The effect of clozapine and MIS416 on demyelination, remyelination and immunomodulation in the murine cuprizone model.

By

Nicola Clair Templeton

A thesis submitted to the Victoria University of Wellington in fulfilment of the requirements for the degree of Doctor of Philosophy.

Victoria University of Wellington 2017.

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Acknowledgements

This PhD could not have been completed without the support of many people, to whom I owe a great deal of gratitude.

Firstly I would like to acknowledge and thank my supervisor Professor Anne La Flamme, for her guidance and knowledge, both of which have been invaluable. I would also like to thank her for the faith she had in me, especially when things weren't going to plan! To Dr Bronwyn Kivell, thank you for all the last minute help with editing and proof reading.

To my lab group members both past and present; Sarrabeth, Jenni, Laura, Lisa, Maddie, Vimal, Yanyan, Pirooz and Carl, thanks for your help with some of the more epic experiments, for training me in flow cytometry and for always being up for tea/coffee and a distraction from science. Also a thank you to the wider lab members who made the office and lab an enjoyable place to work.

To Lou, I couldn't have asked for a better lab minion all those years ago. Giving you boring tasks to do somehow developed in to an amazing friendship both in and out of uni, and I couldn't have done this without you. To Nicole, my uni twin, it has been such a pleasure doing the last 7+ years with you. To Varun, thanks for all the breaks from science and chats about life as a poor PhD student. To my non-uni friends who probably won't read this, thanks, especially you Emily.

Finally, to Mum and Dad. I can't even say thank you enough. Your support has allowed me to finish this thesis and survive the last 4 years, which haven't been easy for any of us. To my siblings Josh and Evie, thanks for your friendship, it's been great living so close to you.

Abstract

Multiple Sclerosis (MS) is a disorder of the central nervous system that affects approximately 2.5 million people worldwide. Due to the heterogeneous nature of the disease, and the want of an identified cause, treatment of MS remains difficult. Treatments are available, however these are limited in efficacy and are not suitable for all forms of MS. Disease pathology is characterised by the formation of demyelinating lesions in the central nervous system (CNS) which lead to cognitive and motor impairments associated with the disease. These CNS lesions can be classified as those with immune cell involvement or those without immune cell infiltrate, which are more commonly seen in progressive forms of MS, and currently, there are no treatments available for progressive MS.

Due to the limited options available for treating progressive MS, this thesis aims to identify the therapeutic effect provided by the immunomodulatory compounds, MIS416 and clozapine, in a non-immune mediated model of MS, which is believed to more closely resemble progressive disease. Both of these compounds have been shown previously to reduce disease burden in an immune-driven animal model of MS. To investigate the effect of immune-modulating therapies on lesions without immune cell infiltrate, the cuprizone model of non-immune demyelination was used.

In summary, the work presented in this thesis found that treatment with MIS416 and clozapine led to improved performance on behavioural assays, although neither agent inhibited cuprizone-induced demyelination or enhanced remyelination. The cellular mechanism behind the observed behavioural improvement is yet to be confirmed. MIS416 was able to maintain its previously identified immunomodulatory properties when administered in this novel setting. Moreover, novel changes to serum growth factors were identified that could provide unexpected benefit to MS patients administered MIS416. In addition to reversing cuprizone-induced behavioural deficits, clozapine reduced LPS-driven inflammatory cytokine production by microglia, indicating that

clozapine has the ability to directly reduce inflammation, which may benefit progressive MS patients.

Protective effects provided by either of these compounds could aid in the development of unique combination therapies to target both the inflammatory immune component and the cellular components seen at different stages of MS. MIS416 induced changes to serum cytokines and growth factors in the periphery could be harnessed to treat not just MS but other auto-immune diseases characterised by a similar cytokine profile.

List of abbreviations

AEC- Animal ethics committee

APC- Antigen Presenting Cell

BBB- blood brain barrier

BCR- B cell receptor

BDNF- Brain derived neurotrophic factor

EAE- experimental autoimmune encephalomyelitis

CAII- carbonic anhydrase II

CNS- central nervous system

ConA- concanavalin A

CTCM- complete T cell media

CTL- cytotoxic T cell

DMEM- Dulbeccos's modified eagle medium

ELISA- enzyme linked immunosorbent assay

FACs- fluorescent activated cell sorting

FCS- foetal calf serum

FoxP3- forkhead box P3

g- gravity

GFAP- glial fibrillary acid protein

HLA- human leucocyte antigen

HRP- horseradish peroxidase

IFN- interferon

IgG- Immunoglobulin G

IHC- immunohistochemistry

IL- interleukin

i.p- intraperitoneal

i.v- intravenous

LFB- luxol fast blue

LPS- lipopolysaccharide

M- moles

MBP- myelin basic protein

MDP- muramyl dipeptide

MDSC- myeloid derived suppressor cell

MHC-major histocompatibility complex

MOG- myelin oligodendrocyte glycoprotein

MS- Multiple Sclerosis

MTT- 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NK- Natural Killer

NKT- Natural killer T cell

NO- nitric oxide

NOD- nucleotide oligomerization domain

NT-3- neurotrophin 3

OG- oligodendrocyte

OPC- oligodendrocyte precursor cell

PBS- potassium buffered saline

PBST- potassium buffered saline with tween or triton

PDL- (1 or 2)- programmed death ligand

PD-1 – programmed death-1

p.i – post injection

PFA- paraformaldehyde

PLP- proteolipid peptide

PPMS- primary progressive multiple sclerosis

RRMS- relapsing remitting multiple sclerosis

SA- strepdavidin

SPMS- secondary progressive multiple sclerosis

TCR- T cell receptor

Th (1,2,17)- T helper cell

TLR- toll like receptor

TNF- tumour necrosis factor

Treg- T regulatory cell

µM- micromolar

µm- micro metre

CHAPTER 1: General Introduction

1 General Introduction

1.1 The Immune System

The key function of the mammalian immune system is to protect the organism from invading pathogens or foreign material. To allow it to carry out this protective function, the immune system must be capable of distinguishing 'self' i.e. material that should be present, versus 'non self' i.e. invading pathogens. To allow efficiency in the protection it provides, the mammalian immune system has developed two branches, or arms: the innate immune system and the adaptive immune system. These systems function in a complementary manner, enabling the immune system to provide the organism with the best overall possible protection (Getz, 2005; Murphy, Travers, & Walport, 2008).

The innate immune system is generally the first system to respond to a pathogen. It is activated rapidly and provides the organism with its first line of defence. The innate system is one we are born with and provides a non-specific response only. Although the adaptive branch of the immune system is slower acting, it is able to provide a more targeted response than the innate immune system. In addition, the adaptive immune system is capable of memory, allowing it to recognise pathogens it has previously encountered and therefore its response is able to improve over time (Murphy et al., 2008).

Since the primary role of the immune system (both innate and adaptive) is to recognise pathogens and distinguish self from non-self, several mechanisms have been developed to prevent the mounting of an unnecessary immune response e.g. a response against self (Murphy et al., 2008). The immune system has developed both central and peripheral mechanisms of tolerance to self. These processes however are not always successful, and failure in these processes can lead to an immune response to self-antigens, in which the immune system attacks the body's cells (Nishimura & Honjo, 2001). A disease in which the immune response has failed to clearly distinguish between self and non-self is multiple sclerosis (MS). Discrimination between self and non-self is confused

in this disease, and this leads to development of an autoimmune disorder. Like other autoimmune disorders, MS is difficult to treat as there are multiple processes occurring and many different cell populations involved in disease onset and progression.

The current study uses an experimental model to investigate cellular processes occurring in MS and whether treatment with test compounds can alter these populations to improve disease outcomes and resolution.

1.2 Adaptive Immune system

Although the adaptive immune system response is much slower to develop than the innate response, once developed, the adaptive response is specific for the pathogen which has been detected. As well as being more specific, immunological memory is developed, so upon further encounters with the same pathogen a response can be mounted more rapidly. The adaptive response works through populations of immune cells, primarily B cells, T cells, NK cells, and antigen presenting cells such as dendritic cells and macrophages, to name a few.

1.2.1 B Cells

The primary role of B cells in immunity is the production and secretion of antibodies; however, B cells are also able to act as antigen presenting cells (APCs) in certain situations (Murphy et al., 2008). Antibodies produced by B cells can function in 3 different ways: by binding to toxins or pathogens to neutralise them, by opsonising targets to allow effective phagocytosis or by activating the complement system (Casadevall & Pirofski, 2004). The majority of mature B cells develop into plasma B cells that continue to secrete large amounts of antibodies, while a smaller portion form memory B cells which are able to respond rapidly if the same antigen is encountered at a later date.

1.2.2 Antigen Presenting Cells

Unlike B cells, which are able to recognise antigens directly through the B cell receptor (BCR), T cells require the antigen to be presented on the major histocompatibility complex (MHC), which in humans is called the human leucocyte antigen (HLA). There are two types of MHC molecule,

class I and class II (Murphy et al., 2008). Antigens derived from an endogenous source i.e intracellular bacteria or viruses, are broken down inside the infected cell and transported to the surface of the cell where they are bound to a MHC class I molecule and presented for recognition by T cells (Vyas, Van der Veen, & Ploegh, 2008). Exogenous antigens are endocytosed by cells and then digested in the endosome by proteases, following which they are presented on MHC class II molecules. In some cases, a process called cross presentation can occur in which exogenous antigens are presented on MHC class I instead of class II. Once the MHC:antigen complex is expressed on the surface of the antigen presenting cell the TCR (T cell receptor) can recognise it and bind. Several cells are capable of antigen presentation and include, but are not limited to dendritic cells, macrophages and B cells (Murphy et al., 2008; Vyas et al., 2008).

1.2.3 T cells

T cells are produced in the bone marrow, from which they travel to the thymus for maturation and positive or negative selection. In this process, T cells that are self-reactive i.e recognise self-antigens are removed which is an important part of the tolerance process (Murphy et al., 2008). T cells are commonly divided into two lineages based on the type of T cell receptor they express. The majority of the T cell population is comprised of αβ T cells while γδ T cells are a small subset of the population. αβ T cells can be further divided based on their expression of the CD4 or CD8 co-receptor, which is capable of recognising antigens presented on MHC class II or class I, respectively. Unique, antigen specific T cell receptors are generated by gene rearrangements which allow for the recognition of multiple antigens presented by MHC molecules.

1.2.4 CD8 cytotoxic T cells

The main role of CD8+ T cells, or cytotoxic T cells (CTL), is the direct killing of cells through multiple receptor interactions. CTLs recognise a cell's endogenous antigens presented on MHC I molecules on the surface of multiple cell types; however, it has been noted that in some cases, CTLs are also able to recognise exogenous antigen presented on MHC I when cross presentation has occurred. (Shen et al., 2006). This process allows CTLs to directly target and kill abnormal and

virus infected cells. This cell killing is mediated primarily through the release of granules containing perforin and granzymes or through Fas-Fas ligand receptor interactions. Cytokines such as TNF-α and IFN-γ released by CTLs can also assist in the killing of the target cells.

1.2.5 CD4 helper T cells

CD4 T cells have multiple roles within the immune system, a key function being the mediation of adaptive immunity through interactions with a range of other immune cells. They are involved in mediating the response of CD8 T cells, regulating the function of macrophages, controlling responses to pathogenic microorganisms and controlling the length and size of the immune response (Zhu, Yamane, & Paul, 2010). Naïve T cells differentiate into a range of T helper lineages that are controlled, in part, by the cytokine environment present during TCR development. The first distinction made amongst CD4 T cells is into Th1 or Th2 T helper cells.

Th1 cells can be identified by their expression of the transcription factor Tbet and by their cytokine production profile which includes the cytokines IFN-γ and TNF-α (Liu et al. 2010). The Th1 lineage of T helper cells is primarily involved in cell mediated immunity and aids in the killing of bacteria or virus infected cells. In contrast to Th1 cells, Th2 cells can be identified by the expression of the transcription factor GATA3 and the production of the cytokines IL-4, IL-5 and IL-13 (Liu et al. 2010; Zhu et al., 2010). This population of T cells is involved in directing responses against extracellular pathogens and therefore plays a role in humoral immunity (Zhu et al., 2010). A third lineage of T helper cells, Th17, produces the cytokines IL-17A, IL-17F, IL-22 and also expresses the transcription factor RORγT. Th17 cells play a role in autoimmunity and neutrophil mediated inflammation (Liu et al., 2010). Treg cells have also been identified as a CD4 T cell lineage. Tregs express transcription factor FoxP3 and produce the cytokines TGF-β and IL-10 (Liu et al., 2010). Further lineages of CD4 cells are under investigation; however, their identifying features and roles in immunity and inflammation are still not fully understood (Zhu et al., 2010). Research by O'Shea and Paul (O'Shea & Paul, 2010) has proposed the idea that CD4 cells commit to a specific lineage;

however, this may need further investigation, as some subsets seem to have plasticity (Yang et al., 2008; Zhou, Chong, & Littman, 2009).

In the context of MS, most research has focused on the TH1/17 subsets as well as the regulatory role of Tregs on inflammatory pathways.

1.2.6 Regulatory T cells

Regulatory T cells (Tregs) are a sub-population of mature T cells produced in the thymus (S Sakaguchi, 2005; S. Sakaguchi et al 1995) and are thought to regulate the immune response and sustain immune tolerance. This subset of cells is CD4+ and also expresses CD25, which is the IL-2 receptor. However, CD25 can also be up regulated on other CD4+ cells, so Tregs need to be further identified by their expression of the transcription factor Forkhead box P3 (FoxP3) (Hori, Nomura, & Sakaguchi, 2003) (Fontenot, Gavin, & Rudensky, 2003; S Sakaguchi, 2005). Tregs are able to suppress T cell activity through multiple mechanisms, primarily mediated through Fox P3, such as secretion of cytokines or through direct cell-cell contact (Chen et al., 2003; S Sakaguchi, 2005). Lack of, or incorrectly functioning, Tregs can lead to the breakdown of self-tolerance mechanisms and development of autoimmune disorders.

1.3 Innate Immune system

The innate immune system is comprised of physical barriers including skin and mucosal membranes, proteins such as complement and immune cells (Murphy et al., 2008) such as natural killer (NK) cells, basophils, eosinophils, neutrophils, mast cells and phagocytic cells (Turvey & Broide, 2010).

1.3.1 Macrophages and Microglia

One of the primary responders to a microbial threat is macrophages, an innate immune cell with dual functions. Macrophages are involved not only in the innate immune response, but also play a key role in in maintaining homeostasis in their tissue environment (Benveniste, 1997; Brück et al., 1996; Hendriks, Teunissen, de Vries, & Dijkstra, 2005). Upon activation, macrophages are able to

phagocytose pathogens and release the inflammatory cytokines IL-12 and TNF-α which are involved in the recruitment and activation of immune cells. Because of their ability to phagocytose pathogens, macrophages, as well as dendritic cells, are able to present peptide fragments via MHC I or MHC II on their cell surface and recruit the adaptive immune system. Microglia are the resident macrophages of the CNS (central nervous system) and their role in MS and the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), appears to be multifaceted, with some papers showing protective roles and others showing a detrimental role (Deng & Sriram, 2005; Jack, Ruffini, Bar-Or, & Antel, 2005; Prineas, Henderson, & Barnett, 2006). As professional phagocytic cells, it is thought that microglia are able to promote neuroregeneration by removing neurotoxic debris and fragments of apoptotic cells, a process that was initially shown in medicinal leeches (Deng & Sriram, 2005). Evidence shown in vertebrates indicates that microglia are also capable of secreting factors to produce an environment conducive to neural regeneration (Deng & Sriram, 2005). In contrast to this reported protective role, other studies demonstrate a damaging effect of microglial activation in MS. Microglia, when acting as antigen presenting cells (APC), are able to activate T cells which leads to the activation of an immune response (Sanders & De Keyser, 2007).

1.3.2 Astrocytes

Astrocytes, discovered by Camillo Golgi in 1871, are a specialized glial cell that largely outnumbers neurons in the CNS (Nair, Frederick, & Miller, 2008). Recently, astrocytes have been identified as having a dual role in CNS inflammatory diseases such as MS, showing both protective and damaging effects in the process of demyelination/remyelination (Barnett and Prineas 2004, Miljković, Timotijević et al. 2011, Burns, Lee Archer et al. 2012, Fischer, Wajant et al. 2014). One of the main functions of astrocytes is to maintain the blood brain barrier (BBB), a barrier that is breached in the case of MS. Astrocytes have been shown to have a protective role by limiting CNS inflammation through the release of soluble factors, as well as providing support to oligodendrocytes (OG), clearing waste and allowing for axonal regeneration (Nair et al., 2008;

Sofroniew & Vinters, 2010). In the absence of trauma or inflammation, astrocytes have also been shown to release brain derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) which indicates that astrocytes promote neuronal survival (Nair et al., 2008). As well as these protective functions, astrocytes have also been implicated in enhancing the immune response and limiting myelin repair, effectively causing further damage to the CNS (Dong & Benveniste, 2001; Nair et al., 2008; Williams, Piaton, & Lubetzki, 2007). These opposing findings have led to the idea that the role of astrocytes in inflammation, namely in MS, depends on the stage of disease, the microenvironment and interactions with other cells present.

1.3.3 Neutrophils

Neutrophils are an innate cell population with a short lifespan, surviving only 6-8 hours in humans (Amulic, Cazalet, Hayes, Metzler, & Zychlinsky, 2012; Kobayashi & DeLeo, 2009). These cells play an important role in innate immunity because of their phagocytic activity and are often one of the first lines of defence against microbes. Neutrophils are granulocytic and are able to secrete granules containing anti-microbial agents to kill pathogens or can directly engulf pathogens (Kantari, Pederzoli-Ribeil, & Witko-Sarsat, 2008; Kobayashi & DeLeo, 2009).

1.3.4 NK cells

Natural killer (NK) cells are cytotoxic lymphocytes that share some similarity in function with T cells. They are able to directly kill virus or tumour infected cells through the release of small molecules such as perforin as well as by being a source of IFN-γ and TNF-α (Bryceson, March, Ljunggren, & Long, 2006). NK cells can further be classified into 2 groups: NK cells and NKT cells. NKT cells differ from NK cells as they possess a TCR that allows them to recognise APCs and secrete T cell related cytokines (Bendelac, Savage, & Teyton, 2007).

1.4 Oligodendrocytes

Oligodendrocytes (OG) are another key cell population involved in demyelination/remyelination. OG produce the myelin sheath that forms the protective covering around neurons allowing for

effective signal transduction. In a pioneering study by Lucchinetti et. al. (Lucchinetti et al., 2000) the lesions of post-mortem MS patients were characterised and classified into 4 distinct groups. Type 1 and type 2 lesions seemed to be primarily immune mediated and were characterised by infiltration of T cells, whereas type 3 and type 4 lesions lacked this T cell infiltration and could be characterised by a loss of OGs. This led to the idea that different stages of disease could be associated with a different cellular process occurring at the lesion sites.

1.5 Myelin

Myelin is the protective sheath that wraps around axons and allows for effective transmission of signals. It is produced by the oligodendrocytes and is composed of multiple proteins, including myelin oligodendrocyte protein (MOG), myelin basic protein (MBP) and myelin proteolipid protein (PLP) (Vercellino et al., 2009). During MS, this protective myelin sheath is destroyed, primarily by T cells and the death of OGs, leading to impaired signal transduction along the axon. This process of myelin and OG destruction is reflected by the loss of motor and neural control seen in MS patients.

1.6 Multiple Sclerosis

MS is an inflammatory disease of the central nervous system and is mediated primarily by the immune system (Barnett & Prineas, 2004). MS affects approximately twice as many women as men, and generally symptoms begin to present themselves in the third or fourth decades of life. Symptoms of MS are varied and can include, but are not limited to cognitive and motor impairment, fatigue, visual disturbances and bladder and bowel problems (Keegan & Noseworthy, 2002).

MS can present in different forms, the most common of which is relapsing remitting MS (RRMS). Approximately 85% of MS patients fall into the RRMS subcategory which features periods of attack/worsening of disease symptoms followed by periods of recovery and gradual improvement. Of the patients diagnosed with RRMS, 10-15% will go on to develop secondary progressive MS

(SPMS) in which the disease becomes continuously progressive with no distinct attacks, just a gradual worsening of symptoms. Another form of MS is primary progressive (PPMS) which is characterised by the development of a progressive onset of disease with no remissions like those seen in RRMS (Bitsch, 2002)

1.6.1 MS aetiology

Extensive research has been conducted into the causes of MS, and although no single causative factor or agent has been identified, several potential risk factors exist. Risk factors that have been a key focus for research into MS include diet, geographical location, smoking, infectious diseases and genetics (Ascherio & Munger, 2007; Handel et al., 2011).

In studies looking at genetic risk factors associated with the development of MS, 2 genes are commonly identified as being involved; HLA-DR and HLA-DQ (Chao et al., 2009; D. A. Dyment et al., 2005; Schmidt, Williamson, & Ashley-Koch, 2007). These genes are part of the human leucocyte antigen molecule, the human version of the MHC molecule. Genes outside the HLA loci have also been identified as risk factors for MS and include cytokine and receptor genes (Hafler et al., 2007). Alongside these genome wide studies, familial studies have also been conducted looking into the rates of MS occurrence among related individuals. In general, there is an increased risk of developing MS the more closely related you are to a family member with MS (David A. Dyment, Ebers, & Dessa Sadovnick, 2004; D. A. Dyment et al., 2005). In adoptive studies it was found that children raised by a parent with MS are no more likely to develop MS than the general population, indicating that risk is genetic and not due to environment.

Geographical location also appears to play a role in the risk of developing MS, as rates of MS vary greatly around the world. Places such as Orkney and the Shetland Islands in Scotland along with Saskatoon in Canada have a very high disease prevalence of around 200 per 100,000 (Alla & Mason, 2014). In contrast to these high rates, cases of MS are very rare in Japanese or African Blacks (Pugliatti, Sotgiu, & Rosati, 2002).

New Zealand is a country with a relatively high incidence of MS. Data collected in the 2006 census showed an age standardised prevalence of 73.1 per 100,000 population, with 75% of cases being recorded in women (Alla & Mason, 2014). In the Maori population, incidence of disease was recorded as being much lower at only 24.2 per 100,000.

A correlation exists between MS levels and latitude, with those living at higher latitudes having an increased risk of developing MS. A meta-analysis conducted by Simpson et. al. (Simpson, Blizzard, Otahal, Van der Mei, & Taylor, 2011) highlighted this correlation, with the exception of two countries, Italy and northern Scandinavia. This variance in levels of MS based on latitude is proposed to be due to the hours of exposure to sunlight, with the protective effects mediated by vitamin D. While the majority of vitamin D is synthesised in the body from exposure to UVB rays, some foods can also provide a source of vitamin D (Ascherio, Munger, & Simon, 2010). Countries with lower than expected rates of MS based on latitude may have their lower MS rates explained by the consumption of foods rich in vitamin D (Pierrot-Deseilligny & Souberbielle, 2013)

One viral agent which has been the focus of MS risk assessment has been the Epstein Barr virus (EBV); however, a strong causative relationship between infection with EBV and subsequent development of MS has not yet been shown (Noseworthy, 1999). A study by Neilson and colleagues (Nielsen, Rostgaard, Nielsen, & et al., 2007) concluded that the risk of developing multiple sclerosis was increased in people with mononucleosis (the infectious form of EBV) regardless of age or sex, and this increased risk persisted up to 30 years after infection. EBV is very widespread and is estimated to infect approximately 90% of the population in a dormant state, with approximately 40% of the population developing infectious mononucleosis. This suggests that infection with EBV is only a small contributing factor to MS since EBV has a high prevalence but a low incidence of MS disease.

1.6.2 MS Immunology

The primary driver of MS is mediated by the immune system. Current treatments aim to modulate the immune response during disease, indicating that although cause remains unknown, the immune system plays a major role in disease development. One of the key cell populations involved in driving disease are CD4+ T cells, which recognise components of the myelin sheath such as MOG, MBP and PLP, as foreign antigens and so mount an immune response to the myelin components. (Herndon, 2003; Sospedra & Martin, 2005). Although self-reactive T cells are present in healthy populations, they are present in higher numbers in people with MS, indicating that in these patients something has caused proliferation and activation of this CD4+ T cell population (Pender & Wolfe, 2002; Racke, Ratts, Arredondo, Perrin, & Lovett-Racke, 2000). Once these self-reactive CD4+ cells have been activated they must migrate to the CNS to cause disease progression. This is referred to as the 'outside-in' theory of MS, and is widely accepted. Once present in the CNS there CD4+ cells direct damage to the myelin sheath leading to inflammatory lesions. During this process other cell populations are recruited/activated including CD8+ T cells, astocytes, microglia and macrophages (Barnett & Prineas, 2004; Noseworthy, 1999). Blood brain barrier (BBB) breakdown also occurs, increasing cell accessibility to the CNS, however it is not clear whether this precedes CD4+ cell invasion or occurs as a result of it. After the initial period of attack, inflammation can resolve leading to regeneration of the lesion site and formation of a glial scar by astrocytes (Dong & Benveniste, 2001; Sofroniew & Vinters, 2010). The level of regeneration occurring will be reflected in the severity of disease symptoms, with periods of remission occurring when this regeneration is successful.

1.6.3 Current treatments

As CD4+ T cell infiltration appears to be a key component of lesion development, multiple current therapies aim to inhibit cell migration into the CNS and immunosuppressive therapies aim at dampening down the immune response. (Bates, 2011; Pender & Wolfe, 2002). Current therapies

can be broadly broken down into two categories: those that modify the immune response such as glatiramer acetate (GA)interferon-β and Ocrelizumab (Ge et al., 2000; Markowitz, 2007) and those that prevent migration of self-reactive cells into the CNS such as natalizumab (Johnson, 2007; Weinstock-Guttman & Jacobs, 2000). Although treatments are available, their efficacy is limited and some are limited in use due to the severity of their side effects. Current therapies have only shown beneficial effects in relapsing remitting forms of disease, with no benefits shown in later progressive forms of disease.

1.7 Disease Models

MS is a multi-faceted disease with no known cause (Herndon, 2003; Lucchinetti et al., 2000), and for this reason creating a disease model to encompass all aspects of the disease is complex. In an attempt to develop or test suitable therapies, several disease models have been proposed, including EAE (Kuerten & Angelov, 2008), the cuprizone model for demyelination (Kipp, Clarner, Dang, Copray, & Beyer, 2009) and a viral model of disease caused by infection with Thieler's encephalomyelitis virus (Tsunoda & Fujinami, 2010). All of these models have their own strengths and weaknesses, and allow research to focus in on a specific area of MS pathology.

1.7.1 EAE

EAE is a model that is commonly used to mimic human MS pathology and to assess the efficacy and safety of potential therapies. The disease model is induced by injecting an animal, usually a rodent or rabbit, with a combination of a myelin peptide and Freunds adjuvant followed by pertussis toxin. This causes the development of myelin peptide specific T-cells which will undergo clonal expansion and provide a pool of CD4+ myelin specific T-cells in the periphery. These autoreactive T-cells are then able to migrate from the periphery into the CNS where they mediate the inflammatory process and ultimately lead to the destruction of the myelin sheath around neurons.

