and stimulating apoptotic caspases (Ruvolo 2003; Jana and

Pahan, 2007).

Glutamate excitotoxicity in MS:

Glutamate excitotoxicity is another primary pathological process essential in the

pathology of MS and can affect Oligodendrocytes which express AMPA and NMDA

type glutamate receptors. Kostic and co (2014) expressed that IL-17, which is a pro-

inflammatory cytokine produced primarily by Th17 cells, has a direct correlation with

glutamate levels in MS patients CSF, which implicates that Th17 cells contributes to

glutamate excitotoxicity (Kostic et al., 2014).

The increase of extracellular glutamate that occurs will lead to an influx of calcium ions

through the AMPA and NMDA receptors found on the oligodendrocyte somata. This

influx triggers a rise in intracellular calcium ions which is responsible for mitochondrial

dysfunction and caspase cascade activation resulting in apoptosis or the more rapid

necrotic injury (Ankarcrona et al., 1995). Calcium is known to initiate apoptosis

through the activation of caspase 12, which activates caspase 3. When mitochondrial

swelling occurs due to Ca^{2+} ions opening the mitochondrial permeability transition

pores, the opening of these pores leads to cytochrome c release. Cytosolic cytochrome

c will bind to APAF-1, allowing APAF-1 protein to bind to ATP, forming the

apoptosome, which activates caspase-9, thereby triggering the caspase cascade,

causing apoptosis and resulting in myelin disruption and neuronal cell death (Matute

et al., 2007). The timescale of injury in this experiment (120 minutes) is too short for

apoptosis to occur, therefore we can deduce that the injury observed is due to

necrosis (Fern, Matute and Stys, 2014). Necrosis is irreversible and can be defined as a

passive process involving in the swelling and lysis of target cells/neurons leading to the

uncontrolled release of the intracellular contents into the extracellular space causing

inflammation. This has been shown in prior studies which concluded that shortly after

exposure to glutamate, neurons had died via necrosis having undergone mitochondrial

dysregulation and lysis of debris into the incubation medium (Ankarcrona et al., 1995).

In relation to our results, the 1 µg-mol LPS concentration has caused significant loss of

myelin, as shown in Figures 7 and 8, as well as significant oligodendrocyte loss, as

shown in figure 10. It has been well established that LPS causes an increased level of

inflammatory cytokines, including IL-1β, IL-17, etc. therefore, glutamate excitotoxicity

is highly likely to have occurred. Activated Th17 cells by IL-17α support the maturation

and recruitment of innate immune cells to the lesion, enhancing the inflammatory

response. Once activated, immune cells such as neutrophils release a high glutamate

concentration via the upregulation of glutaminase. In MS IL-17 has also been implicated in the disruption of the BBB, which causes even more neutrophils to cross into the CNS, leading to an increase of glutamate, thus promoting excitotoxicity. Evidence suggests that this mainly occurs in the early stages of the disease, and neutrophil count significantly decreases as the disease progresses. However, being prominent in the early stages would make it a good target for drug therapy for early-onset MS but not in the later stages of the disease (Kostic et al., 2014; Pitt, Werner and Raine, 2000).

The damage that occurs to the oligodendrocyte will be detrimental to the survival of the myelin as they are the key for myelination of the CNS. This means that remyelination after damage would cease to exist, and the current myelinated axons would perish. Reducing or completely halting the excitatory signals between synapses due to the destruction of the nodes of Ranvier. IL-1β, which is also upregulated in the pathogenesis of MS, is known to cause excitotoxic neurodegeneration via activation of the P53 protein responsible for apoptosis (Rossi et al., 2014).

MS treatments:

As previously stated, the first approved drugs for MS were IFN-beta and glatiramer acetate. IFN-beta's main mechanism of activation is the BBB, wherein decreases the cellular expression of VLA-4 and cleavage VCAM, which are essential adhesion molecules. This will reduce the cell sequestration through the BBB, resulting in fewer lymphocytes and auto-reactive T cells crossing the BBB, therefore, reducing the production of pro-inflammatory cytokines and, in turn, glutamate excitotoxicity, apoptosis and necrosis. IFN-β is also known to have a down-regulatory effect on MHC class II expression present on APC cells which causes T-cells which shifts the production

from Th1 cells to Th2, which produce anti-inflammatory cytokines (Jakimovski et al., 2018).

Glatiramer acetate was FDA approved as a treatment for RRMS. It was tested on the current EAE model for effectiveness with significant results favouring anti-inflammatory. The exact mechanism of action for GA is unknown; however, several potentials are known, all of which contribute to the drug's efficacy (Tselis, Khan and Lisak, 2007). To summarise the immune reaction to myelin in MS, an auto-reactive T cell sensitive to a myelin antigen such as MBP penetrates the BBB where it comes into contact with APC, which presents the antigen on its MHC-II. The T cell will bind to the presented peptide fragment. The APC will dictate the phenotype of the T cells, which in the case of MS Is most commonly Th1 (pro-inflammatory) or Th17. The Th1 pathway will increase IL-12 production, stimulating the secretion of pro-inflammatory cytokines such as IL-2, IL-1β, TNF-α and IFN-γ. These are responsible for elaborating the inflammatory response by damaging the BBB, making it more permeable, resulting in the influx of other inflammatory mediators into the CNS, which results in demyelination and axon injury.