Interestingly, by varying the strain of mouse used, as well as the myelin peptide used to induce disease, EAE with different disease courses can be produced. When SJL mice are injected with the PLP, MOG or MBP, peptide they develop a relapsing remitting form of the disease (Kono et al., 1988). This allows the study of disease progression and demyelination driven primarily by T cell infiltration into the CNS. The clinical and histological similarities seen between this relapsing remitting disease model and the relapsing remitting disease seen in human MS sufferers makes this model a valuable tool for disease study.

In contrast to the SJL strain of mice, the C57BL/6 strain presents with a severe progressive form of disease when immunised with the MOG peptide (Bernard et al., 1997). Having access to a model that can mimic different MS pathologies allows for potential therapies to be assessed in detail and in multiple circumstances.

One of the main criticisms of the EAE model for developing MS therapies, however, has been its failure to predict outcomes when treatments are started in human patients. A key example of this was the blockade of TNF-α with proteins or monoclonal antibodies being predicted to have a protective effect in MS patients based on EAE results. When trialled in MS patients, however, the effects of TNF-α blockade were detrimental to the patients (Steinman & Zamvil, 2005). This in itself is not surprising, as cytokines play a multifaceted role in both healthy and diseased patients, therefore, while blocking a cytokine's effects in a disease setting may also prevent it from acting in other systems that are working in the background to limit disease. For example, TNF-α is a growth factor required for oligodendrocytes, which are required for the growth and repair of myelin.

1.7.2 Theiler's Murine Model for MS

Another model used to assess potential MS therapies is Thieler's murine model, an MS model of viral infection. One widely held idea about MS development is that it results from an interaction of a genetic susceptibility combined with an environmental factor such as a viral infection (Oleszak, Chang, Friedman, Katsetos, & Platsoucas, 2004). Thieler's virus is a picornavirus that induces a

disease resembling EAE in susceptible mouse strains and leads to a chronic demyelinating disease. The virus initially replicates in the grey matter of the brain, with chronic demyelinating disease being evident 4 weeks later. As with MS and EAE, the later stages of disease are characterised by mononuclear cell infiltrate into the CNS, axonal degradation, spinal cord atrophy and the presence of demyelinating lesions (Oleszak et al., 2004). Because the EAE and Thieler's disease models are both mediated by the immune system and share similar patterns in cytokine responses, both are used to model human MS.

1.7.3 Cuprizone Model

Cuprizone, bis(cyclohexanone) oxaldihydrazone, is a copper chelator, that when fed to mice leads to consistent and reproducible demyelination in the central nervous system (Groebe et al., 2009; Gudi et al., 2009). The main cellular target of cuprizone in this model is the mature oligodendrocyte population, and cuprizone selectively targets and kills these cells (Benardais et al., 2013). The mechanism of action for cuprizone is still debated. One of the key features of this model is that if cuprizone is removed from the feed within 12 weeks of its initial administration, spontaneous remyelination will occur (G. K. Matsushima & P. Morell, 2001). Different doses of cuprizone in mouse chow have previously been used and characterised, with most labs carrying out optimisation trials to determine the most effective dose for their needs with the least off-target effects. A 0.1 % cuprizone diet has very minimal effects on demyelination, whilst a 0.5% cuprizone diet causes a high level of mortality. These dose-dependent effects are also strain dependant (Skripuletz et al., 2008).

To avoid confusion between treatment with test compounds/potential therapeutic compounds and 'treatment' with cuprizone to induce the cuprizone model, treatment with cuprizone for disease model induction will be referred to as cuprizone intoxication. This terminology has been used in previous publications (Irvine & Blakemore, 2006; Steelman, Thompson, & Li, 2012) and so will be used here.

1.8 Development of the Cuprizone Model

Some of the first reported experiments using cuprizone as a toxin were carried out by Carlton (Kipp et al., 2009) in the 1960's. Carlton reported that feeding mice cuprizone, a compound used to chelate copper in reagent analyses up until this point, led to the development of microscopic lesions in the brain (Carlton, 1966) accompanied by astrogliosis, demyelination and edema. Carlton fed mice cuprizone mixed into rodent chow at concentrations ranging from 0.2% to 0.5%. Mice showed a dose-dependent growth retardation, with mice receiving the higher dose of cuprizone in their diet (0.5%) having a high associated mortality rate, especially in younger mice. His findings showed a 100% mortality rate in 4 week old mice that was reduced to 20% in 8 week old mice. Based around these earliest studies of the in vivo effects and subsequent toxicity studies, most laboratories now use 6-9 week old mice in their experiments, altering the cuprizone concentration in the chow to produce optimum results in their laboratory. Commonly, dosing is around 0.2%-0.3% but this will vary upon mouse strain, age and desired outcome.

1.8.1 Cuprizone mechanism of action

Cuprizone was initially discovered in the 1950's and described as a copper chelating agent. When mixed with copper, the resulting cuprizone:copper complex produced an easily identifiable peak absorbance centred at 595 nm. It was not until the earlier mentioned studies by Carlton et. al in the late 1960's that the focus on cuprizone shifted to the biomedical field (Kipp et al., 2009). Although the mechanism of cuprizone as a neurotoxicant is not completely understood there are several leading theories, each with its own supporting evidence. It is widely accepted that feeding mice cuprizone results in mega mitochondria being present in the liver tissue. These mega mitochondria indicate a disturbance in metabolism and metabolic injury, resulting in slight alterations to the inner christae of the mitochondria, along with the increased size of the organelle. The presence of mega mitochondria can be explained by two processes – either normally sized mitochondria that enlarge or mitochondria that fuse (Hoppel & Tandler, 1973). Examples of both of these processes have been seen following cuprizone treatment during metabolic studies (Hoppel

& Tandler, 1973; Wakabayashi, Asano, & Kurono, 1974). Another proposed model for the formation is that the mega mitochondria form as a result of an inability of the mitochondria to successfully divide (Flatmark, Kryvi, & Tangeras, 1980). Since cuprizone feeding reliably causes disruption to mitochondrial function, it is proposed that it exerts its neurotoxic effects through a disruption in cellular respiration, a crucial process requiring functional mitochondria.

Cuprizone has also been shown to disrupt the activity of a series of enzymes in the brain and liver. A decrease in the activity of monoamine oxidase and cytochrome oxidase activity was reported by Venturinin (Venturin.G, 1973) following cuprizone treatment of mice. Another enzyme shown to be affected by a cuprizone diet is carbonic anhydrase II (CA II). Levels of this enzyme are seen to decline prior to the occurrence of demyelination in animals exposed to cuprizone (Komoly, Jeyasingham, Pratt, & Lantos, 1987). One of the key roles of CA II is the buffering of pH changes in the brain. Changes in pH can lead to neuronal cell stress and ultimately cell death, including death in the oligodendrocyte population- the cells responsible for producing and maintaining a myelin sheath. To gain further insight into a possible mechanism of cuprizone action, the enzymatic activity of copper-chelated cuprizone treated mice was investigated. Interestingly however, enzymatic activity in these animals was not affected, therefore, chelation of copper, and the resultant loss of available copper for copper-dependent hormones, cannot be solely responsible for the neurotoxic effects seen when mice are fed cuprizone. Despite this finding, cuprizone fed mice treated with copper to restore normal copper levels in the body did not act to replace the copper chelated by the cuprizone and restore the function of those enzymes requiring copper to function (Carlton, 1966) further lending support to the idea that copper chelation alone is not responsible for the neurotoxic effects of cuprizone.

Another possibility put forward for the neurotoxic action of cuprizone is that it chelates other heavy metals including Fe^{2+}, Mn^{2+} or Zn^{2+}; however, a simple deficiency of these metals does not result in the histological or biological changes that are seen with cuprizone treatment, indicating that chelation is again not likely to be the sole cause of disease. (Hoppel & Tandler, 1973; Zatta et

al., 2005). A further idea proposed that a metabolite of cuprizone was causing the effects seen; however, no evidence has been produced that cuprizone metabolites are involved (Kipp et al., 2009).

Work carried out by Pasquini et. al (Pasquini, Calatayud, Bertone Uña, et al., 2007) attempted to further elucidate the neurotoxicant effect of cuprizone through the use of primary rat oligodendrocyte cultures. It was found that cuprizone treatment alone had no detrimental effect on cell viability; however, when cuprizone was combined with certain levels of TNF-α and IFN-γ, survival was affected significantly, indicating that these pro-inflammatory cytokines, commonly secreted by microglia, play a crucial role in oligodendrocyte development (Pasquini, Calatayud, Bertone Uña, et al., 2007). This study also showed that in glial cells isolated from cuprizone treated animals, the mitochondrial function was impaired, indicated by a decreased activity of electron transport complexes of the respiratory chain. A further *in vivo* experiment presented in the same paper showed that by inhibiting microglial activation with minocycline, cuprizone-induced demyelination was prevented. Taken together these results indicate that microglia have a major role in cuprizone-induced demyelination through the release of pro-inflammatory cytokines.

1.8.2 Cuprizone as a model for multiple sclerosis/clinical relevance

The myelin sheath surrounding neurons provides support and protection and has a well-documented history of being able to repair and remyelinate following a demyelination injury (Glenn K. Matsushima & Pierre Morell, 2001). The cellular events involved in demyelination and remyelination however are much more difficult to ascertain, as they are not easily delineated. Remyelination can be occurring at the same time as demyelination, and both processes are complex. Another factor making this a difficult process to study is the variation in location and severity of lesions caused by the EAE model. Lesions are not consistent in a single mouse and can be hard to reproduce, as well as varying greatly between mouse species (Zendedel, Beyer, & Kipp, 2013). In contrast to the variation in lesion development seen in EAE, the cuprizone model can

be utilised to reproducibly induce demyelination on a large scale (Groebe et al., 2009; Glenn K. Matsushima & Pierre Morell, 2001).

One of the key attractions of the cuprizone model as a model for MS is based on the findings of the previously mentioned study by Luccinetti et al, in which lesions were classified into four subtypes based on the profile of the cells involved. Pattern one and two appeared to be autoimmune mediated; however, type three and four lesions fit a different profile, showing a pathology more like that of toxin induced damage. The cuprizone model is useful for investigating the cellular mechanisms of this non-immune-mediated type of lesion similar to categories three and four of Lucchinetti (Lucchinetti et al., 2000; Praet, Guglielmetti, Berneman, Van der Linden, & Ponsaerts, 2014). Some characteristics shared by cuprizone-induced lesions and MS lesions include active demyelinating lesions in the absence of T cell penetration of the CNS, presence of activated microglia and macrophages, as well as hypoxia-like injury (Mahad, Ziabreva, Lassmann, & Turnbull, 2008) and mitochondrial stress, leading eventually to oligodendrocyte apoptosis (Praet et al., 2014). Another structural similarity seen between cuprizone-induced lesions and MS lesions is axonal swelling as a result of redistribution of Na^+ channels along the axonal membrane causing a Na^+ ion imbalance. It was however noted that there was a CD3+ population (thought to be non-contributing to the myelin loss) present in the cuprizone-induced lesions that was absent in the type 3 lesions (Praet et al., 2014) identified by Lucchinetti et al.

MS can be identified by a broad range of motor symptoms that includes loss of bladder and bowel control, muscle weakness and fatigue as well as a loss of balance (Bitsch, 2002; Dutta & Trapp, 2011; Herndon, 2003). In the cuprizone model for demyelination, impaired coordination between left and right paws has been detected, thought to be a result of demyelination in the corpus callosum (Liebetanz & Merkler, 2006). In MS, the level of lesions in the hippocampus and basal ganglia correlates to cognitive impairment, fatigue, and memory deficits and motor skill impairment. Severe hippocampal demyelination and demyelination of the basal ganglia has been reported after cuprizone intoxication (Pott et al., 2009).

In female MS sufferers, pregnancy prevented disease episodes, but this protection was lost by 3 months following delivery. This finding suggests that sex hormones play an important role in disease progression and relapse rates (Confavreux et al., 1998; Praet et al., 2014) (Runmarker & Andersen, 1995). In a study by Patel et al (Patel et al., 2013) it was shown that castrated or ovariectomized mice had a reduced ability to remyelinate following myelin injury caused by administration of a cuprizone diet. Using histological methods, the present study also identified an increase in the oligodendrocyte population in gonadectomised animals when supplemented with estradiol, which could contribute to the remyelination effect seen in the corpus callosum with estradiol supplementation. This beneficial effect of estradiol on remyelination during cuprizone intoxication was shown to be mediated through the G-protein coupled estrogen receptor GPR30, located on oligodendrocytes (Hirahara et al., 2013).

1.8.3 Cuprizone as a model of schizophrenia

A range of the behavioural deficits seen during cuprizone intoxication resemble those seen in patients with schizophrenia, including an impaired spatial memory, sensorimotor deficits and a decrease in social behaviours such as grooming (Haiyun Xu, Yang, & Li, 2014; H. Xu, Yang, & Rose, 2010). Treatment of mice with an antipsychotic drug, quetiapine, was shown to cause a reduction in white matter pathology and a promotion of remyelination when used in the cuprizone model (Haiyun Xu et al., 2014; H. Xu et al., 2010). MS, like schizophrenia, is characterised by inflammation and demyelination, so using the cuprizone model to assess the benefits of antipsychotics commonly used to treat schizophrenia could be a useful approach in an MS disease setting.

1.9 Behavioural assays

1.9.1 Rotating Rod to assess motor coordination

The measurement of motor coordination in mice can be used to gain valuable information about the effects of a test compound on factors such as coordination and balance. These behavioural models can also be useful for assessing the impact or effectiveness of an intervention during disease progression. The rotating rod, or rota-rod is a behavioural assay carried out in many laboratories, mainly to look at effects of neurological impairments or recovery after experimental intervention. The rota-rod is a cylindrical barrel that rotates at a fixed or accelerating speed, as determined by the user. The barrel has grooves marked along it to aid the animals in gripping the barrel, and is flanked by large circular flanges at either end to prevent any environmental distraction of the test animal. After being habituated to the rota-rod the mouse is placed on the rotating rod, and the appropriate experiment is carried out, for example, determining the maximum duration a mouse can remain on the rotating barrel without falling, or noting the speed at which the mouse is able to continue walking at. Brief protocols or starting points can be found in the literature; however, individual labs can adapt the protocols to suit their particular needs (Carter, Morton, & Dunnett, 2001).

1.9.2 Horizontal bars

The horizontal bars are a behavioural assay that can be used to assess grip strength and coordination in mice. A 2 mm steel rod is suspended between two props, approximately 50cm apart and 50 cm above the ground. Mice are placed in the middle of the suspended rod, gripping the bar by their front paws only. The time taken to reach either end of the bar is recorded, with a faster time being associated with an improved performance.

1.10 MIS416

MIS416 is a non-toxic micro-particle derived from the bacterium that causes common acne, *Propionibacterium acnes* (Girvan et al., 2011). The particle is approximately 0.5 nm by 2 nm and is

comprised of a minimal skeleton rich in muramyl dipeptide (MDP) and cross-linked to single stranded bacterial DNA. These components target the internal NOD-2 and TLR-9 receptors, causing the MIS416 particle to have downstream immunostimulatory effects (Girvan et al., 2011). Both of these receptors are intracellular, therefore, cells with phagocytic capabilities are primarily targeted. Fluorescently labelled MIS416 has been shown to be internalised by dendritic cells and macrophages (Girvan et al. 2011). MIS416 was initially developed as a vaccine adjuvant; however, there is now interest in MIS416 as an immunomodulatory agent for treatment of inflammatory disorders (White, Webster, O'Sullivan, Stone, & La Flamme, 2014). After a compassionate use trial in secondary progressive MS patients provided positive anecdotal outcomes, a formal phase 1b/2a trial was conducted to evaluate the safety profile and tolerability of MIS416 in secondary progressive MS following its systemic administration (Luckey, Anderson, Silverman, & Webster, 2015). A maximum tolerated dose was not reached during this trial. Adverse events were most commonly acute flu like symptoms, which were expected based on the known bioactivity of MIS416 (Luckey et al., 2015), and further phase 2 trials based on a 500 mg/week dose were recommended.

1.11 Clozapine

Clozapine is an atypical antipsychotic able to cross the blood brain barrier. It is commonly used to treat schizophrenia(Young, Bowers, & Mazure, 1998) and has been associated with a reduction in inflammation in the CNS , as well as being able to suppress the production of pro inflammatory cytokines in patients as well as *in vitro* (Basta-Kaim et al., 2006; Kato et al., 2011). Data from our lab (submitted for publication) has shown that orally administered clozapine was able to reduce disease severity in EAE as well as alter the CNS cell infiltrate.

1.12 Overall Aims and Objectives

1. To optimise a cuprizone model in C57BL/6 mice capable of producing robust and reproducible demyelination in the corpus callosum, as well as determining which behavioural assays will be sensitive enough to detect differences in motor coordination.

2. Assess any therapeutic effect of MIS416 and clozapine during demyelination, with a focus on myelin, CNS resident cells and peripheral immune cells.

3. Assess the effect of MIS416 and clozapine individually on remyelination after cuprizone induced demyelination on myelin levels, CNS populations and peripheral immune cells.

4. Identify and measure changes to serum cytokines and growth factors as a result of MIS416 treatment and the result of *in vitro* administration of MIS416 and clozapine individually on primary microglial cells.

CHAPTER 2: Methods

2 Methods

2.1 Mice

Animals were housed at the Victoria University of Wellington animal facility, New Zealand. All experimental protocols were approved by the animal ethics committee (AEC) of Victoria University of Wellington**.** The animals were housed in a temperature maintained environment with a light/dark cycle of 12 hours. All handling of animals during experiments was conducted in a laminar flow hood during the dark cycle.

Female C57BL/6J mice were purchased from the biomedical research unit (BRU) at the Malaghan Institute of Medical Research, in Wellington, New Zealand. Animals were bred at this facility, however the initial breeding stock was purchased from Jackson Laboratories (Bar Harbour, ME, USA).

2.2 In vivo techniques

2.2.1 Compounds for *in vivo use*

MIS416 was generously supplied in solution by Innate Immunotherapeutics, Auckland, New Zealand and stored at 4 ºC long term.

2.2.2 Administration of MIS416

MIS416 was injected in indicated concentrations (100 μg or 200 μg) into the lateral tail vein, using a 29 or 31 gauge needle, in a total volume of 100 μL using PBS as a diluent. Prior to injecting, animals were placed under a heat lamp to increase vessel dilation. Doses were selected based on prior work from our lab (White, 2015).

2.2.3 Administration of Clozapine

Clozapine (Medichem, Spain) stored at 4 ºC long term, was dissolved in a 0.1 M acetic acid solution to form a stock drug solution and then diluted in the mice's drinking water at a dose of 60 mg/kg. Clozapine drinking solution was administered in light protected drinking bottles and was made up freshly and replaced every 2-3 days. Dose was determined based on previous work from our lab (Green, Zareie & Templeton et al, 2017).

2.2.4 Preparation of Cuprizone feed

Pelleted animal feed was ground and heated to sterilise it, before the appropriate amount of Cuprizone (Sigma) was mixed through the powdered feed.

2.2.5 Administration of Cuprizone diet

Powdered feed with the indicated Cuprizone concentration was placed in the cage in a petri dish and was readily available. Feed was replaced daily.

2.3 Ex vivo techniques

2.3.1 Dissection and organ removal

Mice were euthanized by CO_2 asphyxiation or with a lethal overdose of sodium pentobarbital (ProVet, New Zealand) and were then washed down with 70% ethanol to make incisions through fur tidier.

To collect blood the peritoneal cavity was opened using scissors and the diaphragm was pierced to expose the heart. A cardiac puncture of the right ventricle was performed using a 1 mL syringe attached to a 27 gauge needle (BD Biosciences) with collection volume ranging between 300-700 μL.

For removal of brains and spinal cords mice were first perfused with 20 mL of 1 x PBS to remove blood cells from the tissues which was done by severing the portal vein and then using a 23 gauge needle attached to a 20 mL syringe. This was then followed by 10 mL 4% paraformaldehyde (PFA) solution to fix the tissues (appendix). For removal of brains for microglia culture and isolation, perfusion with PFA was omitted. Mice were decapitated and the spinal column was severed at the lower lumbar region, spinal cords removed by using a 19 gauge needle attached to a 10 mL syringe containing 1 x PBS to flush the spinal cord out of the spinal column by inserting the needle at the lower end of the spinal column. Brains were removed by using small dissection scissors to sever all connective tissue around the entry point of the spinal cord to the brain. The bone between the eye sockets was cut, then the skull was cut starting at the base of the cerebellum and moving upwards towards the midline. The skull was then peeled back, the optical nerves detached and the brain scooped out and placed in either 4% PFA or sterile 1 x PBS for microglial culture.

2.3.2 Isolation of serum from blood

Blood collected via cardiac puncture was allowed to sit at room temperature for a minimum of 30 minutes or until the blood was visibly clotted. It was then centrifuged at 7800 x g for 10 minutes at 4 degrees Celsius. Serum was removed from the top of the sample and transferred into fresh eppendorf tubes to be stored at -80 degrees Celsius for further analysis.

2.3.3 Single cell suspension preparations from spleens

After isolation, spleens were placed in wash buffer (appendix). Spleens were dissociated by pushing the tissue through a 70 μM cell strainer (BD biosciences) and the remaining cells were washed

through with wash buffer into a 50 mL conical tube. These cells were centrifuged for 770 x g for 5 minutes and then the red blood cells were lysed by resuspending the cells in red blood cell lysis buffer (Sigma) for two minutes. Eight mL of wash buffer was then added and the tubes were again centrifuged at 770 x g for 5 minutes to wash the splenocytes. Splenocytes were resuspended in 10 mL of fresh wash buffer and live cells were counted using the trypan blue exclusion method.

2.3.4 Single cell suspension preparations from brains

After removal, whole brains were dissociated through a 70 μM mesh cell strainer (BD Biosciences) into a 50 mL conical tube. Remaining cells were washed through using sterile 1 x PBS. Tubes were then centrifuged at 760 x g for 5 minutes and were then ready for microglial isolation.

2.4 Microglial Isolation

Supernatant from single cell suspension preparation was poured off and the pellet was resuspended in 10 mL of 70% Percoll, diluted with percoll diluent (appendix) and mixed gently with a 1 mL pipette to dissociate clumps without introducing air bubbles. The 70% Percoll was carefully overlaid with 10 mL 37% percoll added in 1mL increments so that the interface between the two percolls didn't mix. The 37% percoll was then overlaid with 10 mL of 30% percoll, again added slowly in 1 mL increments so that the two percolls didn't mix. The tubes were centrifuged for 30 minutes at 760 x g with low acceleration and no break. After centrifugation the myelin was removed from the top of the 30% percoll using an autopipettor. The microglia were then isolated from the interface of the 70% and 37% percoll solutions and transferred into a new 50 mL conical tube. The cells were diluted at least 3 fold with sterile 1 x PBS and centrifuged at 760 x g for 5 minutes. Supernatant was discarded and the pellet resuspended in 1 mL microglia media (appendix). Cells from individual mice were then pooled and counted using trypan blue before being centrifuged at 760 x g for a further 5 minutes before being resuspended at 250,000 cells/mL. Cells were then plated at 50,000 cells per well and had their media replaced every 3-4 days with

microglia media supplemented with 10 ng/mL M-CSF (Prospec, Rehovot, Israel). After 4 weeks of culture the cells were used for stimulation assays.

2.4.1 Assessing microglial purity by flow cytometry

Microglia were stained using antibodies to CD45 and CD11b. Initially cells were washed with FACs buffer and then resuspended in 2.4G2 and left on ice for 15 minutes in the dark. Cells were washed again with FACs buffer at 400 x g for 5 minutes before resuspending cell pellet in the correct staining mix. Cells were incubated in the dark for 20 minutes before being washed in FACs buffer at 400 x g for 5 minutes and then being resuspended in 300 μL of fresh FACs buffer in a FACs tube. Cells were analysed on a Canto II flow cytometer (BD BioScience) and data was analysed using FLOWJO v 10.0.

2.4.2 Primary Microglial Culture

After isolation microglia were seeded at 250,000 cells per well in a solution of DMEM, L-glutamine, PenStrep, FCS, betamercaptoethanol and recombinant M-CSF. Cells were incubated at 37 degrees Celsius with 5% CO_2 for four weeks, with media being changed every three days. After the four week culture period cells were stimulated with the appropriate compounds and their supernatants harvested for further analysis.

2.5 Trypan Blue Exclusion Assay

To determine live splenocyte numbers, cells were resuspended in wash buffer and counted using 0.4% trypan blue (Sigma) at a 1:10 dilution. 10 μL of the dye and cell mix was placed on a Neubauer haemocytometer (Hawksley, Lancing, UK) and counted using a compound microscope (CX41; Olympus, PA, USA). Microglial isolations were counted in a 1:2 dilution of trypan blue.

2.6 Preparation of single cells for flow cytometry

Single cells isolated from the brain or spleen were suspended in 500 uL of FACs buffer (appendix) and spun at 400 x g for 5 minutes. FACs buffer was removed and the cell pellet was resuspended in 2.4G2 blocking antibody for 15 minutes on ice, protected from the light. 400 μL of FACs buffer was added and cells were pelleted 400 x g for 5 minutes. Supernatant was removed and 50 μL of the appropriate staining mix was added. These were incubated in the dark on ice for 20 minutes. FACs buffer (500 μL) was then added and spun at 400 x g for 5 minutes, supernatant was removed and the cell pellet was resuspended in 300 μL of FACs buffer before being transferred and filtered into FACs tubes. For antibody concentrations see appendix. In experiments with larger sample sizes, staining was carried out in a 96 well plate following a checker-board pattern before being transferred into FACs tubes for Flow analysis.

2.7 Ex vivo restimulation of splenocytes

Splenocytes were plated at 1 million cells per well and stimulated with either LPS (200 ng/mL), ConA (1 μg/mL) MIS416 (10 μg/mL) or a combination of these compounds. Cells were incubated at 37 degrees Celsius with 5% CO_2 for 24 hours and then supernatant was collected and frozen at -20 degrees Celsius.

2.8 Ex vivo restimulation of microglia

Microglial culture media was removed from wells containing microglia and the appropriate volume of Cuprizone (500 μg-50 μg), Clozapine (10 μM), LPS (200 ng/mL), MIS416 or a combination was added. Cells were incubated for 24 hours at 37degrees Celsius with 5% CO_2 and then supernatant was removed and stored at -20 degrees Celsius for further analysis. An MTT cell viability assay was then performed on remaining cells.

2.9 MTT assay

Fifty μL of CTCM (life technologies) was added to each well, followed by 20 μL of MTT solution (appendix). This was then incubated at 37 degrees Celsius for 4 hours. Cell proliferation was stopped by the addition of 50 μL MTT stop solution (appendix) to each well. The plate was then returned to the incubator overnight before having the absorbance read at 570 nm to obtain viability readings.

2.10 Enzyme linked immunosorbent assays (ELISA)

All ELISAs were performed according to the manufacturer's instructions (BD Bioscience) with the appropriate cytokine standard in duplicate. 96 well ELISA plates (BD Bioscience or Thermo Fisher) were coated with 50 μL of the primary 'capture' antibody diluted in a sodium phosphate buffer (appendix) at pH 6 or 9 and left overnight to adhere to the plates at 4 degrees Celsius. The following day capture antibody was removed and the plates were coated with 5% or 10 % FCS in 1 X PBS blocking buffer and left at room temperature for 2 hours. After incubation the blocking buffer as removed and the plates were washed with 1 X PBS containing 0.05% Tween 20 (Sigma). After washing the standards and samples were loaded into the plates in a volume of 50 μL and left for two hours at room temperature or overnight at 4 degrees Celsius. The plates were again washed with the wash buffer and a biotinylated secondary antibody diluted in wash buffer was added to the wells and incubated for a further hour. This secondary detection antibody was removed and the plate washed with wash buffer 6 times and then a strepdavidin conjugated horse radish peroxidise was added and left to incubate in the dark for 1 hour at room temperature. Strepdavidin was then removed and plates were washed 6-8 times, followed by the addition of tetramethyl benzidine (TMB) reagents A and B (BD Bioscience) were mixed in equal volumes then 100 μL of this was added to each well. Once colour had begun to develop 100 μL of stop solution (appendix) was added to each well. Absorbance values were measured using an EnSpire 2300 multi-label plate reader (Perkin Elmer). Absorbance readings were used to construct a standard curve and cytokine concentrations were then calculated. For a list of antibody concentrations see appendix.

2.11 Griess Reaction

Using the Griess assay to measure the levels of nitrite as an indicator of NO levels (NO is unstable and rapidly breaks down into nitrites and nitrates) we were able to get an indication of NO produced in cell culture supernatant. To do this equal volumes of Griess A and Griess B (appendix) reagents were mixed and then 50 μL of this mixture was added to wells in a flat bottomed 96 well plate containing supernatant. A standard for this assay was produced by making serial dilutions of sodium nitrate in duplicate and then constructing a standard curve from the absorbance values of these dilutions. Absorbance was measured at 570 nm using an EnSpire 2300 multi-label plate reader (PerkinElmer) which allowed determination of the nitrite concentration in the supernatant samples. The upper detection limit of this assay was 4 μM.

2.12 Histology

2.12.1 Fixation and sectioning of tissue

After removal, brains were placed in 4% PFA for 24-48 hours, rinsed with 1 x PBS and then transferred into 70% ethanol for long term storage at 4 degrees Celsius. Prior to paraffin processing a brain matrix block (Zivic Instruments, PA, USA) was used to isolate the desired region, commonly 2 mm posterior to bregma. A 2 mm section encompassing this region was then transferred to a tissue processing cassette (Shandon, Thermofisher Scientific). Cassettes containing samples were processed on a Leica TP1020 processor (Leica Biosystems, Wetzlar, Germany). After processing, sections were removed from cassettes and embedded in paraffin using a Leica EG1160 Tissue embedding system (Leica Biosystems, Wetzlar, Germany). Samples were sectioned at 5 – 7 μm and mounted on superfrost slides (thermos fisher) on which they were left to dry overnight before further processing.

2.12.2 Deparaffinising and rehydrating tissue

Prior to all immunohistochemistry or structural staining, sections were deparaffinised using xylene or Histochoice (Sigma) and then rehydrated through a series of decreasing ethanol concentrations ranging from 100% to 70%.

2.12.3 Luxol fast blue staining

Sections were placed in pre warmed luxol fast blue (appendix) (LFB) solution for 3 hours at 60degrees Celsius in an incubator. Once removed from the LFB solution, slides were rinsed with 95% ethanol to remove excess stain, before being placed in 0.1% lithium carbonate (appendix) for 1 minute to differentiate the tissue. Slides were then placed in 70% ethanol for 30 seconds before being rinsed with distilled water. At this point slides were viewed using a compound microscope to check that the grey and white matter regions were defined. Slides were then counterstained in cresylviolet (Sigma) solution for 5 minutes (appendix) and then excess stain was removed by rinsing in distilled water. Slides were placed in 95% ethanol for 5 minutes, 100% ethanol for 5 minutes twice and then transferred to xylene. After a minimum of 10 minutes in xylene slides were removed, DePX mounting media was applied followed by a coverslip (Thermo Fisher). Slides were allowed to dry overnight before being viewed under a microscope.

2.12.4 Hematoxylin Counterstain

After immunohistochemistry, sections were counterstained with Meyers hematoxylin (Sigma) to improve structural resolution of cells in the tissue section. Hematoxylin was applied to slides for 20 seconds before being rinsed off with distilled water. Slides were transferred to acid alcohol (appendix) for 30 seconds, rinsed with water then placed in 0.1% lithium carbonate (appendix) for 40 seconds. Slides were then rinsed with distilled water and placed in 70% ethanol for 5 minutes. Sections were progressively dehydrated up to 100% ethanol, transferred to xylene (Sigma) for 10 minutes and then cover-slipped using DePX mounting media. If Eosin (Sigma) staining was also being carried out, after the 70% ethanol incubation, 0.5% eosin solution was pipetted onto the

slides for a maximum of 5 seconds and then sections were progressively dehydrated following the previously mentioned steps.

2.12.5 Astrocyte Staining

After being deparaffinised and rehydrated, endogenous peroxidise activity was quenched using a 50% methanol and 1% H_2O_2 solution for 20 minutes at room temperature. Slides were then washed 2-3 times in PBST. Following the washes, sections were blocked with a 4% donkey serum (Sigma) in PBST for 60 minutes. Primary antibody, anti-GFAP at a dilution of 1:6000 was applied and sections were incubated overnight at 4 degrees Celsius. Control sections were incubated with immunobuffer alone. Sections were then washed 2-3 times with PBST and the secondary antibody from ABC Vectastain kit was applied according to instructions. After incubation sections were washed with PBST and ABC reagent, made according to manufacturer's instructions was applied for 1 hour. Samples were again washed with PBST and developed by adding vector VIP substrate (Thermo Fisher) before being washed with PBST. Sections were then counterstained with hematoxylin (as described previously (3.8.4) and cover-slipped.

2.12.6 Mature Oligodendrocyte Staining

After being deparaffinised and rehydrated, endogenous peroxidise activity was quenched using a 50% methanol and 1% H_2O_2 solution for 20 minutes at room temperature. Slides were then put in citrate buffer at 80-95º Celsius for 15 minutes before being left to cool. Slides were washed 2-3 times in PBST. Following the washes, sections were blocked with a 4% donkey serum (Sigma) in PBST for 60 minutes. Primary antibody, anti-gst pi transferase at a dilution of 1:500 was applied and sections were incubated overnight at 4 degrees Celsius. Control sections were incubated with immunobuffer alone. Sections were then washed 2-3 times with PBST and the secondary antibody from ABC Vectastain kit was applied according to instructions. After incubation sections were washed with PBST and ABC reagent, made according to manufacturer's instructions was applied for 1 hour. Samples were again washed with PBST and developed by adding vector SG substrate

(thermos-fisher) before being washed with PBST. Sections were then counterstained with methyl green and cover-slipped.

2.12.7 Scoring of Myelin and astrocytes

Photographs of sections to be scored were provided to observers (between 3-6 individuals) who were blinded to the treatment groups. Observers were provided with instructions for scoring as well as an example of some images. To score demyelination, observers were asked to rank the section from a score of 1-3, with 1 being 0-30% demyelination, 2; 30-60% demyelination and 3 being 60-100% demyelination. A similar system was used to look at astrocyte and microglial activation with 1 being low level activation and 3 being strong activation.

2.13 Behavioural Assays

All behavioural assays were carried out in the Victoria University animal facility inside a laminar flow hood and were approved by the Animal Ethics Committee (2013R18).

2.13.1 Rotating Rod Behavioural Assay

Mice were placed on the rotating rod at a fixed speed of 24 rpm. A timer was started as soon as the mouse's tail was released and length of time the mouse was able to remain walking on the rotating rod was recorded. Maximum length of performance on this task was 120 seconds, after which the rod was stopped and the mouse was returned to its home cage. If a mouse fell off, or did 2 consecutive rotations the rod was stopped and the time recorded. If the mouse fell within the first 5 seconds of the test it was probably due to improper placement so was given a brief rest period before attempting to start the test again.

2.13.2 Horizontal Bar Assay

Mice were placed in the middle of a horizontal bar suspended between two props, 45cm above the bench. Time taken for mice to reach either end of the bar was recorded, with the best time of two repeats being used for analysis. Maximal test length was 30 seconds, after which the animal was assigned the maximum score of 30 if it failed to complete the task.

CHAPTER 3: Setting up a robust and reproducible cuprizone model and developing techniques to assess coordination

3 Introduction

One particular model that has been used widely to investigate potential MS therapies is experimental autoimmune encephalomyelitis. In this particular disease model, animals are injected with a protein component of the myelin sheath along with an adjuvant and pertussis toxin, causing the mouse to develop a T cell mediated immune response to the myelin protein (Kuerten, Javeri, Tary-Lehmann, Lehmann, & Angelov, 2008). The onset, severity and course of the disease can be altered by using different combinations of adjuvant and peptide as well as altering sex, age and strain of the mouse being used. The ability to alter these combinations to manipulate disease has allowed researchers to target different aspects of the disease, whether it be delaying the onset of disease, reducing the overall disease burden or altering the immune response during disease (Burkhart, Liu, Anderton, Metzler, & Wraith, 1999; Kuerten et al., 2007).

Previous research has focused on modulating the immune response and cytokine environment to a 'less damaging' phenotype, and this is used by some current MS therapies such as Interferon beta 1-b (Betaseron, Extavia) and Glatiramer Acetate.

These therapies however are only successful at delaying disease course in the relapsing remitting phenotypes of disease. Currently no other therapies are available to treat the progressive forms of disease. Lucchinetti identified a different phenotype of lesions seen in progressive versus relapsing remitting MS patients. In this lesion analysis by Lucchinetti et al (2000), lesions from human MS brain sections were classified into 4 distinct categories using neurobiological and immunological markers. Category 1 and 2 lesions were characterised by a primarily immune cell infiltrate indicating T cell or antibody disease mediation, associated with relapsing remitting forms of disease, whereas type 3 and 4 lesions were characterised by the death of the surrounding oligodendrocytes, associated with progressive MS, which Lucchinetti and colleagues concluded were reminiscent of virus or toxin induced demyelination (Lucchinetti et al., 2000), these findings lend further weight to the idea that MS has multiple neuro biological subtypes, and that therapy therefore will need to be tailored for different cases to successfully target disease.

Due to the development of different types of lesions associated with different pathological pathways involved in disease progression, multiple models are needed to assess potential MS therapies. The cuprizone model for demyelination began to be used as a way to look at demyelination, remyelination and cellular events surrounding these processes. As mentioned previously, the cuprizone model produces a robust and reproducible demyelination throughout the murine brain, with the corpus callosum being an area commonly assessed. The severity and level of demyelination can be controlled by altering the amount of cuprizone included in the diet and by variation of the mouse strain.

Histologically, the demyelination and oligodendrocyte apoptotic lesions seen in the cuprizone model resembles those in human type 3 and 4 MS lesions as described by Lucchineti et al. (2000) Another feature of the cuprizone model, which makes it an interesting model for studying MS, is that the blood brain barrier remains intact (Groebe, 2009), allowing the exclusion of the peripheral immune cells. In relapsing remitting MS there is a clear role for these infiltrating peripheral immune cells, hence use of the EAE model is suitable to study this form of MS. With secondary progressive MS however, there is less infiltration of the peripheral immune cells to the lesion sites and lesions are more readily characterised by the presence of oligodendrocyte apoptosis (Lucchinetti, 2000). By establishing a model that creates a reproducible demyelination, while keeping the blood brain barrier intact, it is possible to analyse events surrounding demyelination whilst excluding the peripheral immune system, similar to those reported as type 3 and 4 lesions by Lucchinetti et al. Currently there are no treatments for progressive MS, so by studying the cellular events involved in this type of lesion it is hoped to be able to characterise any effects of treatment drugs and any protective effects they may provide.

3.1 Administration of Cuprizone

In the cuprizone model, mice are fed a cuprizone containing diet, commonly ranging from 0.1% - 0.5% depending on the desired level of demyelination and the cell population to be investigated (Carlton, 1966; Gudi et al., 2009). Cuprizone selectively targets the mature oligodendrocytes in the

CNS which leads to the loss of the myelin sheaths they provide, resulting in demyelination and apoptosis of the oligodendrocytes (Skripuletz et al., 2008). Oligodendrocyte apoptosis is then followed by the activation of astrocytes and microglia which are involved in the phagocytosis of the myelin proteins and apoptosed cell fragments (Irvine & Blakemore, 2006; Pasquini, Calatayud, Bertone Uña, et al., 2007; Torkildsen, Brunborg, Myhr, & Bo, 2008). Following debris clearance, oligodendrocyte precursor cells invade these demyelinated regions and proliferate in an attempt to repair the region. If the cuprizone is removed from the diet before the end of a 12 week period, an almost complete and spontaneous remyelination occurs to a level seen prior to the start of the cuprizone administration (Torkildsen et al., 2008). This model is useful to assess manipulations and treatments which may prevent the demyelination process or accelerate the remyelination process (Kipp et al., 2009).

3.2 Assessing cuprizone induced co-ordination deficits

The rota-rod was used to evaluate the effect of treatment on motor coordination in animals fed cuprizone. This test is measured by the length of time mice are able to walk on a rotating rod at a fixed speed setting, and based on current literature, a starting speed of 24rpm was chosen. It is known that cuprizone mediated demyelination can lead to weakness of the hind limbs (Franco-Pons, 2007) so developing a method to measure this effect is key if the effect of experimental interventions is to be assessed.

3.3 Aims

The overall aim of this chapter was to set up a cuprizone model that could provide robust and reproducible demyelination in the corpus callosum of C57Bl/6J mice, as well as developing a behavioural monitoring technique, capable of detecting any motor deficits in animals caused by the cuprizone diet. These methods could then be used to assess the effects of test compounds on demyelination, remyelination and motor deficits.

3.3.1 Specific Aims

1. Identify which cuprizone diet provides the most consistent and reproducible demyelination with the lowest off target effects as measured by:
    a. Weight loss
    b. Demyelination
    c. Co-ordination

2. Develop a method for detecting and measuring coordination and behavioural deficits induced by the cuprizone.

3.4 Results

3.4.1 Administration of 0.2% and 0.3% Cuprizone led to weight loss

Effects of cuprizone administration have been shown to vary considerably between labs, even when the same animal strain and percentage of cuprizone feed was used. Thus, it was important to identify a regime that works reproducibly for all further experiments. Based on previous research using the C57Bl/6J mouse strain, a 0.2% or 0.3% cuprizone concentration mixed into ground mouse feed should produce a consistent level of demyelination in the brain, especially the corpus callosum, the region of interest for these experiments. Diets containing 0.4% or 0.5% cuprizone produced a severe demyelination but had a higher associated mortality. At concentrations of 0.2%- 0.3% cuprizone an initial weight loss was expected over the first 10-15 days of the diet, with the weight levelling out over this time. Other expected side effects were a reduction in coordination or a weakness of the hind limbs (Mix, Meyer-Rienecker, Hartung, & Zettl, 2010; Wang et al., 2013).

To determine the best dose of cuprizone, mice were fed 0.2% or 0.3% cuprizone for 6 weeks. As expected, mice receiving cuprizone experienced rapid weight loss upon switching to the cuprizone diet (Figure 3.1). Initially this weight loss could be in part due to the presentation of food in a 'novel' manner, however after a few days weight loss persisted, which can be explained by the presence of cuprizone in the feed. The control mice receiving food in a powdered 'novel' manner also had an initial decrease in weight, however began to steadily gain weight, lending further support to the consistent weight loss seen in the cuprizone fed animals being a result of the cuprizone, not the novel manner in which the food was presented. Mice were weighed and observed daily throughout the administration of the cuprizone diet to monitor their overall health. Mice on both the 0.2% and 0.3% cuprizone diet experienced a steady decline in weight over the first 10 days of the diet, followed by a stabilisation of body weight around day 12, which was then

maintained for the duration of the experiment. Mice in the 0.2% cuprizone group lost an average of 0.5 g over the course of the experiment, whilst mice on the 0.3% cuprizone diet lost on average 2.52 g**.** In contrast to this, mice on the control diet gained an average of 0.9 g. Although mice in both cuprizone groups lost weight as expected, the mice in the 0.3% group followed a tighter pattern of weight loss (Figure 3.1e,f), i.e. all animals had very similar weight changes over the duration of the experiment; the 0.2% treatment group had a less uniform pattern of weight loss as seen in Figure 3.1 c and d.

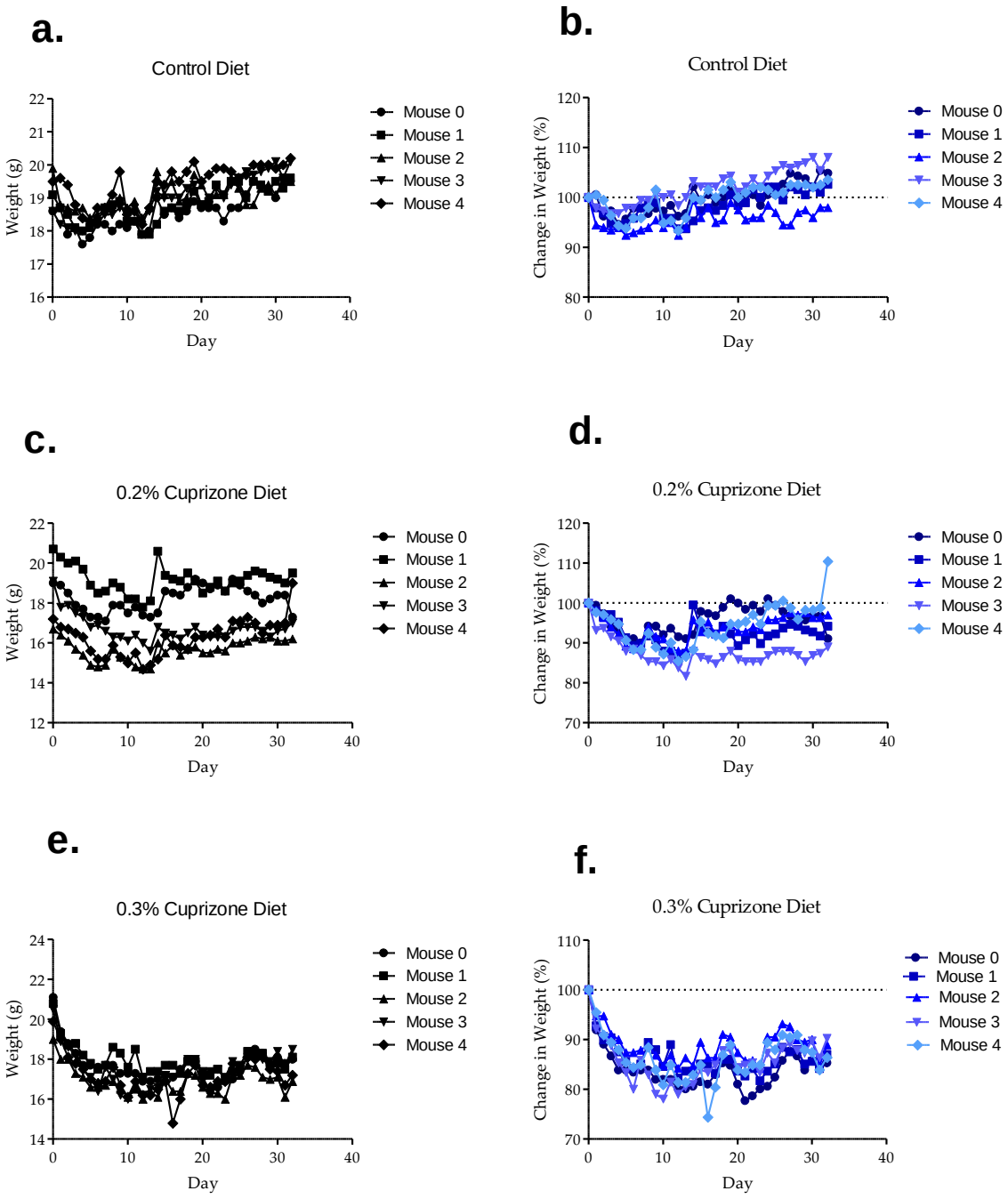

Figure 3.1: Both 0.2% and 0.3% cuprizone in the diet led to weight loss.
Female 6-8 week old C57Bl/6J mice were fed ground mouse chow or cuprizone mixed into ground mouse chow (0.2% or 0.3%). Food was replaced daily and water was available ad libitum. Animals were weighed daily as an indicator of their overall health. Animals in the 0.2% and 0.3% cuprizone groups lost weight rapidly over the first 10 days before weight stabilised. Control animals initially lost weight then gradually continued to gain weight, with a showing absolute values and b showing % change from baseline. n=5 per group.

When all treatment groups are compared (Figure 3.2a) the absolute weight of both cuprizone treated groups are similar, with both being significantly different from the control group. No significant difference was seen in weight loss when comparing the 0.2% and 0.3% cuprizone groups (Figure 3.2a). When the change in weight from baseline is measured there is still no significant difference between the 0.2% and 0.3% cuprizone groups. However, when data are viewed this way there is a significant difference between the 0.2% group and control from day 10 and a significant change in weight between the 0.3% and control group beginning day 9 and lasting the duration of the experiment in both cases (Figure 3.2b).

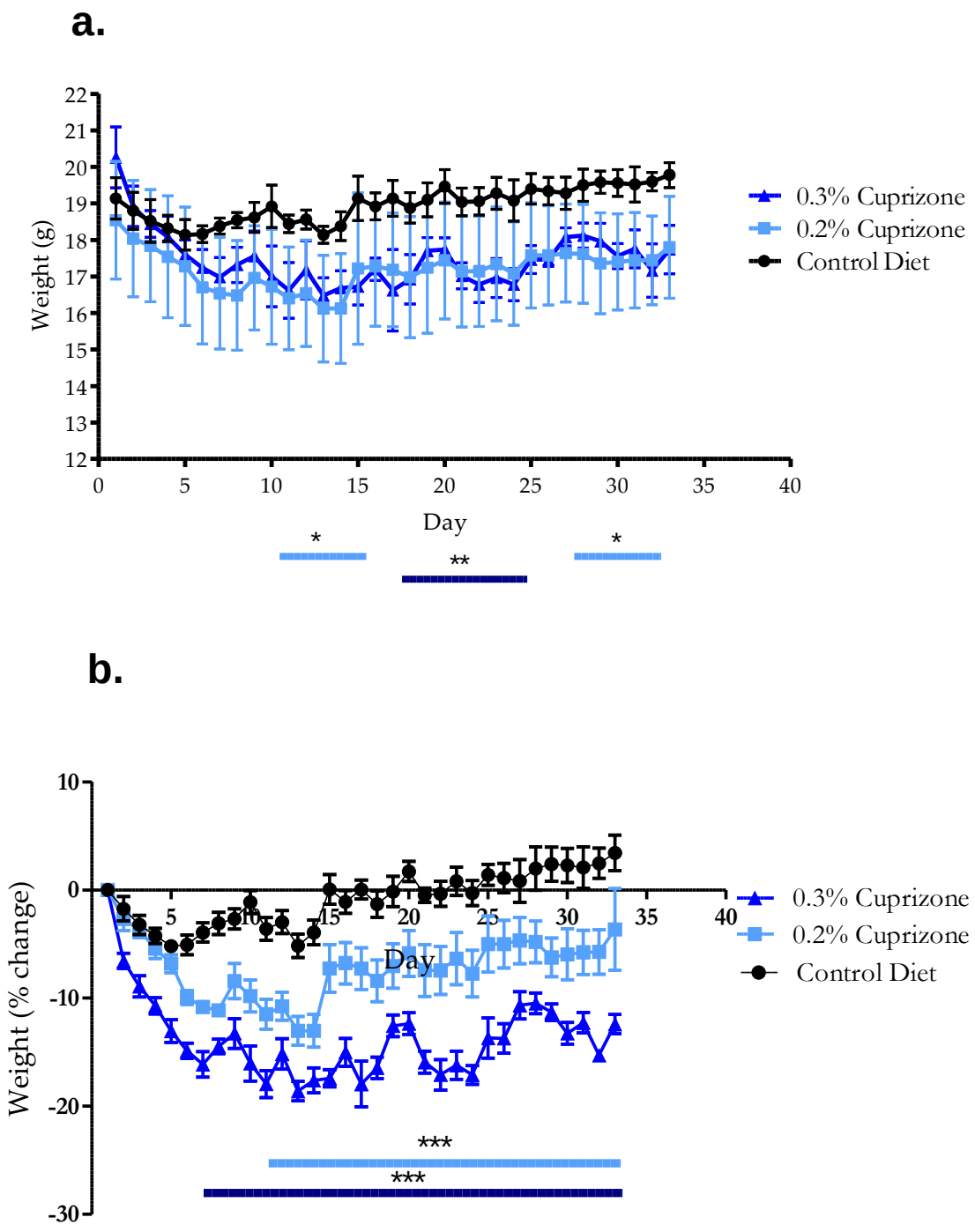

Figure 3.2: 0.3% cuprizone led to greater weight loss than 0.2% cuprizone.
C57Bl/6 mice aged 6-8 weeks were fed a diet of either 0.2% or 0.3% cuprizone mixed into ground mouse chow for 6 weeks. Animals were monitored and weighed daily to assess the effect of the diet on health. A diet of 0.3% cuprizone led to a larger change in overall % of body weight (c). n= 5 for each condition. ***p<0.001 with 2 way ANOVA and Bonferroni's post-test.

Overall, mice receiving either 0.2% or 0.3% cuprizone both experienced a significant weight loss relative to control animals (Figure 3.3). The 0.2% cuprizone fed group showed a larger range of weight loss than the 0.3% cuprizone fed group, which showed a very uniform pattern of total weight loss.

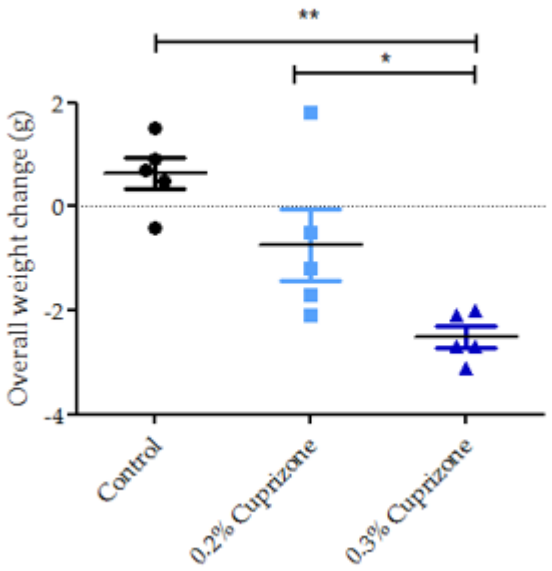

Figure 3.3: 0.3% cuprizone intoxication led to a more uniform pattern of weight loss.
Female C57Bl/6 mice aged 6-8 weeks were fed a diet of either 0.2% or 0.3% cuprizone mixed into ground mouse chow for 6 weeks. Animals were monitored and weighed daily to assess the effect of the diet on weight loss. Overall weight loss during the 6 week experiment were calculated. n= 5 for each condition. *$p<0.05$, **$p<0.01$ with 1-way ANOVA with Bonferroni's multiple comparison post-test.