There are three potential ways the treatment GA will prevent injury;

Firstly, the GA may bind to the MHC molecule on the APC, displacing the fragment of MBP from the APC, preventing the binding of the appropriate T cell preventing the cascade of cytokines and apoptotic cascades from occurring. However, the limitations of this theory are that subcutaneously injected GA would have difficulty in crossing the BBB, considering this would have to happen in the CNS to stop the formation of lesions (Tselis, Khan and Lisak, 2007).

Secondly, it could be possible that the GA-MHC-II complex binds to the T cell receptor, becoming a competitive inhibitor competing with MBP-MHC-II complexes, thereby blocking inflammatory response (Tselis, Khan and Lisak, 2007).

Thirdly the GA-MHC-II complex binds to the T cell receptors and partially activates the T cell, which induces a tolerance by an altered peptide ligand mechanism. The GA then induces the Th1 immune response to a Th2 immune response (pro-inflammatory to anti-inflammatory), producing IL-4, IL-5, IL-10, and TGF-β (Farina et al. 2001). This would prove the clinical improvements in animals with EAE in which the CNS still contained inflammatory lesions after treatment. This process has been called a 'bystander protection' in which GA induces MBP reactive Th2 cells to release the protective anti-inflammatory cytokines, which downregulate the response of the reactive Th1 cells (Sampaio-Baptista and Johansen-Berg, 2017).

Methylprednisolone:

Methylprednisolone is a popular corticosteroid that has strong anti-inflammatory effects. Its mechanism is similar to anti-inflammatory treatments. It prevents BBB damage, thereby reducing the influx of pro-inflammatory cytokines into the CNS; it also acts to initiate the production of anti-inflammatory cytokines and reduce T cell activation, all of which help suppress inflammation and ease MS symptoms (Frohman et al., 2007). In relation to our results, as shown by figure 9, MP has a strong anti-inflammatory effect on the myelin not only has high fluorescence, which indicates that minimal damage has occurred by the structure of the myelin being easily recognised in comparison to figure 5 in which the myelin structure has been significantly impaired. Not only does this support the efficacy of MP as an anti-inflammatory, but it supports the use of LPS as a model of inflammation for speedy drug testing, as MP counters pro-

inflammatory cytokine production and has successfully prevented damage from occurring. Although this has been established, it is still unclear what effect the treatment would have on the oligodendrocytes and if it can prevent the damage shown in figure 8. If COVID-19 had not occurred, this area would have been explored further supporting the use of LPS in a model of MS inflammation.

AMPA antagonists:

AMPA receptors are tetrameric ion channels responsible for excitatory neurotransmission in the CNS and other glutamate receptors. AMPA receptors consist of 4 subunits, gluR1-4, and each subunit is responsible for a different function (Pellegrini-Giampietro, 1997). These receptors can be found at excitatory synapses and have recently been proved to be found on oligodendrocytes. These receptors are activated once the neurotransmitter glutamate binds to them. AMPA receptors undergo rapid transmission, which leads to the depolarization of the postsynaptic.

AMPA activation is responsible for the removal of Mg^{2+} ions that usually block the voltage-dependent channel (Mayer et al., 1984); this induces calcium ion influx which leads to long-term potentiation (LTP), which is an increase in synaptic efficacy due to an elevated level of AMPA receptors present on the postsynaptic membrane. Thereby making the stimulation and tracking of AMPA receptors a vital factor in synaptic plasticity (Lu and Roche, 2012).

Due to COVID-19 research on the effects of AMPA antagonists on the acute treatment of LPS on optic nerves was not carried out, this aspect of research would have given an insight into whether oligodendrocyte damage that occurs in figure 10 involved glutamate excitotoxicity which would strengthen the support for the use of LPS in a

model of MS. The treatment would likely be successful as oligodendrocytes express AMPA receptors (Krieger et al., 2014).

 In theory, the AMPA antagonists, when applied to the optic nerve, would reduce the shown damage in the figure 8. As mentioned above, glutamate excitotoxicity is another key factor in the pathogenesis of MS; therefore, it is vital to find a way to prevent this from occurring. AMPA antagonists act as a non-competitive inhibitor, thereby competing with glutamate for the AMPA receptors. This competitive inhibition will inhibit the excess calcium flow through AMPA receptors and NMDA receptors as AMPA activation leads to NMDA activation. This prevents the apoptotic cascade via mitochondrial swelling. This would also lead to fewer pro-inflammatory cytokines being produced as fewer leukocytes will be recruited to damage sites. AMPA antagonists have been proved to reduce both acute and chronic lesions in MS and increase oligodendrocyte survival and reduce axonal injury (Malekzadeh et al., 2018).