3.4.2 0.2% and 0.3% Cuprizone caused demyelination of the corpus callosum

Using online brain atlases and The Mouse Brain Atlas (Paxinos & Franklin, 2013) a region of interest in the corpus callosum was identified and attempts were made to reproducibly isolate sections from within this region. This region was approximately 2 mm posterior to bregma and covered a distance of 320 μm. Figure 3.4 shows a range of example sections from this region to help identify and locate the sections collected from our trial brains. (a) is anterior to section (b) followed by section (c), with an enlarged version of the corpus callosum located next to the corresponding section.

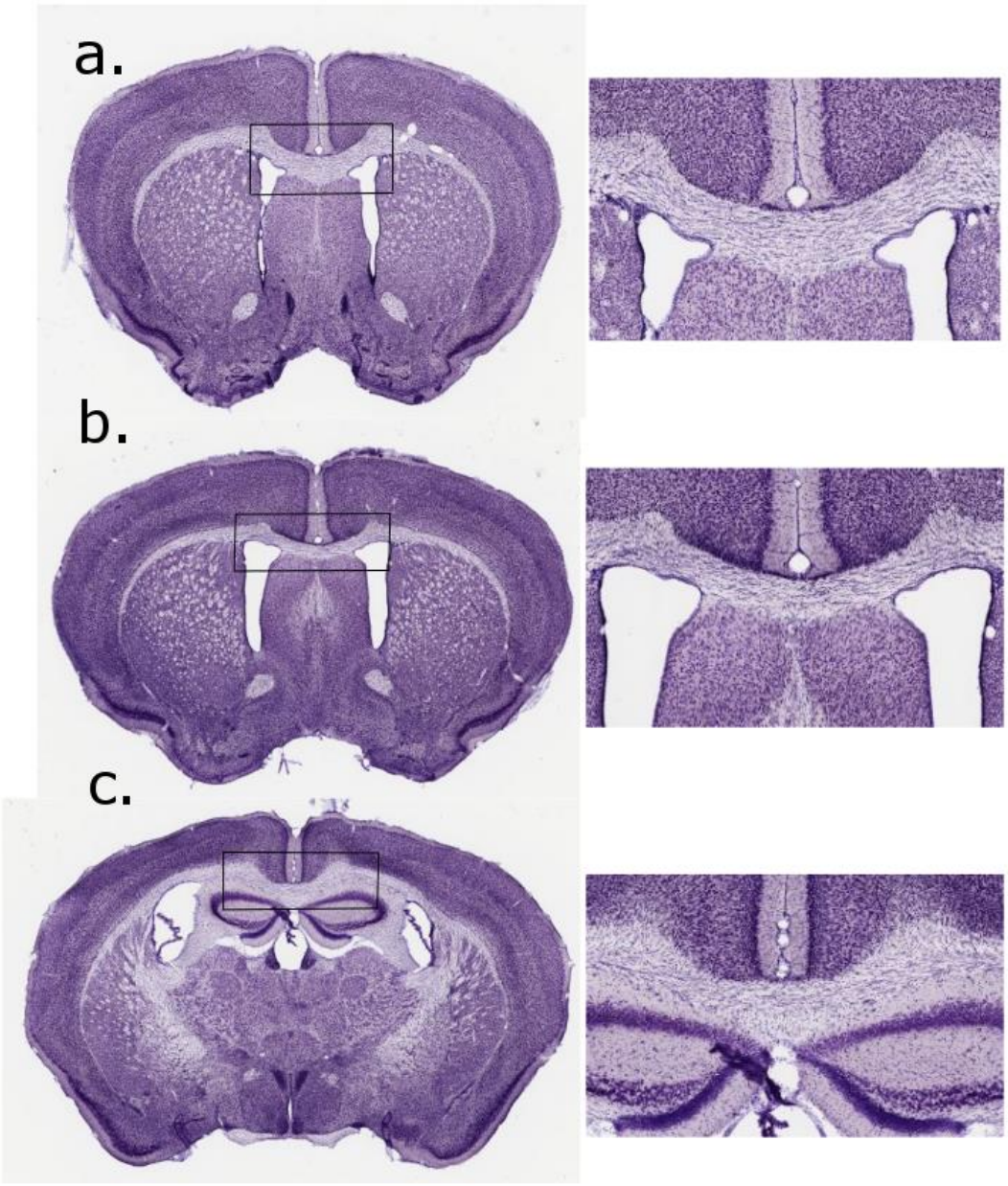

Figure 3.4: Identifying the region of interest for further analysis.
Images are taken from brain atlas found at http://brainmaps.org/ajax-viewer.php?datid=38&sname=26. Sections were taken from snap frozen tissue, at a thickness of 40 µm and then stained with Nissl stain. (a) to (c) represent a snapshot of sections taken from the region of interest for this study and cover a distance of 320 µm, with enlarged sections of the corpus callosum placed next to each image.

As seen in Figure 3.5 (a) and (d) corpus callosum isolated and stained with LFB from control animals showed intact myelin (d is enlargement of a) indicated by the strong blue staining. Sections (b) and (e) have a much lower level of myelin staining in the corpus callosum and are from animals which received the 0.2% cuprizone diet (e is enlargement of b), with (c) and (f- enlargement) being from animals receiving the 0.3% cuprizone diet and showing the lowest level of myelin staining. The images chosen in Figure 3.5 are representative for each test group (n=5).

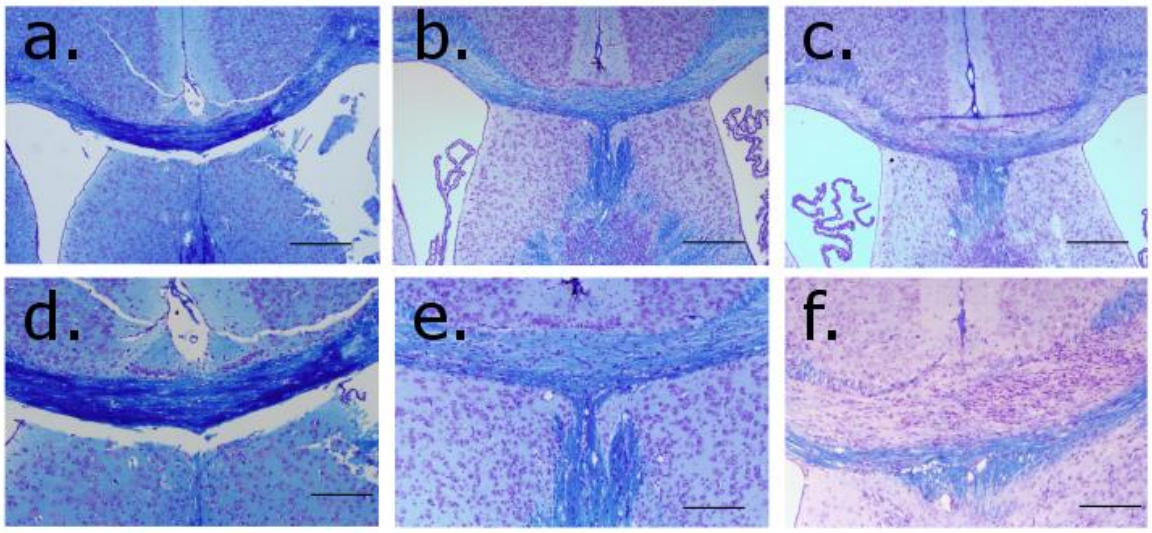

Figure 3.5: More consistent demyelination was achieved with 0.3% cuprizone intoxication.
After 6 weeks of cuprizone intoxication, brains were collected and processed for paraffin sectioning. 5-7 µm sections were collected and stained with luxol fast blue and cresyl violet. No demyelination was seen in the control animals (a and d) with varying levels of demyelination seen in the 0.2% cuprizone group (b and e) and the 0.3% group (c and f). d, e and f are enlarged regions of a, b and c, scale 100 µm and 50 µm respectively.

In the control animals no demyelination was seen as expected, evidenced by the solid blue staining in the corpus callosum. In the 0.2% cuprizone diet there was a much larger variation in the level of demyelination occurring in the corpus callosum (Figure 3.6). Sections from animals in the 0.3% cuprizone treatment group had a much more consistent level of demyelination in the corpus callosum across individual animals, which ties in with the more uniform pattern of weight loss seen in this treatment group. The range of weight loss in the 0.2% group could be an indicator of the range in level of demyelination occurring for this group, with some animals not responding as strongly to this lower level of cuprizone in the diet.

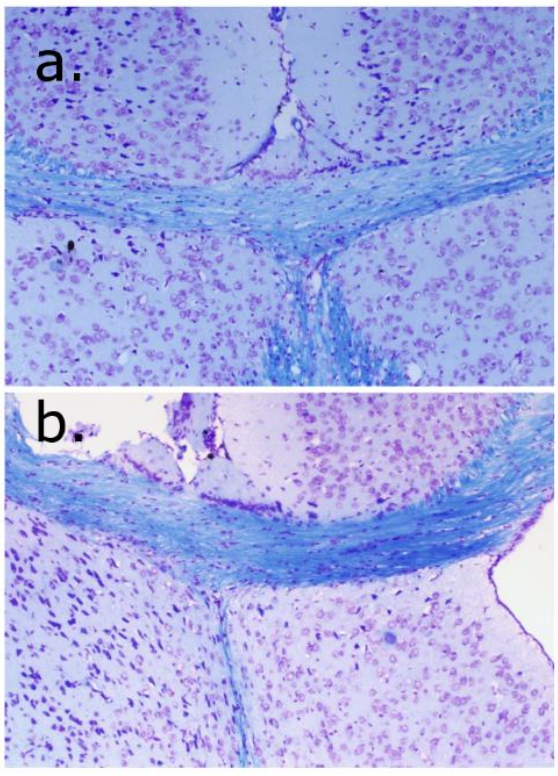

Figure 3.6: Variation in demyelination was seen with 0.2% cuprizone intoxication.
Female C57Bl/6 mice aged 6-8 weeks were fed a diet of 0.2% cuprizone for 6 weeks which led to demyelination of the corpus callosum. In this 0.2% treatment group there were higher levels of variation in myelin with some animals (a) developing more demyelination than others (b) whilst on the 0.2% cuprizone diet.

3.4.3 0.3% Cuprizone diet led to more consistent demyelination

After being stained and photographed using Cellsens software, images of the corpus callosum, similar to those seen in Figure 3.5 and 3.6 above, were presented to individuals blinded to their treatment groups. Individuals were asked to rank the sections using a scale of 0-3 to indicate the level of myelin present, with 3 being the highest level of myelin (no demyelination) and 0 being the lowest level of myelin (high demyelination) (Xiang Gao et al., 2000; Groebe et al., 2009). When sections were ranked using this system it was clear that the 0.3% cuprizone diet caused a much higher level of demyelination than 0.2% cuprizone (Figure 3.7), based on the lower myelin scores given to animals in this group. The difference in demyelination seen in 0.2% and 0.3% diets also indicated that the method of food preparation and drug incorporation was successful, as the group with the higher levels of cuprizone in the diet produced the higher levels of demyelination.

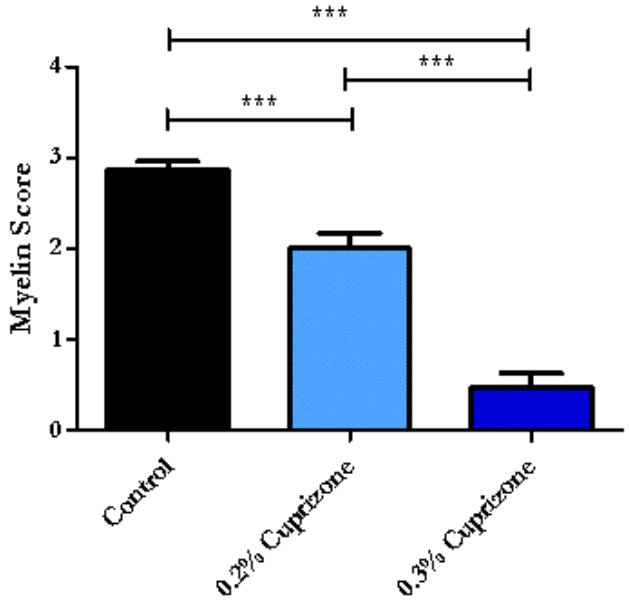

Figure 3.7: Level of demyelination in both 0.2% and 0.3%significant.
C57BL/6 mice were fed either 0.2% or 0.3% cuprizone for 6 weeks, before brains were collected and fixed in PFA. Brains were paraffin embedded and sectioned at 5-7 μm then stained with LFB to detect demyelination in the corpus callosum. Sections were then photographed and presented to individuals blinded to treatment for their scoring, with 0 being complete demyelination of the corpus callosum and 3 being no demyelination/high level of myelin still present. n=5. ***$p<0.001$with 1-way ANOVA and Bonferroni's multiple comparison post-test.

3.4.4 Rotating Rod detected cuprizone induced coordination deficits

To assess the effect of the different cuprizone diets on coordination the rotating rod was used. In this behavioural assay the barrel is set to rotate at either a fixed speed or an accelerating pattern determined by the user, and can be optimised to pick up motor deficits. The time at which the animal falls off the rod or is unable to continue walking forwards and begins to do rotations is then recorded.

For this initial trial a fixed speed of 24 RPM was used and the time at which the animal fell off or did 2 consecutive rotations was recorded. On the day prior to the onset of the experiment mice were given a trial run, to familiarise them with the equipment.

Every 2 days for the duration of the experiment, mice were placed on the rota-rod and performance times recorded. The performance of animals in both the 0.2% and 0.3% cuprizone groups was comparable to animals in the control group when this rota-rod protocol was used. The majority of animals in each group consistently remained on the rotating rod for the full duration of the test (120 seconds) with the exception of a few animals in the 0.2% cuprizone group, however this difference in performance was not significant. No difference in the motility or grooming behaviours of any of the animals was observed throughout the duration of the experiment, indicating that their co-ordination was probably not being significantly impacted with these low doses of cuprizone, so failure to pick up any difference in coordination using this rota-rod protocol is not unusual. Due to the duration of this experiment it is inevitable that as well as coordination playing a role in performance, learning will also play a role.

Since no difference was picked up with this protocol several adjustments were made. Instead of behaviour being assessed on alternate days, a final performance at the end of the demyelination period was recorded and the speed was increased from 24RPM to 28RPM. When tested using this method a clear difference was observed between cuprizone fed animals and healthy controls as

seen in Figure 3.9. Control animals were able to remain on the rotating rod for approximately 90 seconds, whereas cuprizone fed animals only remained on for an average of 10 seconds.

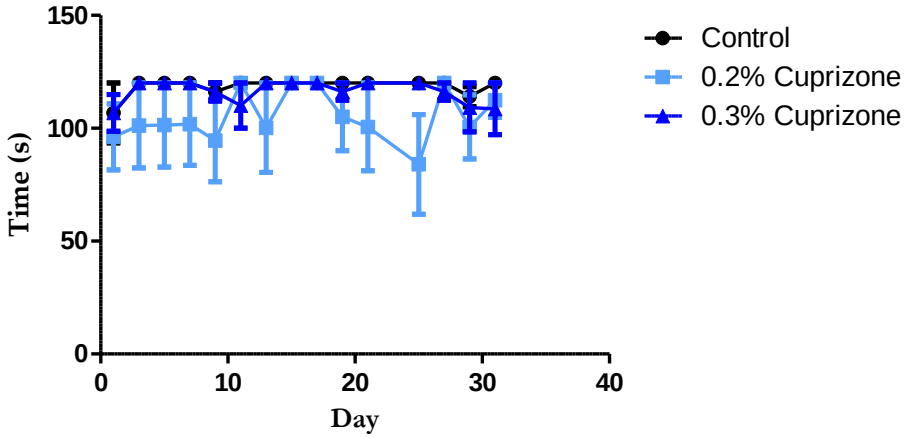

Figure 3.8: No effect on motor co-ordination was observed using the rotating rod behavioural assay at 24RPM.
Coordination was assessed every second day during the 6 week cuprizone diet using the rota rod at a fixed speed of 24 RPM. Briefly, mice were placed on the rotating barrel and time taken to fall, or do a rotation was recorded. No significant differences were recorded between treatment groups. n= 5 per group.

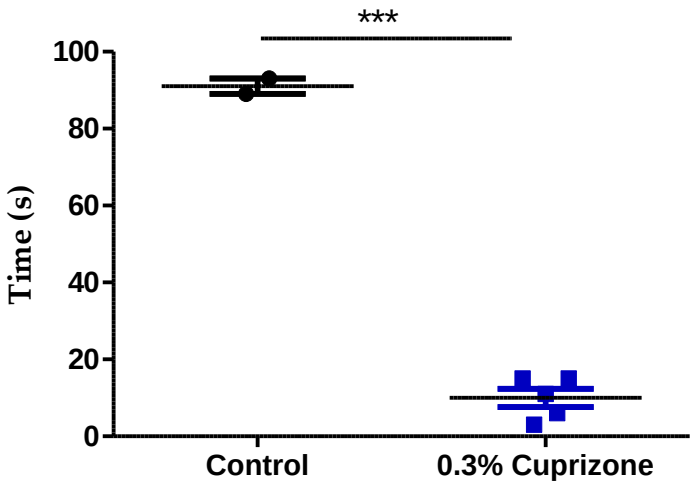

Figure 3.9: Clear differences were seen in coordination when using the rota rod at 28 RPM.
Coordination was assessed at the end of the 6 week experiment using the rotating rod at a fixed speed of 28RPM. ***p<0.0001 using an unpaired t-test.

3.4.5 Quantifying astrocyte activation using ImageJ

As well as scoring sections by hand, attempts were made to develop a macro add on in ImageJ as a means of rapidly quantifying astrocyte staining based on the difference in colour intensity of the astrocytes and the background staining. Scores acquired using this ImageJ script are shown alongside scores provided by individuals for comparison (Figure 3.10). Although the range of scores (arbitrary units) differ, the pattern seen in level of activation is the same between the hand scored sections and the sections scored using ImageJ.

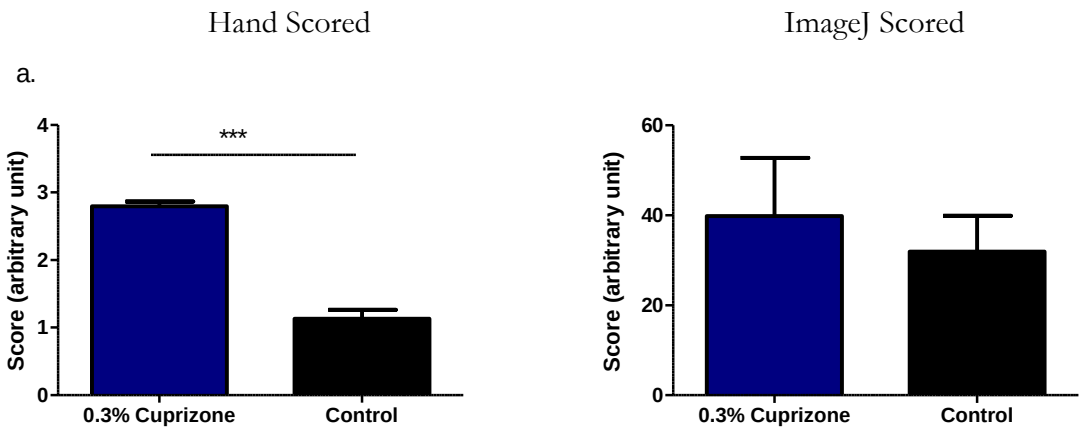

Figure 3.10: ImageJ can be used to rapidly quantify astrocyte activation.
As well as sections stained for GFAP being scored by hand (a), photographs of sections were processed with an ImageJ script to detect astrocytes (b). Although values differ (arbitrary units) the same pattern in scoring was seen. ***$p<0.05$ with an unpaired t-test.

3.5 Discussion

Multiple sclerosis is a disease affecting a large number of people in western countries, with females being more affected than males. Disease course has 2 major forms of presentation; either in discreet episodes of disease which is classified as relapsing remitting disease, or a build-up of symptoms continuously over time, which is classified as progressive MS. The majority of patients initially present with a relapsing remitting disease course, however over time this can develop into progressive forms of disease (Torkildsen et al., 2008).

EAE is a well characterised model for studying MS and potential therapies, and this model produces lesions which closely mimic human type 1 and II lesions as described and identified by Lucchinetti (Lucchinetti et al., 2000). Lucchinetti et al also identified another category of lesions, type 3 and 4, which were not accurately represented by EAE lesions, as type 3 and 4 lesions lack the immune infiltrate associated with EAE lesions. These type 3 and 4 lesions are also characterised by oligodendrocyte death, a feature not commonly associated with EAE lesions. For this reason it is important to use other models alongside EAE to assess potential therapies, as MS lesions can vary considerably between individuals.

The cuprizone model has been accepted as a model for studying MS type lesions which are primarily mediated by oligodendrocyte loss, more closely mimicking the type 3 and 4 lesions identified by Lucchinetti and colleagues (2000). These lesion types are seen more commonly in progressive MS, a form of disease that currently has no treatment options.

A distinct advantage of the cuprizone model over EAE is the reproducibility of the model. When the correct cuprizone diet is established, the level and location of demyelinating lesions can be reproduced across individual animals with minimal individual variation. As the level of cuprizone in the diet increases, the level of demyelination in the corpus callosum will also increase (Carlton, 1966) and this was able to be reproduced in the current experiment. It was important to ensure that cuprizone levels were sufficient to cause demyelination but were not causing off target side

effects such as edema or mortality, which can be seen in some more concentrated diets (Carlton, 1966).

As well as causing demyelination, some behavioural effects of cuprizone feeding have been reported previously. In a study by Franco-Pons et al (Franco-Pons, Torrente, Colomina, & Vilella, 2007) mice on a 0.2% cuprizone diet were subjected to a range of behavioural assays. A functional observational battery of tests (measuring 18 different parameters) picked up several differences between cuprizone fed animals and their untreated control animals. Signs of ataxia and splayed hind limbs were recorded after 5 weeks of cuprizone intoxication, as well as freezing in response to an auditory signal, a response that was used to gage sensorimotor activity. The present study also showed that following 5 weeks of cuprizone intoxication, motor coordination was impaired as assessed by performance on the rotating rod, and that mice which were treated with cuprizone for 6 weeks and then allowed to recover for 6 weeks still performed more poorly when compared to control groups.

No evidence of impaired performance was detected using the rota rod for either the 0.2% or 0.3% cuprizone diets when set at 24RPM, however there are many factors which could explain this. Individual labs tailor rota-rod protocols to meet their specific needs so a general 'go-to' protocol for measuring coordination across different laboratories is not established. After surveying current literature it was decided to use a fixed speed protocol starting at the onset of cuprizone administration so that any decline in coordination could be picked up. As no significant decline was seen in either cuprizone group it can be concluded that this 24RPM setting was not sensitive enough to detect a motor deficit. Further investigation determined that using the rotating rod at 28RPM a clear and significant difference can be detected between cuprizone fed animals and healthy controls at the end of the 6 week period. Following this finding, future experiments will use this 28RPM protocol to assess motor coordination deficits.

Previous work in the cuprizone model has shown a consistent and reproducible demyelination occurring in the corpus callosum, hippocampus and in some areas of the cerebellum (Benetti et al., 2010; Groebe et al., 2009; Skripuletz et al., 2008). Experiments carried out in this chapter found that with the female C57BL/6 mice on both a 0.2% and 0.3% cuprizone diet demyelination of the corpus callosum could be produced in a manner matching reports of other studies. Since an effective means for causing demyelination has been established, focus can now be placed on running compounds through this model to assess their effects on demyelination and any action that may be exerted on other cell populations within the CNS.

As well as assessing levels of demyelination using an arbitrary scoring unit (Skripuletz et al., 2008) other cellular populations can provide useful information about events surrounding demyelination and remyelination. Alongside myelin staining with luxol fast blue, IHC for myelin peptides such as MBP or PLP are often carried out as another way to confirm patterns of demyelination around lesions (Du et al., 2016; Glenn K. Matsushima & Pierre Morell, 2001), and can also be used for absolute quantification of myelin via western blotting.

Two cells that play a key role and are of significant interest for further experiments are astrocytes and microglia. Both have been shown to play an active role in demyelination and remyelination, by releasing pro and anti-inflammatory cytokines as well as phagocytosing myelin debris which allows for the influx of oligodendrocyte precursor cells (OPC's) and allows their development into myelinating oligodendrocytes. A study by Tanaka et. al. showed that inhibition of microglia with minocycline prevented remyelination after cuprizone intoxication (Tanaka, Murakami, Bando, & Yoshida, 2013). Yet other studies have shown that microglia activation during the demyelinating phase of disease is detrimental due to their release of pro inflammatory cytokines (Deng & Sriram, 2005; Raivich & Banati, 2004; Sanders & De Keyser, 2007). This report of microglia being both protective and damaging during cuprizone intoxication is likely due to the fact that demyelination and remyelination are not distinct events in which one directly follows the other. Both processes

are occurring simultaneously at different rates, so teasing out cellular events associated specifically with demyelination or remyelination is difficult.

One approach that has been taken to tease out these two events is by looking at oligodendrocytes in isolation. The role of oligodendrocytes and the effect of clozapine and MIS416 administration on this population will be discussed in chapter 6.

3.6 Summary

When taken together the results presented in this chapter indicate that using a 0.3% cuprizone diet in 6-8 week old C57BL/6 produces a more consistent level of demyelination than the 0.2% cuprizone diet. The corpus callosum is able to be reproducibly identified, and staining using luxol fast blue successfully stains the myelin in the collected sections. Scoring of these sections by individuals blinded to the treatment groups is an effective way to quantify the differences in level of demyelination caused and so this method will be continued for further experiments. Importantly, at these cuprizone doses no off target or harmful effects are seen in mice as indicated by their performance on coordination tasks.

CHAPTER 4: Effects of MIS416 and clozapine on demyelination

4 Introduction

The previous chapter identified that both 0.2% and 0.3% cuprizone, administered over a 6 week period caused demyelination of the corpus callosum in female C57BL/6 female mice. Although both doses caused changes that were detectable with a luxol fast blue stain for myelin, the 0.3% cuprizone diet provided a much more consistent demyelination in the corpus callosum than 0.2%. The current chapter will use this model to investigate the effects of two immunomodulatory agents, MIS416 and clozapine, on cellular changes in the CNS throughout the demyelination period. Both of these agents have been chosen because they have been shown to reduce CNS disease in the mouse model of MS, experimental autoimmune encephalomyelitis and because they appear to mediate their effects by targeting the innate immune system.

Previous work from our group (White, 2015) has shown that MIS416 is taken up by peripheral myeloid populations and causes alterations to the T cell populations during EAE. The direct pathways for MIS416 action are still under investigation although it is known to act through TLR-9 and NOD-2 receptors, with IFN-γ being essential for its protective effects (White et al., 2014).

Clozapine is an atypical antipsychotic prescribed to treat schizophrenia and acts by antagonizing the dopamine D_2 and serotonin $5HT_{2a}$ receptors (MacDowell et al., 2013; O'Sullivan et al., 2014). As well as being effective for the treatment of psychiatric disorders, research has shown the potential immunomodulatory effects of atypical antipsychotics, including clozapine (Basta-Kaim et al., 2006; O'Sullivan et al., 2014; Zheng et al., 2008). Due to the previously reported role of atypical antipsychotics in modulating the immune system, the effects of clozapine will be examined in the non-immune mediated cuprizone model to determine if the protective effects seen in EAE are maintained (O'Sullivan et al., 2014).

One of the key features seen in the MS lesion is a loss of the myelin sheath surrounding axons (Bando et al., 2015; Dhib-Jalbut, 2007), which leads to impaired signal transduction and results in some of the motor deficits observed in MS (Barnett & Prineas, 2004). Therapeutically, being able

to reduce or halt the rate of demyelination would provide a novel mechanism to treat MS lesions and potentially prevent relapses where disease is already established. The myelin sheath, composed of multiple proteins is supplied by mature oligodendrocyte cells (Miron, Kuhlmann, & Antel, 2011) and wraps around axons in a bandage-like manner. During cuprizone intoxication the mature oligodendrocytes are destroyed (Pasquini, Calatayud, Bertone Uña, et al., 2007) and the myelin growth is no longer supported, leading to its breakdown. Protecting this oligodendrocyte population or recruiting new oligodendrocyte precursors could also have exciting implications for therapies.

The presence of myelin debris can lead to the activation of many CNS cell types, primarily those involved in clearance of debris. Microglia, the macrophages of the CNS as well as astrocytes have been shown to have a dual role in MS progression (Miljković, Timotijević, & Mostarica Stojković, 2011; Williams, Piaton & Lubetzki, 2007), with some studies reporting a protective role, with others claiming a detrimental role upon their activation. Skripuletz (2013) showed that astrocyte activation was necessary to recruit microglia to lesion sites to remove myelin debris, without which remyelination events were delayed (Skripuletz et al., 2013). Although a clear consensus on the benefit of astrocyte activation has not been reached, it is clear that the astrocyte population has a very important role to play in MS.

The primary aim of this chapter is to identify whether treatment with either clozapine or MIS416 can alter or prevent the demyelination caused by cuprizone intoxication as well as to characterise any changes to the CNS cellular populations.

4.1 Aims

The overall aim for this chapter was to determine the effect of MIS416 or clozapine administration on demyelination as well as characterising changes in the CNS. Cell populations of interest included astrocytes based on their roles in inflammation and the potential of the immunomodulatory compounds clozapine and MIS416 to alter their responses.

4.1.1 Specific Aims

1. Determine the effect of clozapine and MIS416 on demyelination in the corpus callosum

2. Characterise changes to CNS cell populations including astrocytes after treatment with clozapine and MIS416

3. Assess the effect of clozapine and MIS416 administration on motor coordination

4. Identify any changes occurring in peripheral immune populations after treatment with MIS416

4.2 Results

4.2.1 Clozapine treatment did not protect from cuprizone induced weight loss

As in previous experiments, mice were fed a 0.3% cuprizone diet mixed into ground chow for 6 weeks. Animals were monitored and weighed throughout the experiment, with no signs of stress (e.g. lack of grooming behaviour). As expected (Benetti et al., 2010; Carlton, 1966) mice that received the cuprizone diet rapidly lost weight over the first 10 days before their weight stabilised, while animals in the control group (i.e. no cuprizone) did not experience this initial weight loss and continued to gain weight throughout the duration of the experiment. The weight loss which occurred over the final few days of the experiment was likely to be as a result of stress caused by the behavioural testing which occurred at these time points, although all attempts were made to cause as little disruption to the animals as possible. Treatment with 60 mg/kg of clozapine (for duration of the cuprizone diet) did not prevent the weight loss caused by the 0.3% cuprizone diet, and since the clozapine vehicle (0.1 M acetic acid) and clozapine alone (no cuprizone diet) had no negative impact on the animals' weight, the weight loss seen in this experiment is likely a result of the cuprizone and not the clozapine treatment. Weight loss in animals receiving cuprizone alone or cuprizone + 60 mg/kg of clozapine was significantly different when compared to control, vehicle control and clozapine vehicle treated animals (Figure 4.1a). When viewed as a percentage change in weight from onset of the experiment, the cuprizone only animals and the cuprizone + 60mg/kg clozapine animals had a significantly greater loss of weight when compared to the animals not receiving cuprizone (Figure 4.1b, one-way ANOVA $p<0.0001$.

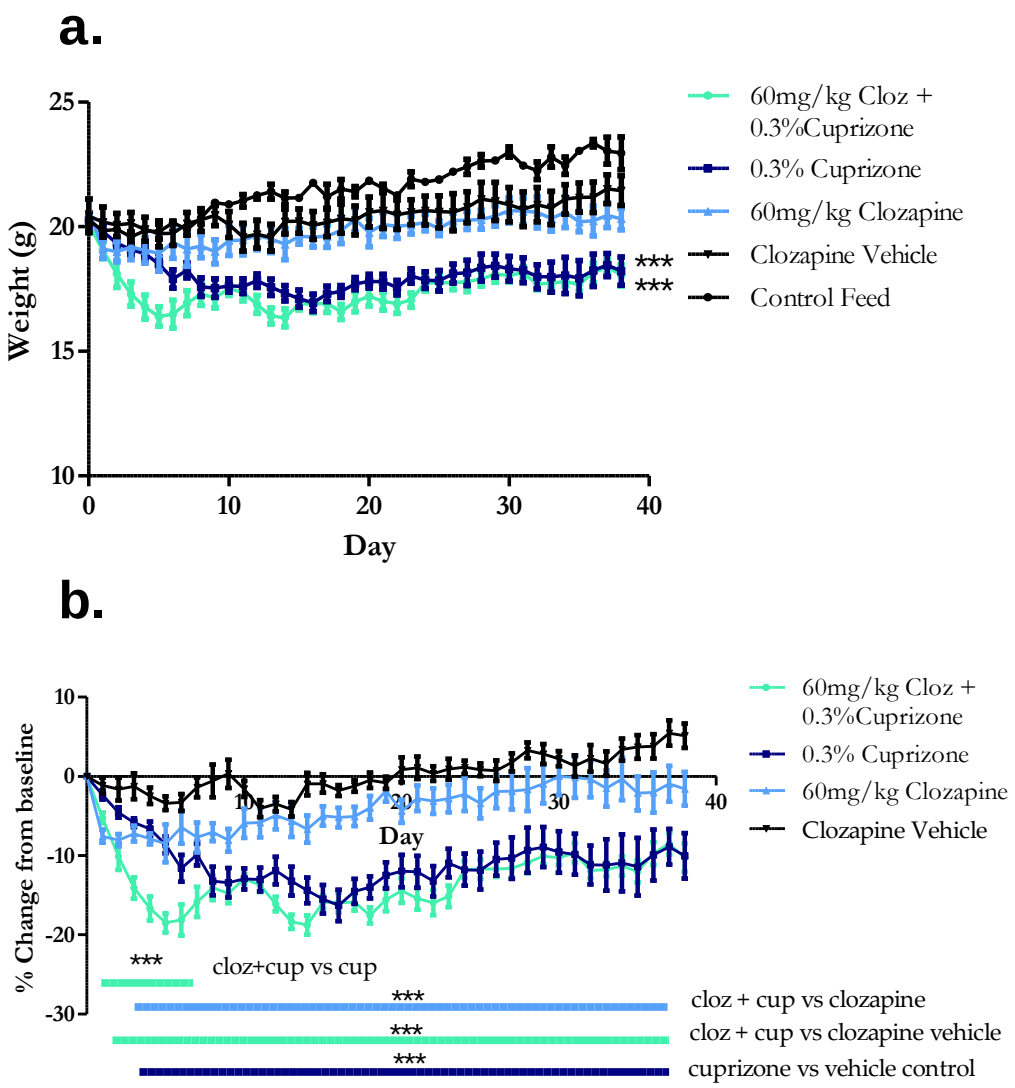

Figure 4.1: Clozapine treatment did not prevent cuprizone induced weight loss.
Female C57BL/6 mice were fed a diet containing 0.3% cuprizone ground into standard mouse chow. Food was available ad libitum and was refreshed daily. Animals were weighed and observed daily to monitor health. Animals receiving cuprizone rapidly lost weight at the onset of the diet. (a) shows absolute values for weight with (b) showing the percent change from baseline. Shown are the means and SEM of individual animals (n=4-10/group) from two experiments. (a) ***p<0.001 with two way ANOVA and Bonferroni's multiple comparison post-test compared to clozapine, clozapine vehicle and control. (b) ***p<0.001 by two way ANOVA with Bonferroni's multiple comparison post-test.

4.2.2 Cuprizone mediated loss of motor coordination was not prevented by clozapine

On the final 2 days of the experiment, after 6 weeks of a demyelination-inducing diet, the rota rod was used to assess coordination. Cuprizone treatment has been shown to cause a reduction in coordination, specifically seen as a hind limb weakness (Franco-Pons et al., 2007). In the current study, feeding with 0.3% cuprizone reduced the length of time animals were able to remain on the rota rod at a fixed speed of 28 RPM and because no significant difference in performance was found between control animals and clozapine vehicle animals fed cuprizone, these groups were pooled for analysis (Figure 4.2a). Mice receiving 0.3% cuprizone had a significantly decreased performance compared to the no cuprizone control group, and this decreased performance was not improved significantly by clozapine (Figure 4.2b). The maximum performance time allowed for the test was 120 seconds, and this was reached by the majority of animals in the control groups. Average performance for animals in the clozapine-treated cuprizone group was 43.4 seconds, with the untreated cuprizone fed group achieving an average score of 28.3 seconds, but this difference did not reach statistical significance. Clozapine treatment alone recorded an average performance of 67 seconds, slightly lower than the control average of 91 seconds. This difference may be due to the slight sedative affects associated with clozapine treatment (Daniel et al., 1996; Young et al., 1998).

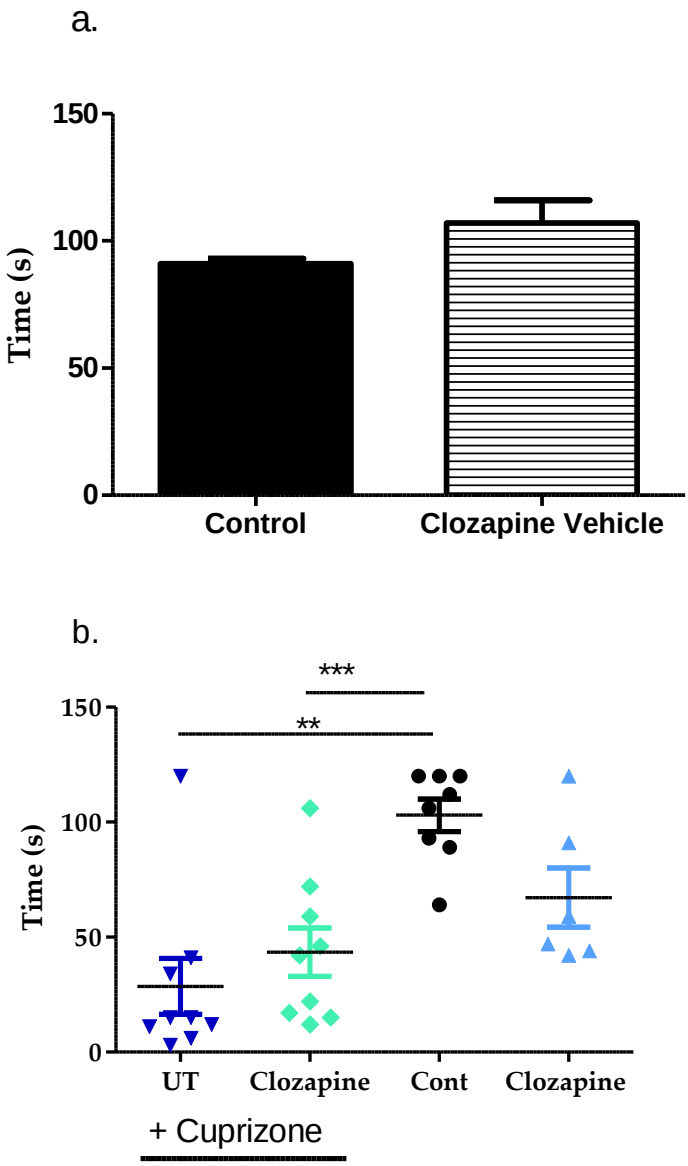

Figure 4.2: 0.3% Cuprizone led to impaired performance on the rotating rod behavioural assay.
2 days prior to cull mice were placed on the rotating rod at a fixed speed of 28 RPM, the first day was considered trial/habituation day with results not being recorded. On the day prior to cull animals were again placed on the rota-rod at a fixed speed of 28 RPM and the time taken to fall was recorded, with maximum test duration being 120 seconds. This was repeated twice for each animal after a sufficient recovery interval (minimum 5 minutes), with the best performance being recorded. UT= untreated cuprizone fed animals. Shown are the means and SEM of individual mice (n=6-9/group) from 2 independent experiments ** $p<0.01$ and ***$p<0.001$ by 1-way ANOVA and Bonferroni's multiple comparison post-test.

4.2.3 Clozapine did not prevent cuprizone induced demyelination

To assess the effect of clozapine administration on demyelination, sections were stained with Luxol Fast Blue and counterstained with cresyl violet to show cell nuclei. The area of interest was the corpus callosum, as this area is reproducibly targeted with cuprizone administration (Groebe et al., 2009).

In the control animals not receiving cuprizone in their diet, the corpus callosum in the region of interest stains deep blue, indicating the presence of myelin (Figure 4.3 d). Treatment with the clozapine vehicle alone (no cuprizone) did not have any effect on the myelin sheath in the corpus callosum, with myelin staining similar to that of the control group (Figure 4.3 c). When compared to the visible demyelination observed in mice treated with cuprizone alone, clozapine treatment did not alter the cuprizone-induced demyelination (Figure 4.3 a & b).

Once sections were stained they were photographed and presented to a group of individuals blinded to the treatment groups for scoring. Ratings of 0-3 were used to score the level of demyelination which had occurred in each section (1 section per animal) and these values were averaged and graphed to compare give the level of demyelination. In the cuprizone only animals and the clozapine-treated cuprizone animals, there is an increase in demyelination seen, with an average score of 2.8 and 2.5 respectively (arbitrary units) shown in Figure 4.4. One-way ANOVA with Bonferroni's multiple comparison test showed that the level of demyelination in these two groups was significantly higher than the control group, clozapine vehicle group and clozapine only treatment group, indicating that clozapine or clozapine vehicle themselves did not have any effect on myelin levels in the absence of cuprizone.

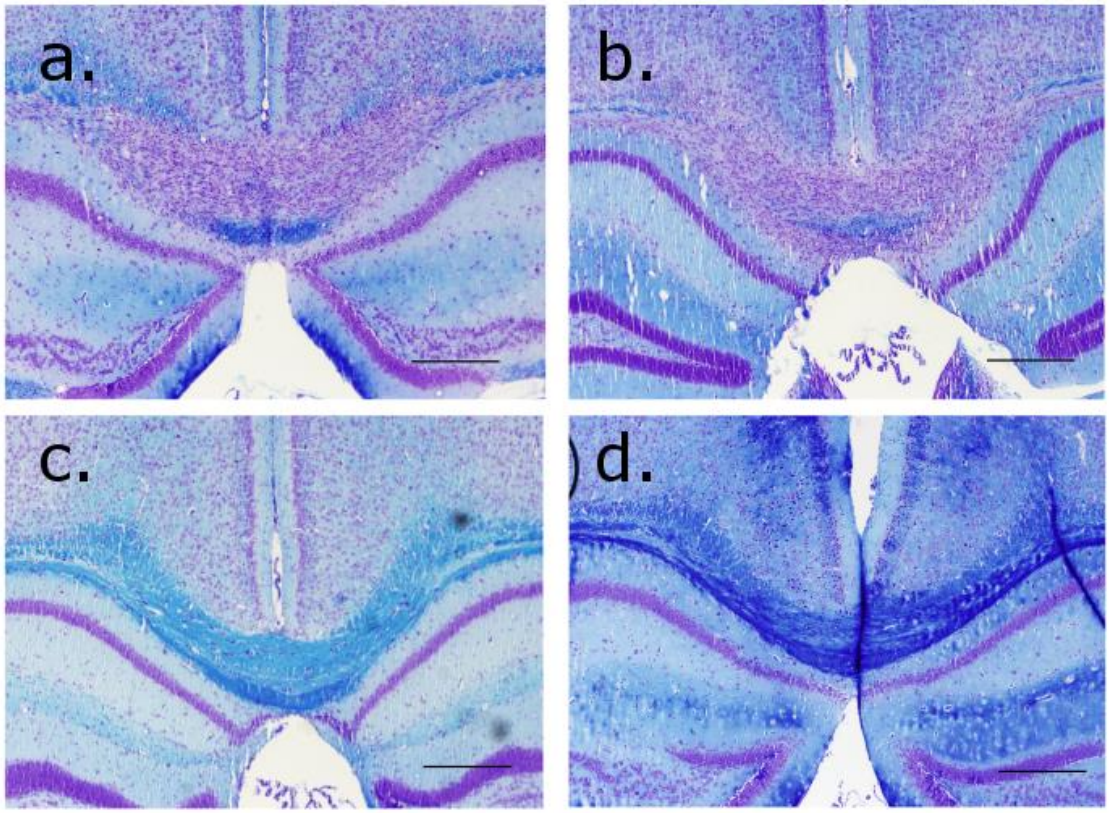

Figure 4.3: Clozapine did not prevent 0.3% cuprizone induced demyelination of the corpus callosum.
6-8 week old C57BL/6J mice were fed a diet containing 0.3% cuprizone for 6 weeks and effects on demyelination were assessed. Brains were fixed with 4% PFA and embedded in paraffin before being sectioned at 5-7 μm. Sections were mounted on slides and rehydrated before staining with LFB and cresyl violet counterstain. Sections above are representative of treatment groups, with (a) showing cuprizone alone, (b) cuprizone and 60 mg/kg clozapine, (c) clozapine vehicle and (d) normal. Scale bar 100 μm.

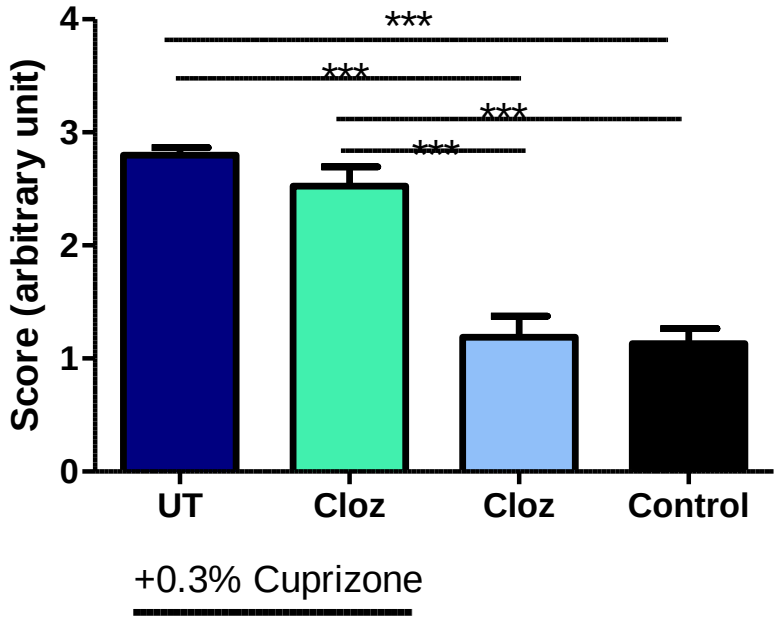

Figure 4.4: Clozapine treatment did not significantly alter levels of demyelination in the corpus callosum. Sections stained with Luxol fast blue from individual animals were presented to individuals blinded to treatment groups for scoring. Lower numbers indicated little to no demyelination had occurred whilst higher numbers indicated greater levels of demyelination. Shown are the means and SEM of individual mice (n=4-10/group) from 2 independent experiments *** $p<0.001$ with 1 -way ANOVA and Bonferroni's multiple comparison test.

4.2.4 Clozapine did not prevent cuprizone induced astrogliosis

As well as staining for myelin levels, consecutive sections were assessed for astrocyte activity by GFAP. Similar to LFB sections, GFAP stained sections were photographed (1 per animal) and presented in a random fashion to individuals blinded to the treatment groups. Individuals were asked to score sections, focusing on the staining within the corpus callosum only. Numerical values were assigned to sections ranging from 0 (no visible astrocytes) to 3 (strong astrocyte activation). Average values were graphed per section to assess astrocyte activation across the different treatment groups.

As well as scoring manually, ImageJ was used to analyse GFAP staining. Some artefacts were picked up when sections were scored using ImageJ such as small folds or creases in the tissue being picked up as darker regions of staining and therefore being counted as astrocytes. It was however possible to go through by hand and alter these images to almost completely exclude these regions, allowing the ImageJ script to be used as a quick way to quantify any differences in astrocyte staining. Scores by blinded observers and using imageJ can be seen alongside each other in Figure 4.6.

Treatment with cuprizone led to mass activation of astrocytes as measured by GFAP expression (Figure 4.5 and 4.6a), and treatment of cuprizone fed animals with clozapine did not reduce the level of astrogliosis (Figure 4.5 and 4.6a). Treatment with clozapine vehicle alone led to a slight increase of astrocyte activation however this increase was not significantly different to the control animals. Taken together these results show that clozapine vehicle has no effect on astrocyte activation level, and that clozapine treatment is unable to prevent cuprizone-induced demyelination and astrocyte activation.

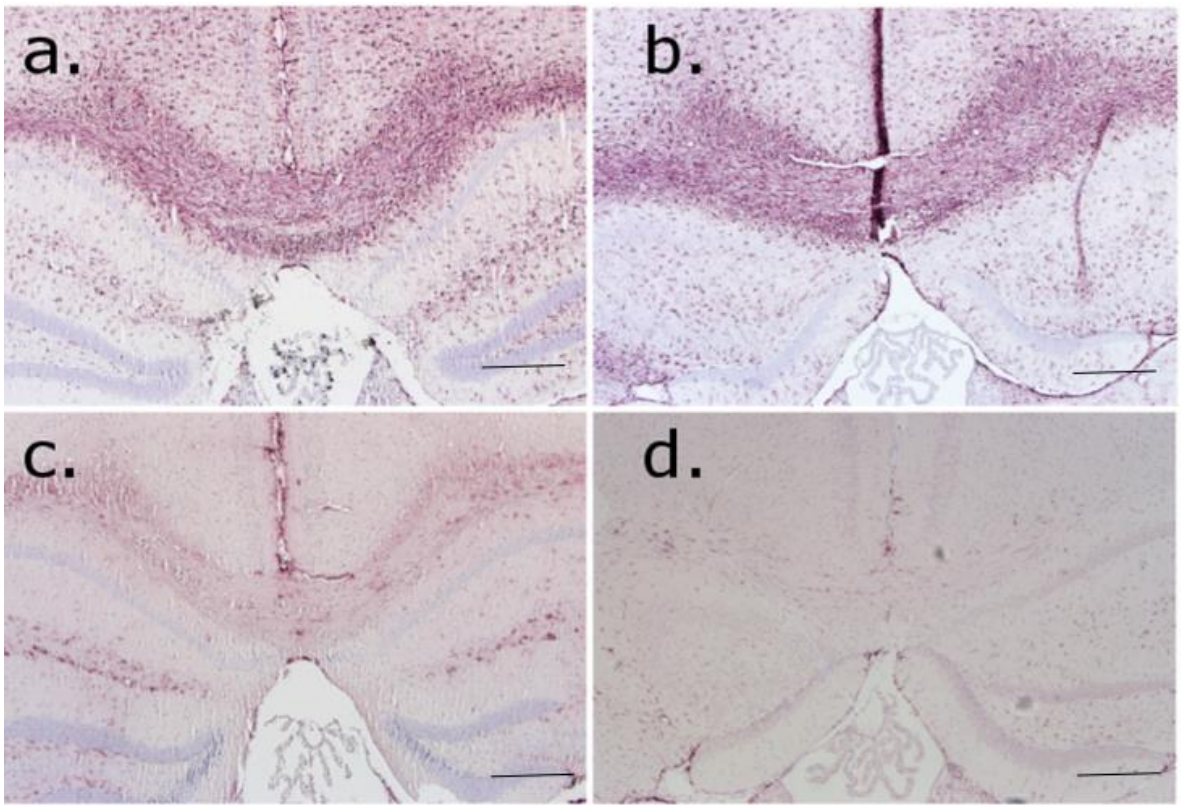

Figure 4.5: Assessment of astrocyte activation using IHC for GFAP.
5-7 μm paraffin embedded sections were rehydrated and stained for GFAP, followed by counterstain with haematoxylin to show tissue structure. Representative images show (a) 0.3% cuprizone, (b) 0.3% cuprizone + 60 mg/kg clozapine, (c) clozapine vehicle and (d) control. n=4-10. Scale bar = 100 μm.

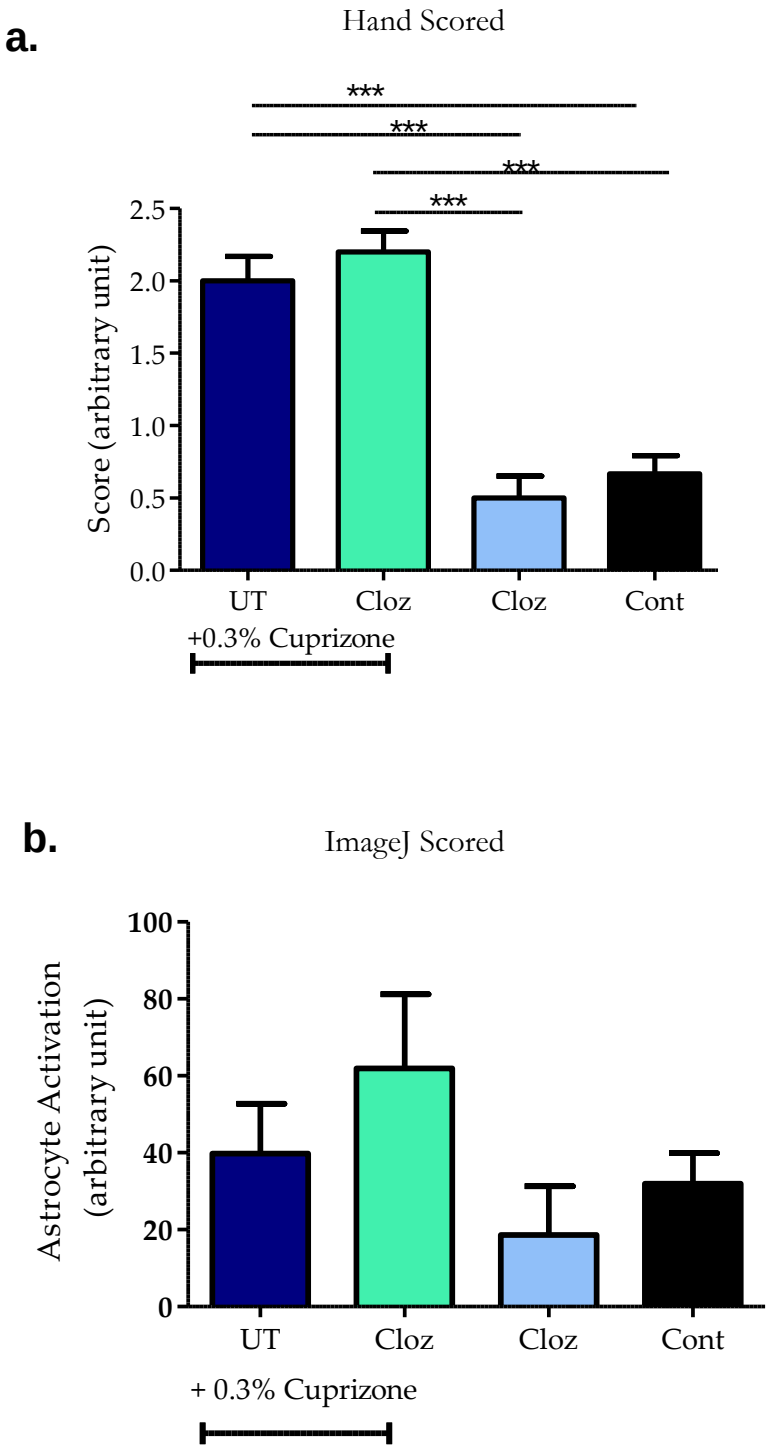

Figure 4.6: Treatment with 0.3% cuprizone led to increased astrocyte activation which was not reduced by clozapine treatment.
5-7 µm sections from cuprizone fed mice were scored for astrocyte activation (GFAP) either by individuals blinded to their treatments (a) or using a script developed in ImageJ (b). Both methods showed a similar pattern of scoring however significance was only reached using manual scoring (a). Mean and SEM are shown from 2 individual experiments with n=4-10 per treatment group. Clozapine vehicle and control animals were combined. One way ANOVA ***$p<0.001$, with Bonferroni's post-test.