MP has also shown to be effective in the treatment of optic neuritis, which can be defined as an acute inflammatory demyelinating disorder of the optic nerve with a similar pathology to MS to regards to it being immune-mediated; however, the exact mechanism is unknown with it ultimately resulting in painful visual loss. Optic neuritis has shown to be initially present in over 20% of MS patients, making it an important possible biomarker for the onset of MS (Kale, 2016). This information consolidates the use of optic nerves in the experiment and validates them as a successful candidate for an MS model.

Limitations of this study:

LPS is widely used and is renown for aiding in the understanding of the relationship between neuroinflammation and the progression of many neurodegenerative diseases, however there are various different serotypes of LPS available which may give a wide range of results, therefore, repeating this study with various different serotypes would give a much stronger argument for the use of LPS as a model of neuroinflammation. The dose and methodology of LPS application should also be considered due to the potential varied responses that may be caused by this endotoxin. The pathology of MS also occurs throughout varying regions of the brain. Although optic nerves effectively explore the demyelinating effects, future studies should aim to consolidate the same results with different brain regions such as the corpus callosum, to identify if LPS has any different effects on varied CNS structures.. The administration of fluoromyelin such as dose, time-length and application method could also be explored to solidify the current significance of myelin disruption. Along with other myelin stains being used to see if the significant myelin disruption discovered in this project reflects through other lipid staining methods such as luxol-fast blue (LFB) staining, which is a myelin sheath stain that stains phospholipids.

The sample size for this study could be larger, which would make the results more reliable in further studies. I would aim to improve the sample size and look into the effects of AMPA antagonists at varying concentrations as well as the use of different concentrations of MP to solidify the findings so far. Due to COVID-19 restrictions, this research was performed by myself alone as social distancing needed to be respected and followed during most of my research period. Therefore, the results were not blinded, which would have improved the reliability and accuracy of the stated results.

Again, due to COVID-19 and equipment availability, confocal images on the PLP-GFP

positive mice were limited to 3 per nerve. Although I believe this is sufficient in

portraying the experiment's effects, in the future, an increase in this number would

have given more substantial results. Due to MS having a preference for females, the

gender of mice could have been noted and compared, which could have given more

specific effects per experiment, however as the aim of the study was to argue for the

use of LPS in a model of inflammation in relation to MS I believe this was not

necessary.

Concluding remarks:

Although COVID-19 had severely impacted the original plan, I believe the current

experiments portrayed in this thesis gives a solid stance for the use of LPS as a model

of Inflammation in MS. The primary pathogenesis of MS being the production of

inflammatory cytokines, leading to inflammation and demyelination has been strongly

supported. The demyelination that occurred due to acute LPS exposure, as shown in

figure 7, 8 and 10 being down to activation of the MyD88 dependent pathways, with

studies supporting the presence of MyD88 in MS as well as other successful MS models

such as EAE. The use of Methylprednisolone had not only solidified its use as an anti-

inflammatory but also proved that LPS acts on an inflammatory basis of cytokine

production, concluding that both LPS exposure and MS pathogenesis is very similar as

both can be treated with Methylprednisolone. Oligodendrocyte damage has also

proven to be affected by exposure to LPS through figure 10 which ties in with other

literature where oligodendrocyte damage has occurred in MS patients, in-vivo MyD88

stimulation and EAE models with all leading to crucial effects on remyelination

capabilities and the detrimental myelin disruption. This research could have been

expanded through the use of PLP-GFP mice with Methylprednisolone; Unfortunately, COVID-19 halted this progress. Other pathological effects such as oxidative stress and glutamate excitotoxicity have also been strongly implicated with both MS and the EAE models of MS (Stojanovic, Kostic and Ljubisavljevic, 2014). In theory, the stimulation and inflammatory effects of MyD88 result in the production of reactive oxygen species and increased levels of extracellular glutamate, causing excitotoxicity as it does in MS. Future experiments would aim to look at the effects of AMPA-antagonists' LPS exposure to white matter. If the results are non-significant like that of MP in figure 7, then this will tell us that glutamate excitotoxicity is present in acute LPS exposure further proving that the main demyelinating effects in MS can be accurately replicated in this LPS model. The main downside is that the introduction of auto-reactive T cells, and the permeability of the BBB, both of which contribute to the pathogenesis of MS and can be measures in models such as EAE and cuprizone, cannot be replicated in this model. However, it can allow faster generation dose-response relationships, rapid drug testing, which will be beneficial for the testing of new therapies and reduce animal experimentation, reducing animal distress.

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