4.2.5 Clozapine did not prevent 0.2% cuprizone induced weight loss

As no protective effect was seen with clozapine, it was decided to assess whether a lower concentration of cuprizone in the feed might show a treatment-mediated effect in case the 0.3% cuprizone was driving the demyelination too hard for clozapine to have an effect. Thus, animals fed 0.2% cuprizone and treated with clozapine were weighed and monitored daily to detect any signs of distress. All animals receiving cuprizone in the diet experienced rapid weight loss over the first 10-12 days, with weight stabilising after this period. The animals receiving cuprizone and clozapine treatment initially had a greater weight loss but then returned to levels similar to the other cuprizone fed groups (Figure 4.7). The control group and the clozapine vehicle control group continued gaining weight at a steady duration throughout the 6 week experiment and did not experience weight loss, indicating that the clozapine vehicle does not have any impact on animal weight. When viewed as a percentage change in weight the clozapine treated cuprizone fed animals also showed an increased weight loss over the first 10-12 days of the experiment.

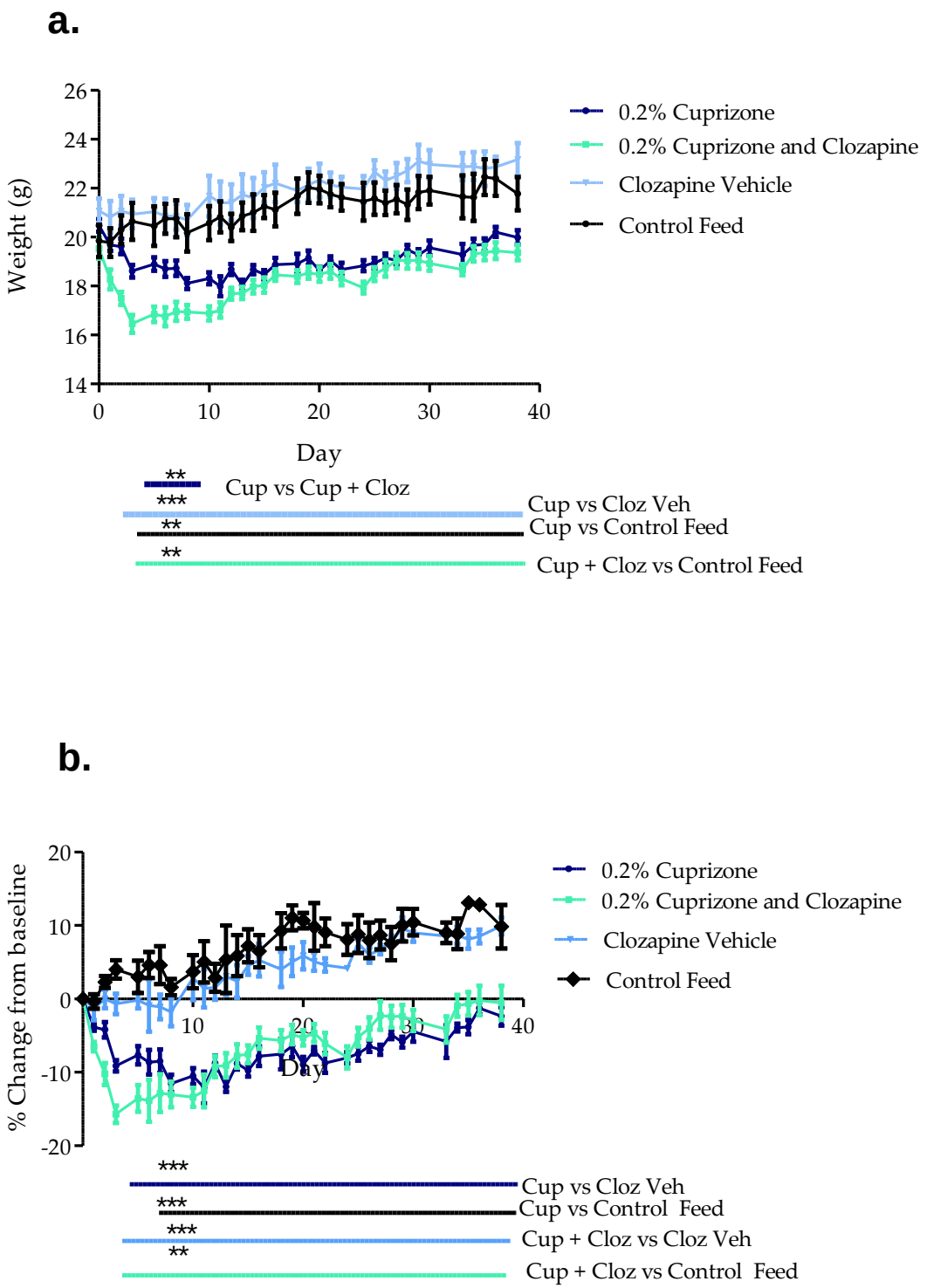

Figure 4.7: Clozapine treatment did not protect from 0.2% cuprizone induced weight loss.
6-8 week old female C57BL/6 mice were fed a diet containing 0.2% cuprizone which was refreshed daily. Water was available ad libatum. Mice were weighed and monitored daily. (a). 02% cuprizone administration led to weight loss over the initial 10-12 days of the experiment with animals in never reaching the same weight as control animals. 60 mg/kg clozapine did not protect from weight loss with (a) showing absolute values and (b) showing percentage change from starting weight. Mean and SEM are shown from two individual experiments with n= 4-10 per group. **p<0.01, ***p<0.001 with 2 way ANOVA and Bonferronis post-test.

4.2.6 Clozapine did not significantly improve motor coordination after 0.2% cuprizone intoxication.

The 0.2% cuprizone diet led to a trend towards a decreased performance time, indicating a reduction in coordination. Clozapine treated cuprizone animals showed a trend towards improved performance, with all but one animal achieving the maximum performance time. Interestingly the animals receiving the clozapine vehicle and normal feed showed a trend towards a decreased performance on this round of testing which cannot be explained by cuprizone as they were not exposed to it.

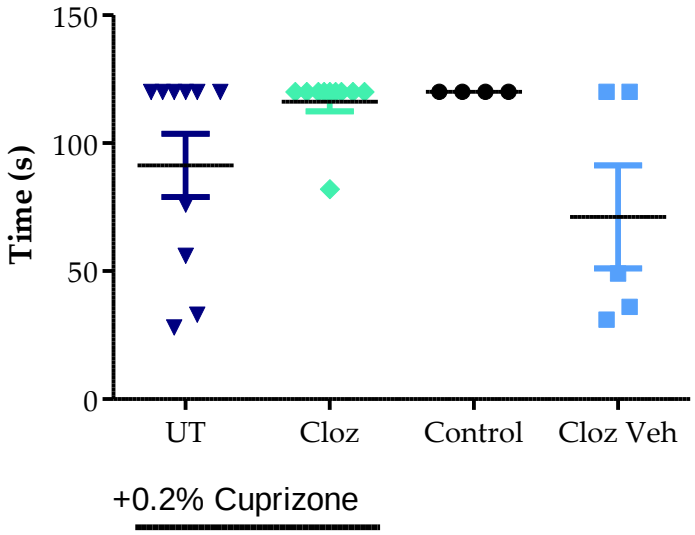

Figure 4.8: 0.2% cuprizone led to impaired performance on the rotating rod assay.
Mice were placed on the rota-rod at a fixed speed of 28RPM and maximum performance time was recorded. Prior to test day mice were exposed to the rota-rod however performance was not recorded. On test day the longest time out of two attempts was recorded. No significant difference was seen between treatment groups.

4.2.7 Clozapine did not significantly alter rates of 0.2% cuprizone induced demyelination

As in previous experiments, after 6 weeks of a 0.2% cuprizone containing diet, animals were sacrificed and brains were collected and processed for fixation in paraffin. After sectioning and staining with luxol fast blue sections were photographed and presented to individuals blinded to their treatment group in a random order. Pictures in Figure 4.9 are representative images from different treatment groups. Treatment with clozapine did not significantly reduce cuprizone induced demyelination (Figure 4.10) at the 6 week time point.

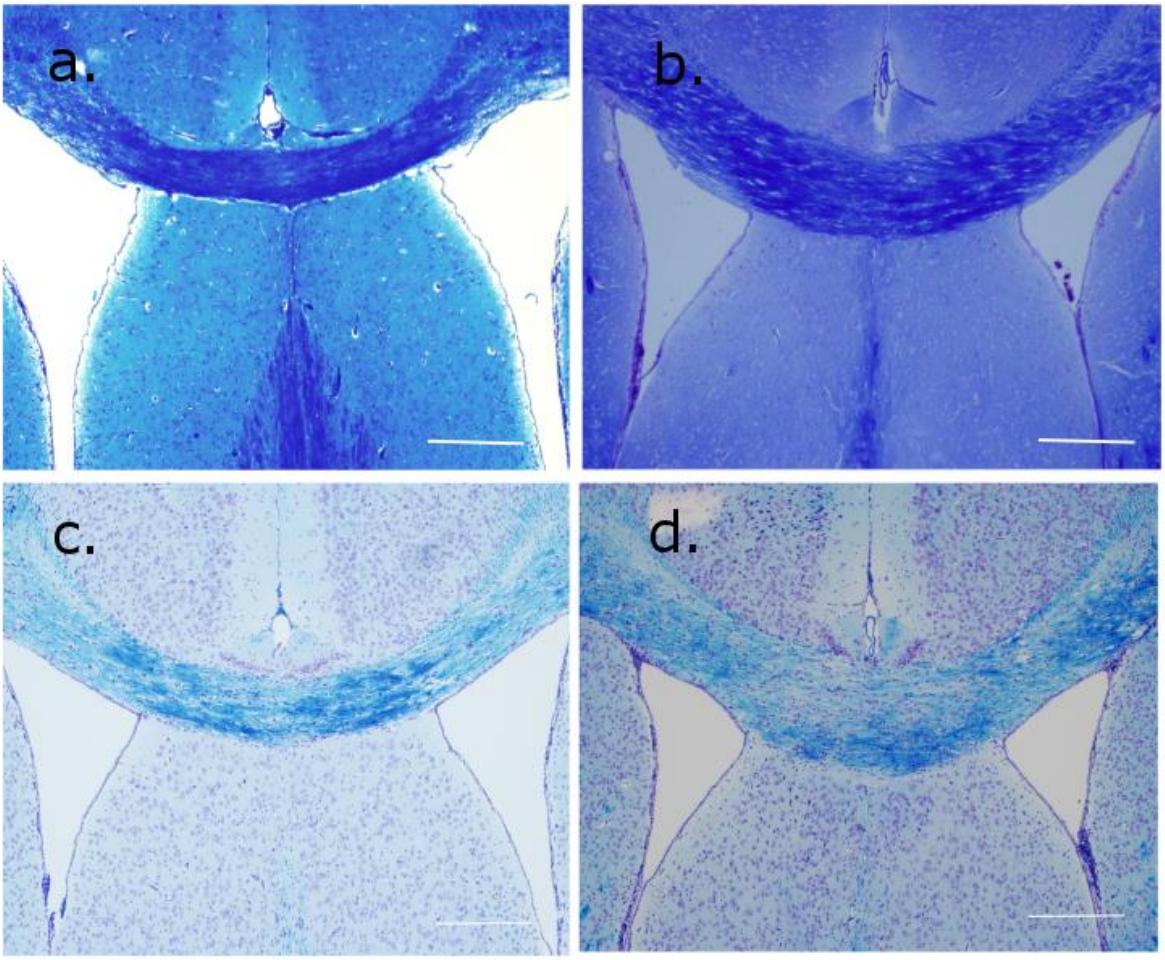

Figure 4.9: Level of demyelination was assessed by staining myelin with luxol fast blue.
5-7 µm sections were stained with luxol fast blue and counterstained with cresyl violet to visualise myelin in the corpus callosum approximately 2mm posterior to bregma. Images in this figure are representative with (a) showing control, (b) clozapine vehicle, (c) 0.2% cuprizone treatment, and (d) 0.2% cuprizone + 60 mg/kg clozapine. n =4-10 per group. Scale bar = 100 µm.

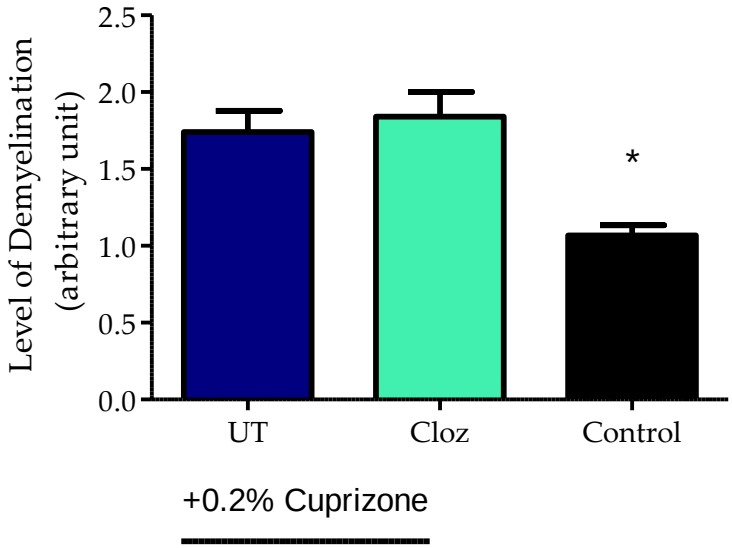

Figure 4.10: Clozapine treatment did not significantly reduce the level of demyelination occurring after 0.2% cuprizone intoxication.
Photographed sections from each mouse were presented in a random order to individuals blinded to their treatments. Sections were scored based on the level of demyelination, with a higher number indicating a greater level of demyelination. Significance was not reached with this lower 0.2% cuprizone diet. *p=0.05 with one way ANOVA and Bonferroni's post-test.

Again sections were stained with luxol fast blue and scored by individuals blinded to their treatment. Included in this experiment were sections from mice that were culled after only three weeks of demyelination to determine if clozapine was having any effect on myelin levels at an earlier time point (Figure 4.11). Clozapine treatment did not significantly reduce cuprizone induced demyelination (no difference between 0.3% cuprizone versus 0.2% cuprizone + clozapine).

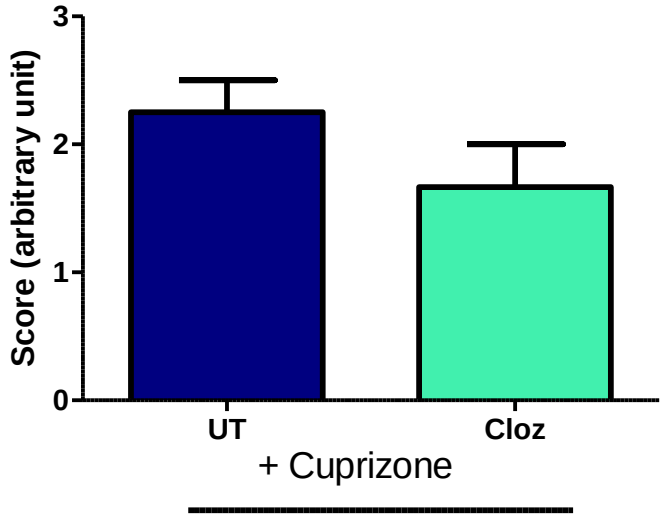

Figure 4.11: Clozapine treatment had no effect on myelin levels after 3 weeks of a 0.2% cuprizone diet. To assess the potential effect of clozapine on demyelination at an earlier time point, animals were sacrificed after 3 weeks and sections were stained for myelin. No significant difference was seen in myelin levels at this earlier time point. n=3.

4.2.8 Clozapine did not alter 0.2% cuprizone induced astrogliosis

To look at astrocyte activation, as measured by staining for GFAP, 3-4 sections per mouse were stained and the most complete section photographed for analysis. Pictures (one per animal) were presented to individuals blinded to their treatment groups and given a score from 0 (low/no astrocyte activation) to 3 (strong astrocyte activation). Again, assessors were asked to focus only on the staining within the corpus callosum rather than the whole section. As in the 0.3% cuprizone experiments presented earlier, the 0.2% cuprizone diet induced activation of astrocytes in the corpus callosum (Figure 4.12 c). 60 mg/kg of clozapine (Figure 4.12 d) did not significantly alter the level of astrocyte activation relative to the untreated group (Figure 4.12 c). This pattern is difficult to see when only viewing the representative images shown in Figure 4.12 as there was some variation between animals which is accounted for when looking at group scores in Figure 4.13. Scoring of photographs by ImageJ was also carried out alongside the manual scoring of

activation and the same pattern of scoring was recorded, further indicating that this method is effective for rapidly quantifying astrocyte activation using an arbitrary score.

Clozapine treatment did not lead to a significant change in astrocyte activation when compared to the untreated 0.2% cuprizone test group. Overall these experiments indicate that clozapine is not preventing demyelination or altering astrocyte activation during the demyelination phase of the cuprizone model in the corpus callosum.

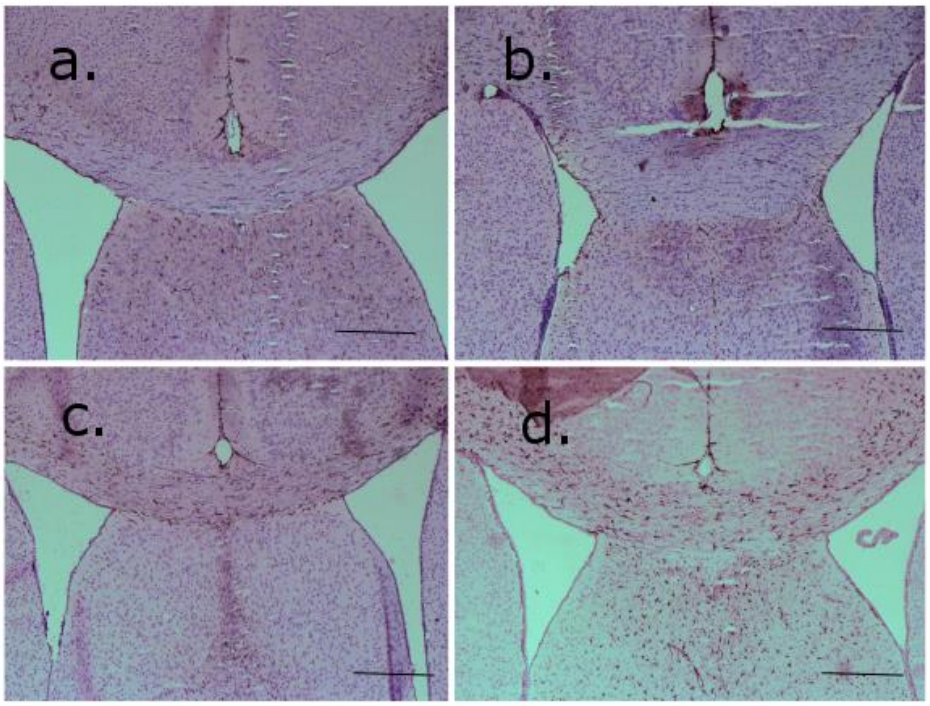

Figure 4.12: Astrocyte activation in response to Clozapine treatment was assessed by IHC for GFAP.
After 6 weeks of treatment 5-7 μm paraffin embedded sections were stained for the astrocyte marker GFAP, which is upregulated on activated astrocytes and then counter stained with haematoxylin to visualise tissue structure. This figure contains representative images for the groups (a) control, (b) clozapine vehicle, (c) 0.2% cuprizone and (d) 0.2% cuprizone + 60 mg/kg clozapine. Scale bar = 100 μm.

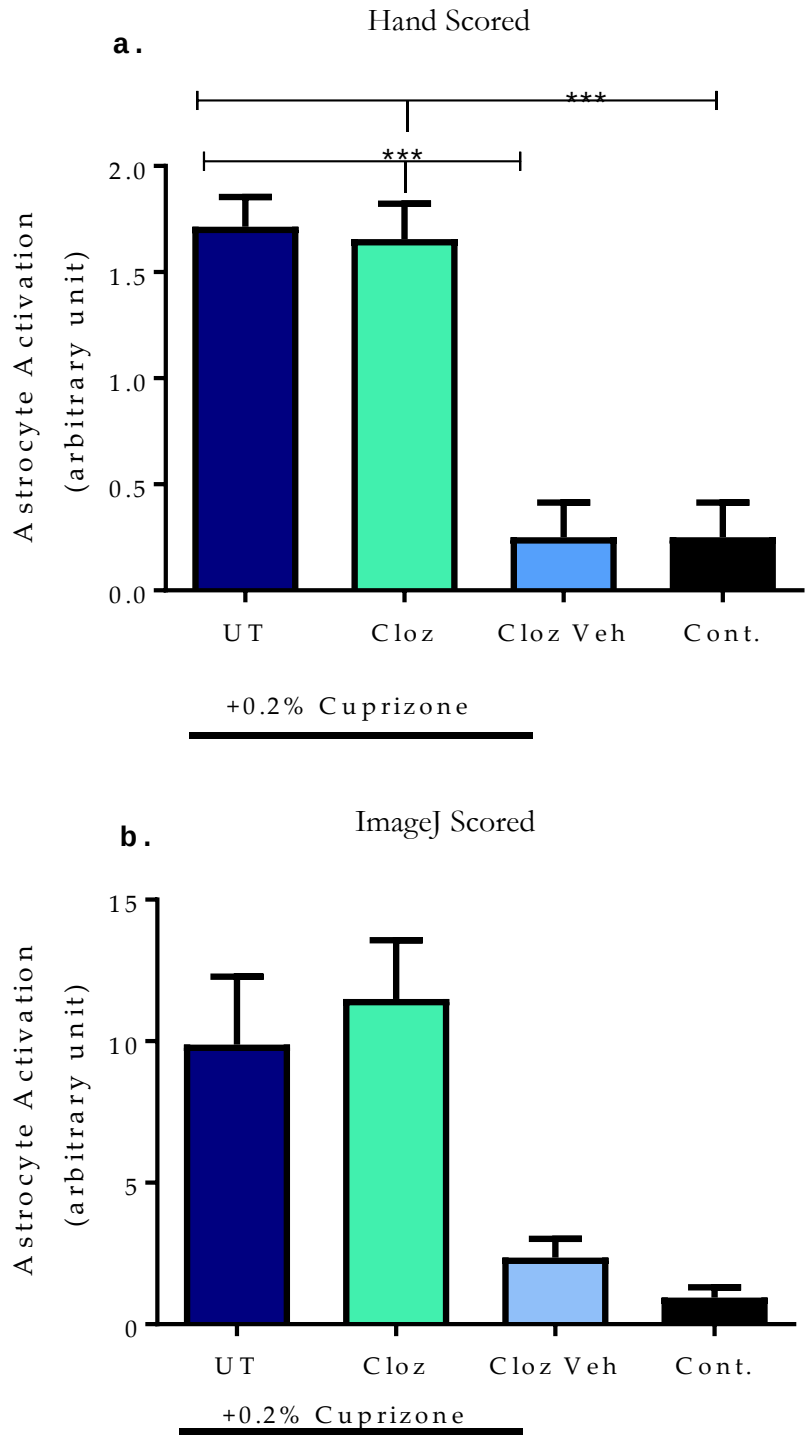

Figure 4.13: Cuprizone intoxication significantly increased the activation of astrocytes.
Sections were scored for astrocyte activation (GFAP) either by individuals blinded to their treatments (a) or using a script developed in ImageJ (b). Both methods showed a similar pattern of scoring however significance was only reached using manual scoring (a). n=4-10 per treatment group. One way ANOVA ***$p<0.0001$, with Bonferroni's multiple comparison test.

4.2.9 MIS416 did not prevent cuprizone induced weight loss

Mice were fed 0.2% cuprizone mixed into ground chow, and were treated with 100 μg MIS416 i.v weekly. Animals were monitored daily and weighed for the duration of the experiment. Cuprizone intoxication led to a significant change in weight from starting weight after 10 days in both groups fed cuprizone (Figure 14 b), and administration of MIS416 weekly was unable to prevent the cuprizone-induced weight loss. Treatment with MIS416 alone (i.e. no cuprizone in diet) did not lead to any weight loss (Figure 14 b), with animals following the same trend of weight gain as that seen in healthy control animals, indicating that the weight loss seen in the cuprizone and MIS416 treated group is as a result of the cuprizone and not the weekly MIS416 administration.

Using the same method as described for clozapine-treated animals, MIS416-treated animals had coordination assessed using the rota-rod at a fixed speed of 28 RPM. Treatment with 100 μg of MIS416 i.v. weekly did not significantly improve performance on the rota-rod relative to untreated cuprizone fed animals, however the presence of cuprizone in the diet led to a trend in decreased rota-rod performance, which did not reach significance due to individual animal variability in performance at this lower dose of cuprizone (Figure 4.15).

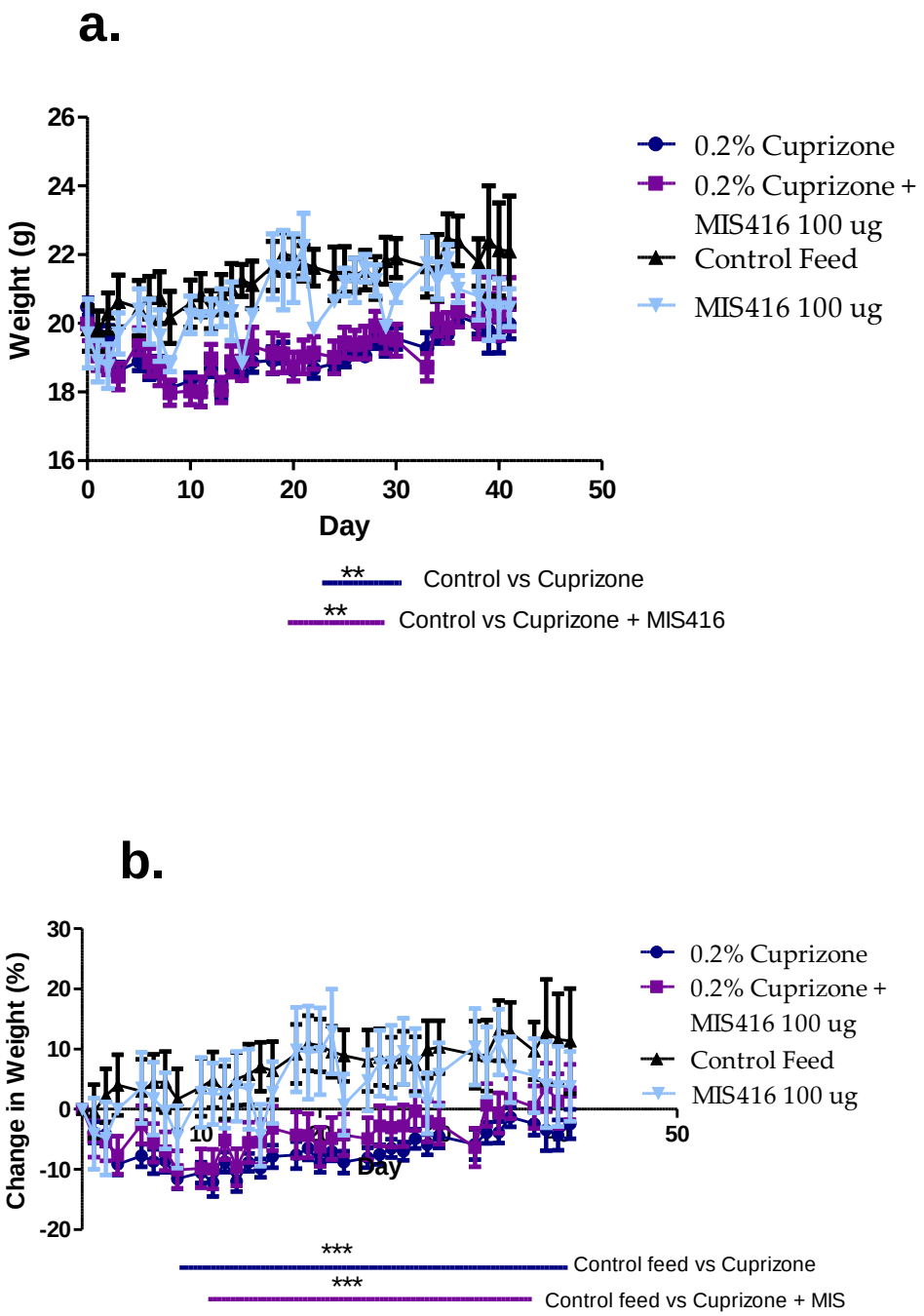

Figure 4.14: MIS416 treatment did not prevent 0.2% cuprizone induced weight loss.
Female C57Bl/6 mice were fed either control diet or cuprizone mixed into ground mouse feed at 0.2% for 6 weeks. Mice were weighed throughout the duration of the experiment. MIS416 was administered weekly by i.v. n=4-8 per group. Data shown is mean and SEM of 2 individual experiments.***p<0.0001 with two-way ANOVA and Bonferroni's post-test.

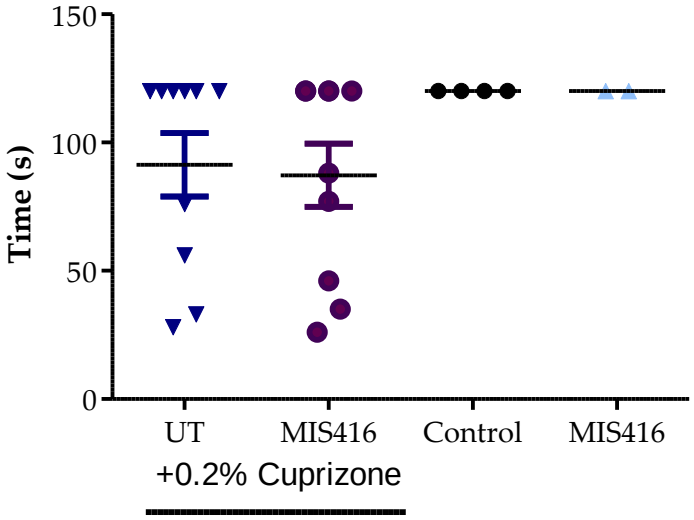

Figure 4.15: MIS416 did not improve performance on the rota rod after cuprizone intoxication.
Mice were placed on the rota-rod at a fixed speed of 28RPM and the time taken to fall was recorded. Max test time was 120 seconds.

4.2.10 MIS416 did not prevent cuprizone induced demyelination

In this model of demyelination with 0.2% cuprizone, the difference in demyelination mediated by cuprizone did not reach significance nor did treatment with MIS416 weekly prevent demyelination from occurring in the corpus callosum (Figure 4.16 and 4.17). Treatment with MIS416 alone (no cuprizone) had no impact on myelin (Figure 4.16), with representative images from each experimental group shown in Figure 4.16.

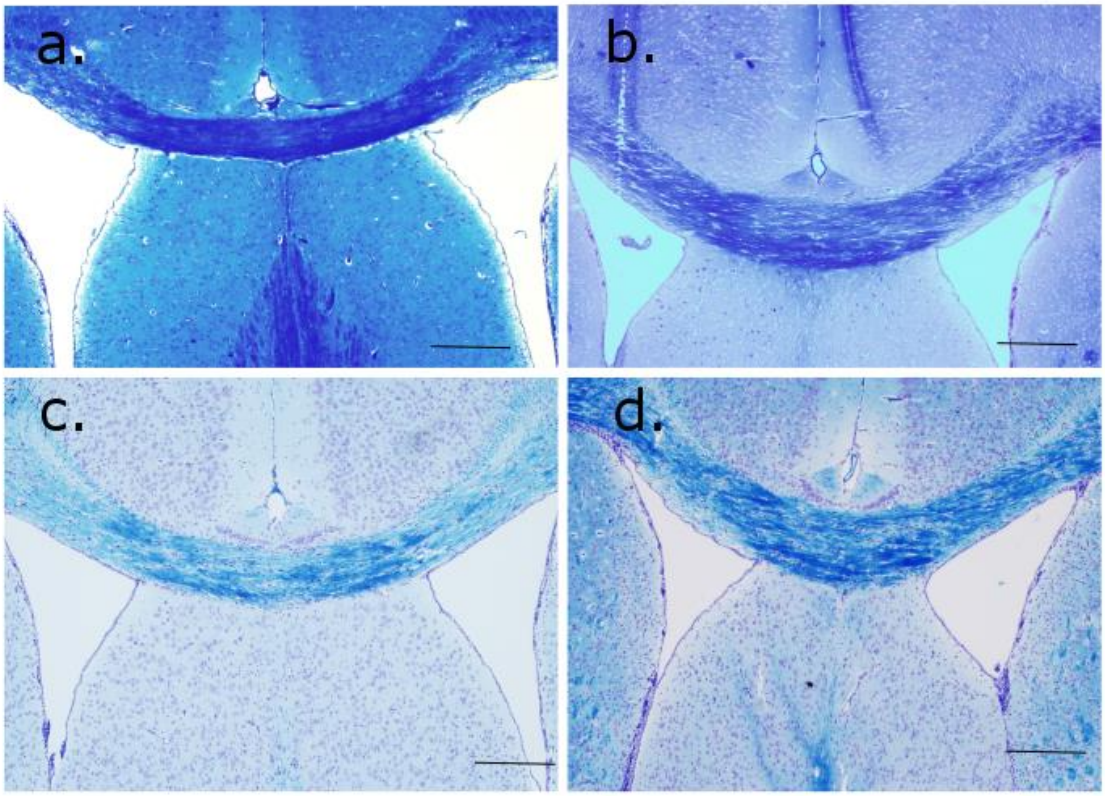

Figure 4.16: MIS416 did not prevent demyelination of the corpus callosum.
5-7 μm paraffin embedded sections were collected and stained for myelin using luxol fast blue. Control healthy animals (a) show intact myelin in the corpus callosum, MIS416 alone did not disrupt myelin (b), whilst 0.2% cuprizone (c) and 0.2% cuprizone and MIS416 (d) weekly showed small regions of demyelination in the corpus callosum. Scale bare = 100 μm.

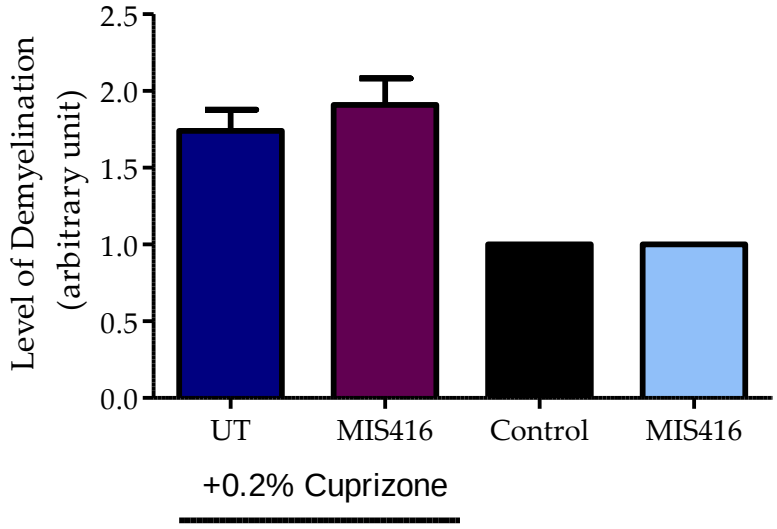

Figure 4.17: Demyelination score after MIS416 treatment was not significant.
Individuals blinded to treatment groups scored images of myelin in the corpus callosum from 1- 3 with one being low/no demyelination and 3 being high levels of demyelination. One section per animal was scored. n=4-8. Treatment with MIS416 did not cause significant changes to demyelination.

4.2.11 Astrogliosis was not prevented by MIS416

MIS416 treatment alone (Figure 4.18 b and 4.19) did not have a significant impact on astrocyte activation in the corpus callosum relative to untreated 'normal' animals (Figure 4.18a and 4.19), indicating that MIS416 itself has no direct effect on astrocyte activation in the absence of demyelination. All groups receiving 0.2% cuprizone (cuprizone only, + MIS416) had significantly increased astrocyte activation compared to the untreated control and MIS416 only groups, but MIS416 treatment of cuprizone-treated animals did not alter astrocyte activation compared to cuprizone only (Figure 4.19a).

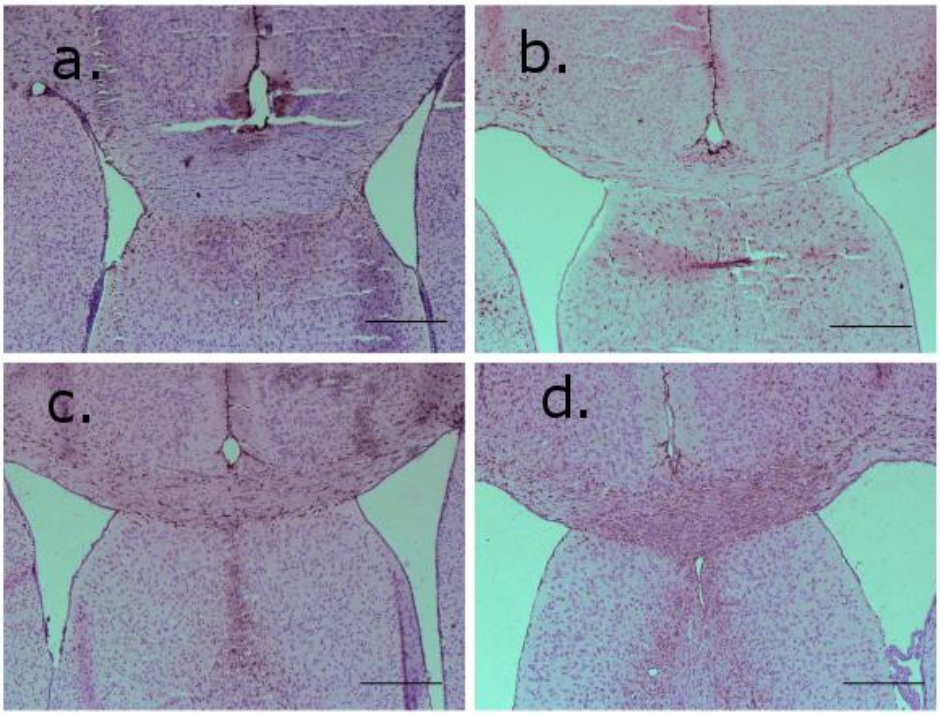

Figure 4.18: MIS416 treatment did not reduce 0.2% cuprizone induced astrogliosis.
Individuals blinded to treatment groups scored one section per animal on a scale of 1-3 with 1 being low astrogliosis and 3 being high astrogliosis. (a) shows a control animal, (b) is MIS416 treatment alone, (c) is cuprizone alone with (d) showing cuprizone and MIS416. Images shown above are representative of treatment groups. Scale bar = 100 μm.

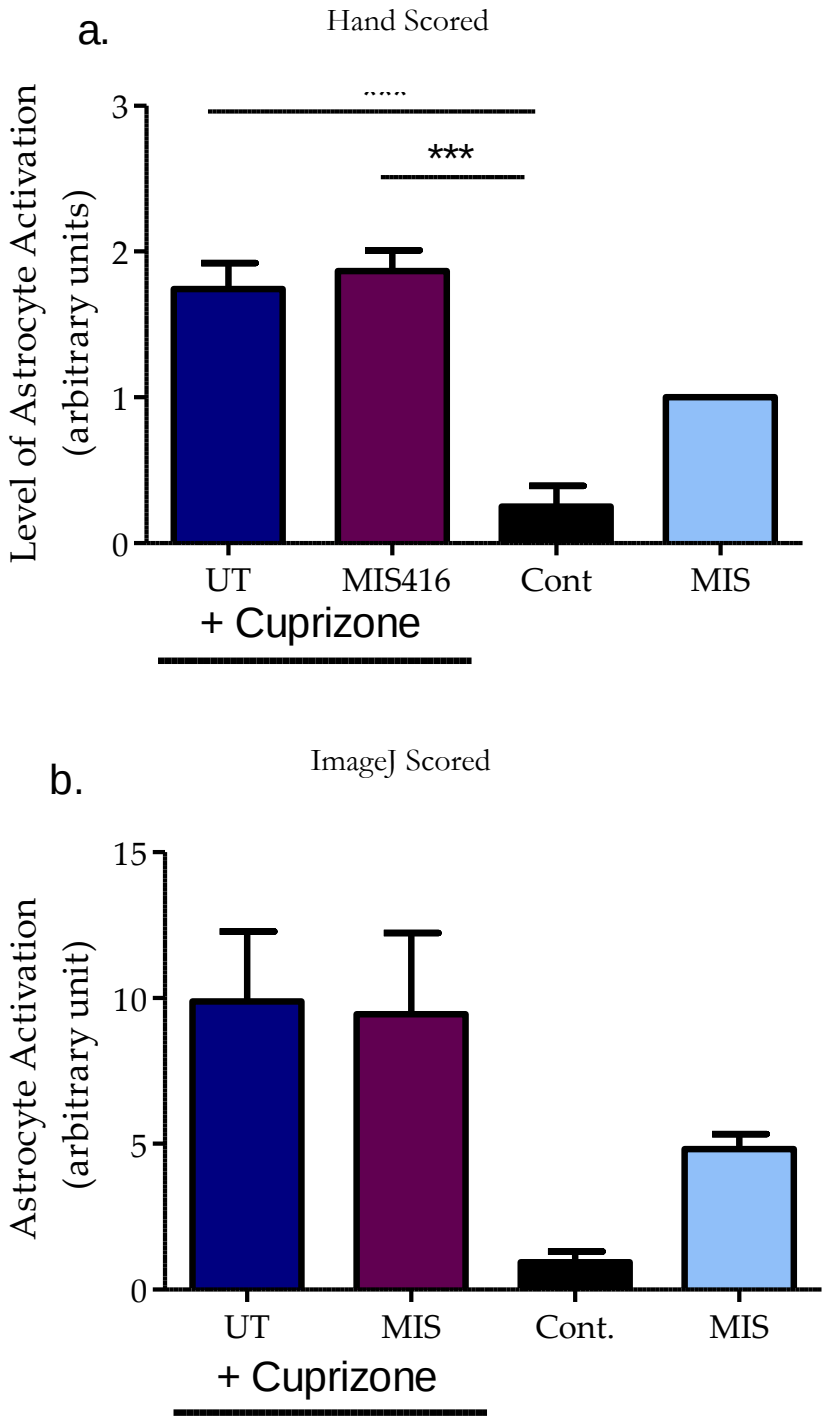

Figure 4.19: MIS416 did not reduce cuprizone associated astrogliosis.
Sections were scored by individuals blinded to treatment (a) or using a script developed in ImageJ (b). The same pattern is seen using both methods, however significance is only reached in a. One-way ANOVA ***$p<0.001$, with Bonferroni's post-test. Mean and SEM shown from 2 individual experiments with n= 3-8.

4.2.12 MIS416 treatment led to spleen enlargement

Previous work from our lab has identified some key immune effects of MIS416 administration including increased splenocyte count, increase in the splenic Treg population and increased cytokine production including IFN-γ (White, 2015). To determine if MIS416 was having the same effect in the cuprizone model, splenocytes were characterized by flow cytometry and restimulated *in vitro* to assess changes to immune function. In line with other work conducted in our laboratory, MIS416 treatment led to an increased splenocyte count (Figure 4.20) although this increase did not reach significance, primarily due to the small sample size. In the 0.2% cuprizone fed group, MIS416 administration continued to cause an increase in splenocyte populations, which was not reduced by the presence of cuprizone in the diet.

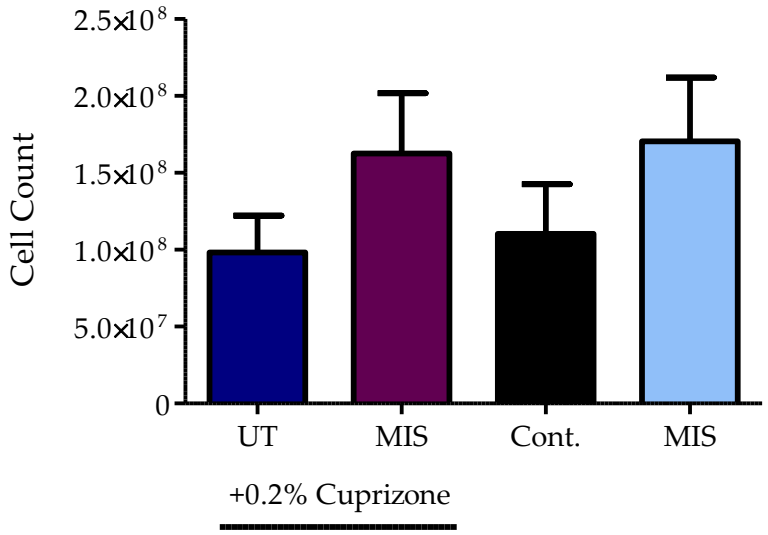

Figure 4.20: MIS416 treatment caused enlargement of the spleen.
After isolation of spleens a single cell suspension was prepared, followed by lysis of red blood cells. Live cells were counted and the total cell count was calculated. Mean and SEM from two individual experiments with n= 2-5 per group.

4.2.13 MIS416 caused reduction in CD8, Treg and NK splenocyte populations

Following preparation of single cell suspensions, splenocytes were prepared and stained for flow cytometry. Of the live single cells, CD45 (common leucocyte antigen) was used to narrow down populations of interest before CD8 T cells, CD4 T cells, regulatory T Cells (Treg) and natural killer (NK) cells were identified. Cuprizone treatment alone had no effect on either percentage or total cell number for CD4, CD8, Treg or NK cells (Figures 4.21 & 4.22). MIS416 treatment did not alter the splenic CD4+ T cell population relative to the 0.2% cuprizone alone when viewed as either absolute cell counts or percent of live cells (Figure 4.21a & 4.22a). In contrast, CD8 T cells, Treg, and NK cells were reduced in both percentage and total number relative to untreated 0.2% cuprizone fed animals by MIS416 although the total number of CD8 T cells does not reach significance (Figure 4.21b-d & 4.22b-d). Furthermore, treatment with MIS416 also led to a significant reduction in the total number of Treg and NK cells and the frequency of NK cells relative to untreated 0.2% animals (Figure 4.21c & d and 4.22d) Finally, MIS416 treatment was not found to increase any of the splenic myeloid populations in the cuprizone model, which is in contrast to previous findings in healthy and EAE-immunized mice (Figure 4.23).

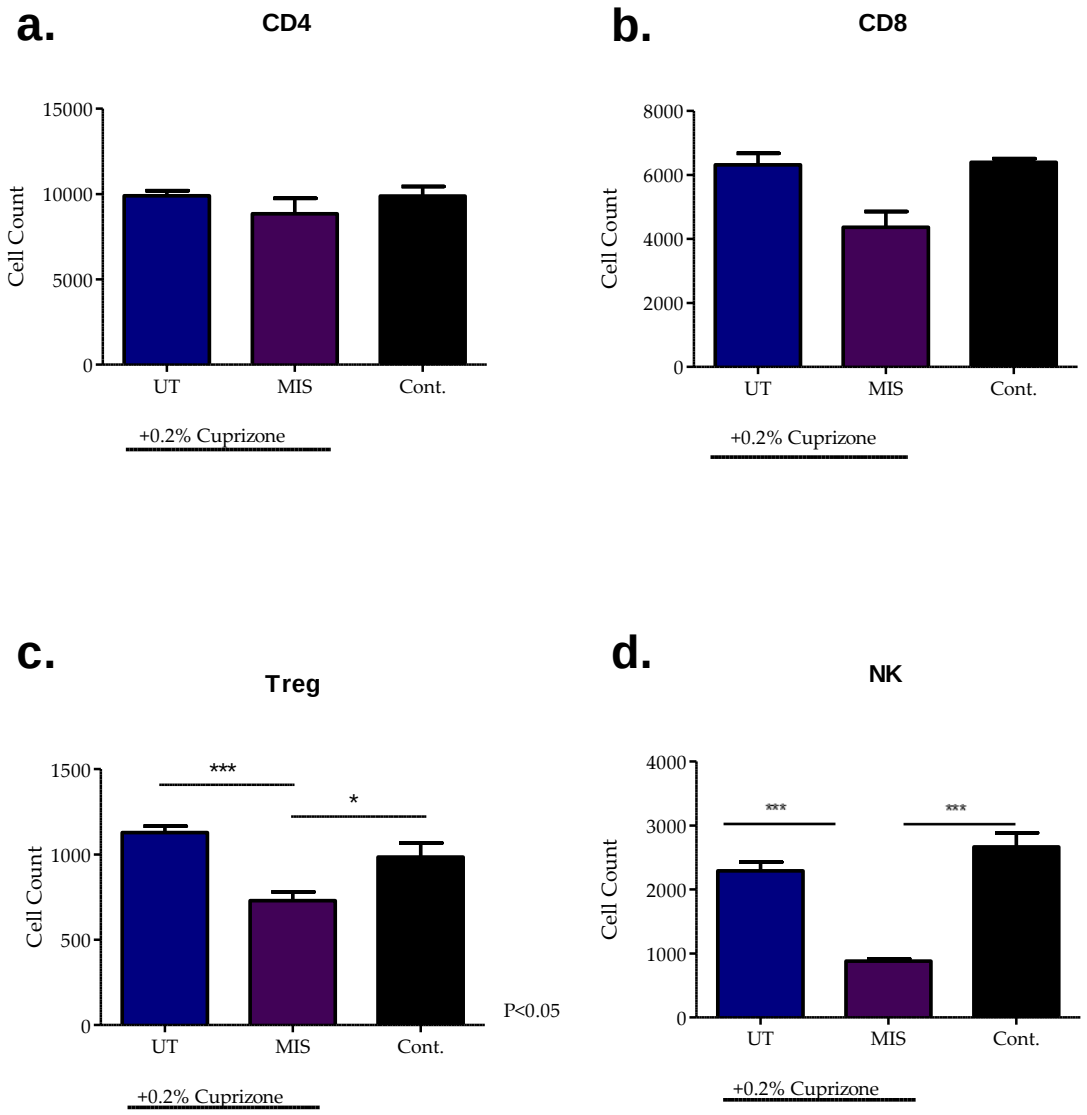

Figure 4.21: MIS416 treatment altered peripheral immune cell populations
Single cell suspensions were prepared from spleens isolated from animals upon completion of the experiment. Cells were stained with the appropriate mixture of antibodies and data were collected using FACs Diva software, prior to analysis on FlowJo v.10. Data shown are mean and SEM. n=2 to 5. One way ANOVA with Bonferroni's post-test *p<0.05, ***p<0.001.

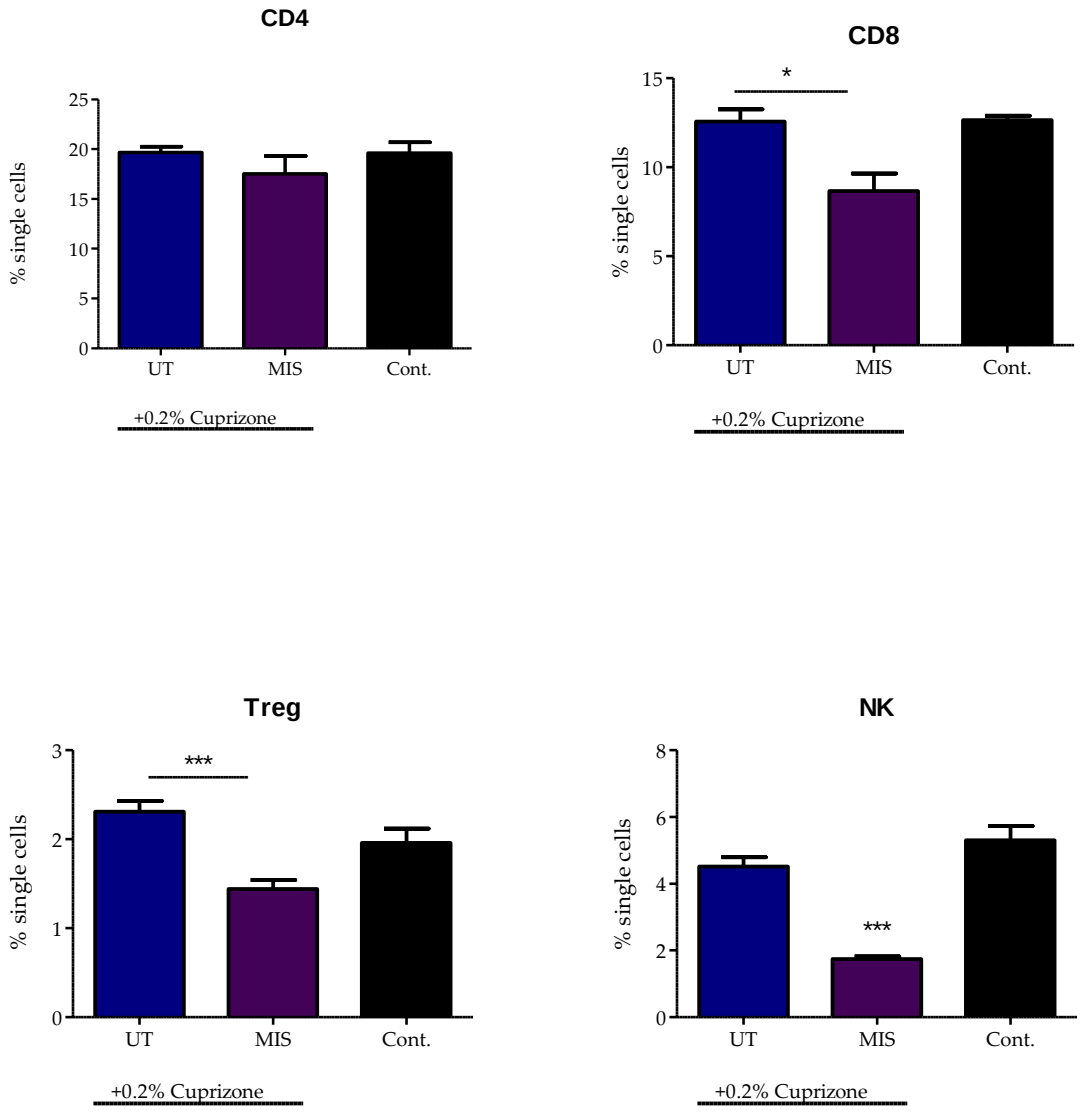

Figure 4.22: MIS416 treatment altered peripheral immune cell populations
Single cell suspensions were prepared from spleens isolated from animals upon completion of the experiment. Cells were stained with the appropriate mixture of antibodies and data were collected using FACs Diva software, prior to analysis on FlowJo v.10. Data shown are mean and SEM. n=2 to 5. One way ANOVA *$p<0.05$, ***$p<0.0001$ and Bonferroni's post-test.

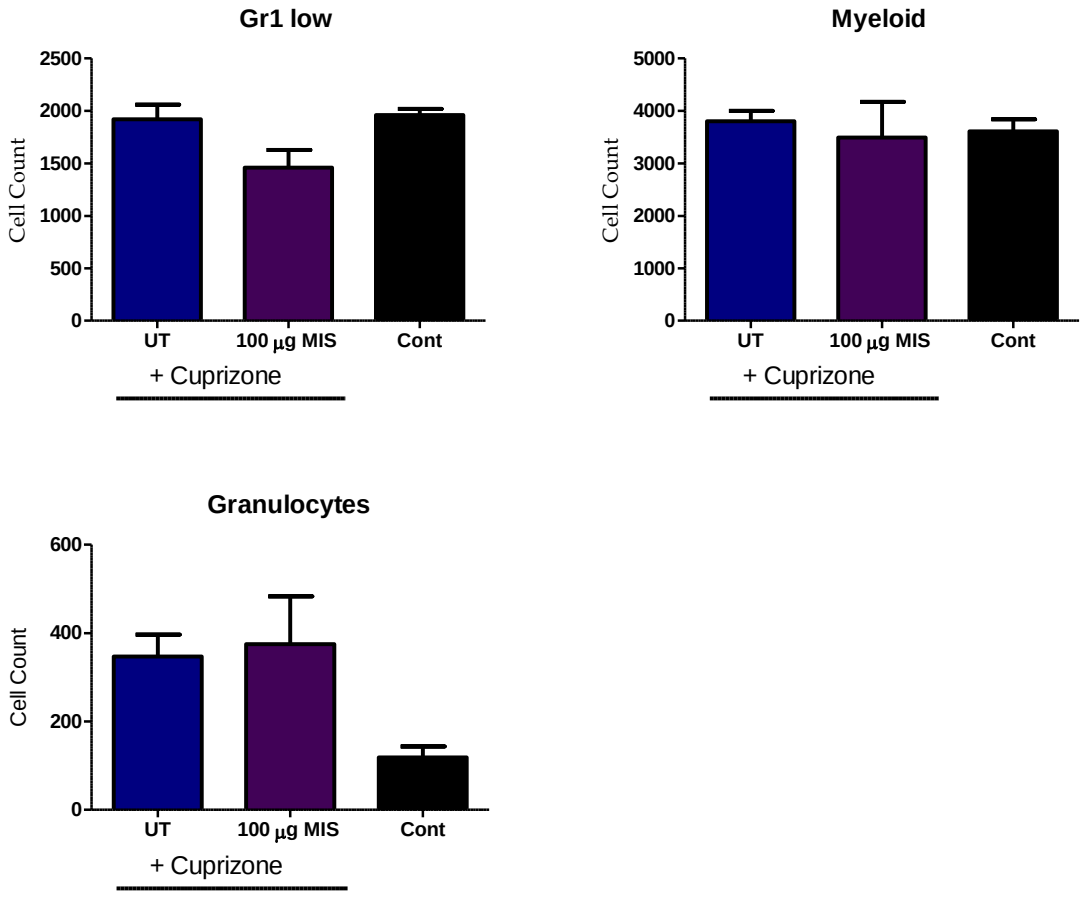

Figure 4.23: MIS416 did not cause changes to peripheral cell populations.
After 6 weeks of a cuprizone diet. Mice were culled and single cell suspensions were prepared for flow cytometry from the spleen. Cells were analysed using flow cytometry and gated into Gr1 low cells, myeloid cells and granulocytes. Changes were not significant due to low sample numbers, shown are mean and SEM with n=2-5 per group.

4.2.14 MIS416 altered MIS416 stimulated production of cytokines by splenocytes

As previous work from our laboratory has shown, red pulp macrophages are key responders to MIS416**.** Once activated by MIS416, macrophages and T cells will increase levels of activation markers and produce cytokines in response to a stimulus. To determine the effect of MIS416 treatment on the cytokine profile of splenocytes, a splenocyte restimulation assay was performed. At the conclusion of the 6 week demyelination experiment, splenocytes were isolated and restimulated with LPS, known to activate macrophages, Con-A, a T cell mitogen and MIS416. Cytokines of interest after restimulation included TNF-α, IL-12 and IFN-γ.

Splenocytes from MIS416-treated mice, restimulated with ConA produced significantly higher levels of IFN-γ and IL-12 relative to untreated control mice and in cuprizone-treated animals, IL-12 but not IFN-γ was enhanced (Figure 4.24). When restimulated with MIS416, splenocytes from MIS416-treated mice produced significantly higher levels of IFN-γ and TNF-α but not IL-12 (Figure 4.24) and in the presence of cuprizone all cytokines were elevated by MIS416 treatment with the greatest effect found on TNF-α and IL-12 (Figure 4.24). The response to restimulation with LPS was similar amongst all treatment groups. These data indicate that treatment with MIS416 primes splenocytes to respond differently to restimulation, relative to cuprizone mice which is determined by their altered cytokine profile.

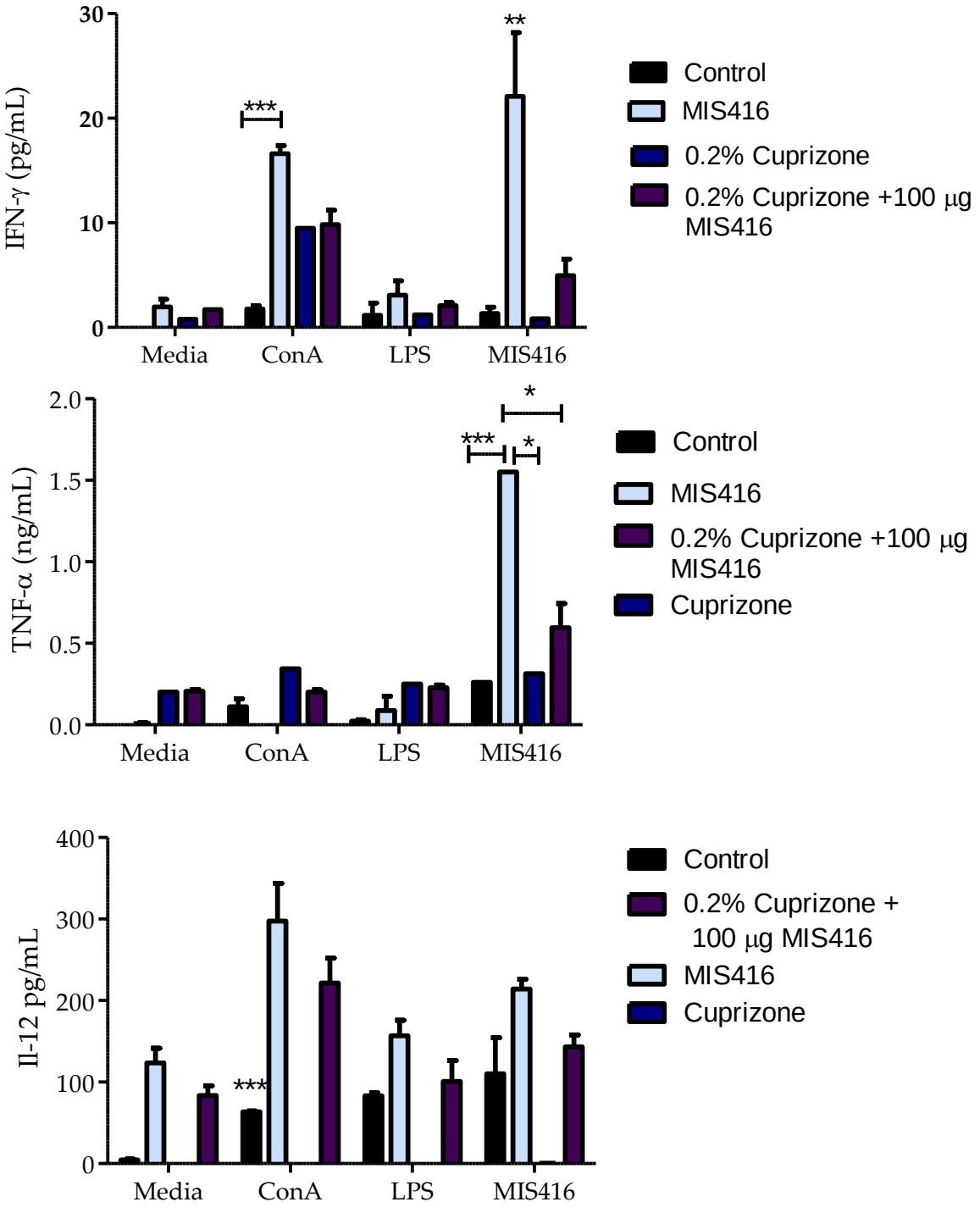

Figure 4.24: MIS416 enhanced MIS416 stimulated production of TNF-α and IFN-γ.
After cull, single cell suspensions were produced and plated at 10^6 cells per well. Cultures were treated with either media, ConA, LPS or MIS416 for 48 hours and then supernatant was harvested. ELISA's were then carried out on supernatant to determine cytokine levels. Two way ANOVA *p<0.05, **p<0.01, ***p<0.001 with Bonferroni's post-test. Stimulation was performed in triplicate.

4.3 Discussion

Experiments carried out in this chapter aimed to identify whether clozapine or MIS416 could prevent demyelination in the cuprizone model, or provide some therapeutic effect by modulating the immune environment. Previous work from our lab has shown both of these compounds to provide protection in the EAE model. Neither clozapine nor MIS416 was able to prevent demyelination in this experimental set up, and astrogliosis was not altered. MIS416 treatment caused similar peripheral immune cell changes to those seen previously by our lab in the EAE model.

Administration of cuprizone to C57BL/6 mice has been shown previously to cause a consistent demyelination of the corpus callosum when administered daily over a 6 week period (Skripuletz et al., 2008; Torkildsen et al., 2008). Having a distinct and reproducible region of demyelination has allowed for the assessment of clozapine and MIS416 as potential therapies to prevent demyelination from occurring in the cuprizone model.

Using luxol fast blue as a means to stain for myelin provides rapid and easily interpretable data about the levels of myelin present in the selected region. As seen in the results section of this chapter, both clozapine and MIS416 treatment failed to prevent demyelination at either a 0.3% cuprizone diet or a lower 0.2% cuprizone diet.

The process of demyelination using the cuprizone model is a gradual process with myelin loss beginning to occur at 2-3 weeks after inclusion of cuprizone into the diet. Maintenance of the cuprizone diet until the 6 week time point has been identified as causing the most complete demyelination of the corpus callosum (Mix et al., 2010; Wang et al., 2013). Behavioural tests were also used while the experiments were ongoing to provide extra information. When neurons lose their protective myelin coat, their ability to transmit signals becomes impaired (Sun et al., 2006). Using behavioural tasks which relied on motor coordination can provide information about the level of demyelination and axonal disruption occurring during the experiment, without having to

sacrifice animals at earlier time points for histology. In all cuprizone treated groups there was a decrease in performance on the rota rod relative to untreated animals and this was not improved by treatment with either clozapine or MIS416. During the experiments (i.e before sacrifice and subsequent histology) this failure to show improvement on behavioural assays after therapeutic intervention was interpreted as meaning that the demyelination was not being prevented by either of the test compounds. This could have been due to multiple explanations including the dose not being high enough, the selected level of cuprizone administered was driving demyelination too strongly or the compounds simply not having any impact on the processes involved in demyelination. To provide further information on coordination and behavioural differences, another test, the horizontal bars, will be carried out alongside the rota rod assay for future experiments. By adding in another behavioural test it is hoped that an increased sensitivity in detecting motor deficits will be possible. The horizontal bars is a rapid and reproducible assay used commonly to assess grip strength and coordination in mice.

One aim of these experiments was to investigate whether MIS416 or clozapine would push the astrocyte population into an activation state that would lessen demyelination by providing extra support to oligodendrocytes or by altering the cytokine environment into a state more favourable to preventing damage. As shown above, treatment with clozapine or MIS416 did not lead to a reduction or an increase of astrocyte activation relative to an untreated cuprizone fed animal. Astrocytes were clearly activated when compared to a healthy control, but due to the dual role reported for astrocytes in disease it is difficult to define this activation as being protective or detrimental to the underlying neuronal structures (Miljković et al., 2011). The observed astrogliosis could be functioning to recruit microglia to clear debris and promote an environment conducive to repair, similar to the effect reported by Skripuletz et al or could be producing high levels of TNF-α and MMP which promote inflammation and induce breakdown of the BBB to allow inflammatory cell invasion (Nair et al., 2008). The latter however is unlikely in this case as invading cells around blood vessels were not seen with nuclear staining.

As demyelination was occurring with these experiments, by extension the mature oligodendrocyte population was also being killed or severely damaged, as the myelin is produced and supported by mature oligodendrocytes (Keirstead, Levine, & Blakemore, 1998; Miron et al., 2011). Future experiments will look into identifying the oligodendrocyte populations, both mature and immature, and will be discussed in a later chapter.

In secondary progressive MS, patients present with lesions with a different phenotype when compared to the inflammatory lesions seen in relapsing remitting MS. The lesions in SP-MS can be characterised by a lack of immune infiltrate as well as death of mature oligodendrocytes (Lucchinetti et al., 2000). Since the oligodendrocyte primarily functions to produce myelin sheath to support and protect neurons, any therapy which protects this cell population could be used in a therapy for preventing demyelination. The cuprizone model for demyelination selectively targets the mature oligodendrocyte population (mechanism still debated- see introduction) and is therefore an ideal model to identify any treatment able to prevent demyelination.

As no effects of MIS416 were seen on demyelination or astrogliosis, we investigated whether MIS416 was having the same immunomodulatory effects in the cuprizone model as it does in EAE and healthy animals. When MIS416 is administered, one of the key peripheral effects identified is an enlarged spleen. This has been shown in previous work from our group (White et al., 2014) using the EAE model and in healthy controls, and was again seen with the cuprizone model. Work using the EAE model identified macrophages as a key population of cells responding to MIS416 treatment through their TLR-9 and NOD2 receptors, and the red pulp macrophage population of the spleen was shown to increase in proportion of total spleen cells and increase in number as well (White et al., 2014). Similar results were seen in this study, with MIS416 treatment leading to an increase in overall spleen size, however this did not reach significance due to initial small sample numbers. When broken down by cell type using flow cytometry gating strategies to pull out specific populations, MIS416, was shown to significantly reduce the number and percentage of NK1.1+

and Treg cells in the context of cuprizone intoxication. While MIS416 has been shown to reduce NK cell previously, Tregs have been found to be expanded by MIS416 treatment of healthy and EAE mice. Because these analyses did not include an MIS416 only treatment group, it is difficult to determine if that lack of Treg expansion is unique to MIS416 treatment during cuprizone intoxication or if it is a consequence of the long term MIS416 administration used in this model.

4.4 Summary

This chapter has shown that in a 0.2% and 0.3% cuprizone model, administration of 60 mg/kg of clozapine does not prevent demyelination, as measured by luxol fast blue staining. Clozapine also had no significant effect on the level of astrogliosis. Similarly, MIS416 did not prevent demyelination in the 0.2% cuprizone diet, nor did it prevent astrogliosis. These experiments have shown that clozapine or MIS416 are unable to provide protection from cuprizone induced demyelination. Demyelination however is not occurring in isolation, remyelination is also occurring at the same time, so the ability of clozapine or MIS416 to alter remyelination also must be assessed.

CHAPTER 5: Effect of clozapine and MIS416 on remyelination

5 Introduction

Lesion formation in multiple sclerosis is a dynamic process, with multiple events occurring at the same time in a very precise location. While the previous chapter looked at events surrounding demyelination of the active lesions, the current chapter will focus on events surrounding remyelination, a process that is occurring spontaneously and concurrently with demyelination.

When evaluating old lesions histologically, areas of remyelination can often be identified as this is a process that will occur naturally. These regions of remyelination within an active lesion are called shadow plaques, and stain differently to 'older' myelin using luxol fast blue. This new myelin sheath is much thinner when it is first produced, showing up as light blue patches. This endogenous process of remyelination occurs spontaneously in patients, however as disease develops the ability to remyelinate in active lesions is often decreased or lost all together.

A healthy pool of oligodendrocyte progenitor cells and a mature oligodendrocyte population are necessary for the production of the myelin sheath; however activity of this cell population alone is not enough. A study by Neumann and colleagues (Neumann, Kotter, & Franklin, 2009) showed that remyelination is unable to occur efficiently unless microglia and macrophages are activated to phagocytose and remove the debris during the demyelination period. Li et al (Li, Setzu, Zhao, & Franklin, 2005) used minocycline, a compound commonly used to inhibit microglial populations, to investigate the link between microglial activation and remyelination. When treated with minocycline, female rats had significantly lower levels of remyelination than PBS-treated controls. They further examined whether the decrease in remyelination was due only to the interaction between microglia and oligodendrocytes or whether there was a direct effect of minocycline on mature oligodendrocyte populations as well. It was found that treatment with minocycline reduced the oligodendrocyte (OPC) population directly following demyelination, and that in an oligodendrocyte culture, treatment with minocycline reduced proliferation in a dose dependent manner, as measured by BrdU incorporation

Spontaneous remyelination occurring in some MS lesions does occur, and has been correlated with the number of oligodendrocytes at the lesion site (Hans Lassmann, Brück, Lucchinetti, & Rodriguez, 1997). On the other hand, some MS patients, particularly those with the progressive forms of disease do not show this spontaneous remyelination. Since the repair of old and newly forming lesions has been shown to be decreased in some MS patients, targeting the remyelination process provides another potential therapeutic route. As current treatments target the immune pathways involved in MS, a therapy that targets remyelination could be used in combination with the current immune-mediated therapies to provide a powerful therapy for MS patients, especially those with progressive forms of MS, where the ability to remyelinate lesions has almost been lost entirely (Hans Lassmann et al., 1997).

In progressive MS, lesions differ from relapsing-remitting MS and can be characterised by oligodendrocyte death and the lack of the immune infiltrate that is associated with lesions in RR-MS patients (Lucchinetti et al., 2000). Multiple sclerosis therapies utilising the endogenous remyelination seen in normal tissue and targeting the oligodendrocyte population are now becoming a highly discussed area as alternative therapies for progressive forms of MS. A review published by Zhang et al (J. Zhang et al., 2011) looked at the targeting of oligodendrocytes and remyelination in multiple sclerosis, highlighting the amount of research currently occurring in this area. Several molecules including neurotrophins, insulin-like growth factor and ciliary neurotrophic factor have been shown to have protective effects on both the differentiation and maturation of oligodendrocytes into mature oligodendrocytes capable of producing structurally sound myelin. Researchers are also identifying molecules involved in the prevention of OPC development and attempting to identify ways to target these in an attempt to promote remyelination (Chamberlain, Nanescu, Psachoulia, & Huang, 2016).

MIS416 is currently in a phase 2b trial for patients with secondary progressive MS (SP-MS). As SP-MS is associated with impaired remyelination, the effect of MIS416 on events surrounding remyelination are of interest. Previous work has shown that MIS416 alters the cytokine

environment, potentially creating an environment more suitable for repair and regeneration (White, O'Sullivan, Webster, & LaFlamme, 2011; White et al., 2014). Analysis of patient serum samples following a phase 2a clinical trial with MIS416 showed a transient increase in serum IL-10 levels, an anti-inflammatory cytokine, directly following administration of MIS416 (Webster, Sim, La Flamme, & Mayo, 2016). Since IL-10 and other anti-inflammatory mediators were able to be detected in measureable quantities in the serum, it raises the question of whether these molecules are able to access the CNS and provide an effect on inflammatory pathways at lesion sites. Importantly, it was also shown that administration of MIS416 did not lead to systemic inflammation or activation of old lesions, and preliminary studies demonstrated that MIS416 treatment induces licencing factors that correlate with enhanced CNS non-inflammatory myeloid cell trafficking and recruitment of MIS416+ myeloid cells (White, 2015).

In contrast to MIS416, which is known to harness the peripheral innate immune system and modify CNS inflammation, clozapine is directly CNS active and alters neuroinflammation. Although the results from the previous chapter indicate that clozapine did not prevent demyelination, given its known effects on microglia, it has the potential to alter remyelination events in this model.

5.1 Aims

Given that previous studies indicate that both clozapine and MIS416 have the potential ability to regulate oligodendrocyte survival in the cuprizone model and given the functional role of oligodendrocytes along with the support of microglia and astrocytes in remyelinating axons, the overall aim of this chapter was to investigate whether clozapine or MIS416 promoted remyelination in the cuprizone model. To assess remyelination and recovery, we exposed mice to cuprizone for 6 weeks, before removing cuprizone from the diet and treating orally with clozapine or i.v with MIS416.

5.1.1 Specific Aims

1. Investigate the effects of clozapine and MIS416 on motor coordination using rota-rod and horizontal bar behavioural tests.

2. Identify the effect of clozapine and MIS416 administration on remyelination after removal of cuprizone from the diet

3. Determine the effect of clozapine and MIS416 on CNS cell populations

4. Characterise any peripheral changes to immune cell populations occurring after MIS416 administration

5.2 Results

5.2.1 Clozapine did not alter cuprizone-induced weight loss

As in the previous chapter, 6-8 week old female C57Bl/6 mice were fed a diet containing 0.3% cuprizone mixed into ground chow. As expected, mice receiving a diet of 0.3% cuprizone rapidly lost weight over the first 10-12 days before gradually maintaining a stable, lower weight. Control animals (untreated, healthy mice) gained weight at a steady rate throughout the experiment. After 6 weeks of receiving a cuprizone-containing diet, cuprizone was removed from the feed and animals were returned to a normal, cuprizone-free diet to model the recovery phase (Figure 5.1A). As expected, the return to a normal diet led to rapid weight gain in all animals. None of the cuprizone-fed animals reached the weight of the control animals; however, the weight difference decreased from an average of 5 g less to only 1.5 g.

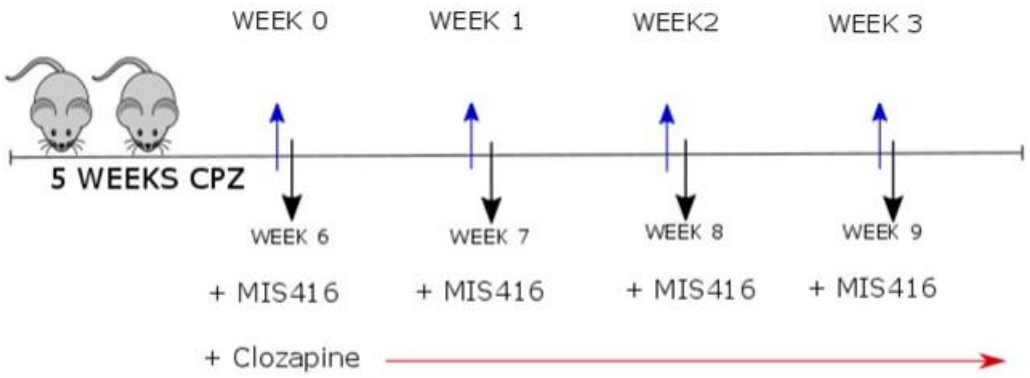

Figure 5.1: Schematic representation of experimental timeline and treatment administration
Mice were continuously fed cuprizone for 5 weeks, after which 60 mg/kg clozapine treatment in the drinking water was administered or 100 μg MIS416 was administered i.v weekly. Cuprizone was removed from the diet after 6 weeks.

5.2.2 Clozapine altered rota-rod performance during recovery period

Figure 1 shows a schematic of how treatment and behavioural assays fit together. Briefly, after 5 weeks of a cuprizone diet, the first rota-rod assay was performed 1 day prior to the start of the 6th week. The next day, at the start of the 6th week, clozapine or its vehicle was introduced into the drinking water of selected animals, and at the end of the 6th week, cuprizone was removed from the diet. This set up allowed treatment to begin during the disease course to more closely mimic a therapeutic setting.

As shown in Figure 2b, five weeks of cuprizone intoxication significantly reduced performance of mice compared to healthy controls, which achieved between 100 and 120 seconds consistently, and removal of cuprizone lead to a progressive improvement over the recovery period (Figure 2b). As in Chapter 4, no difference in the performance was found in cuprizone-fed animals treated with the clozapine vehicle or left untreated and so these groups were combined as a cuprizone control group. Treatment with clozapine lead to a significant improvement in motor coordination, which is most evident at the one week time point, compared to the cuprizone control group.

As an additional way to detect any behavioural improvement associated with clozapine treatment, data were analysed to determine the week in which each individual animal achieved its peak performance time on the rota-rod. This week (0 through 3) was then assigned as the week which the animal 'recovered'. As shown (Figure 5.2c) clozapine-treated, cuprizone animals recovered significantly earlier (weeks 1-2) than untreated cuprizone animals (weeks 2-3). Control healthy animals were scored as recovering in week 0 or 1, as most animals in this group performed slightly better in the second week of testing, despite having had no therapeutic intervention or manipulation.

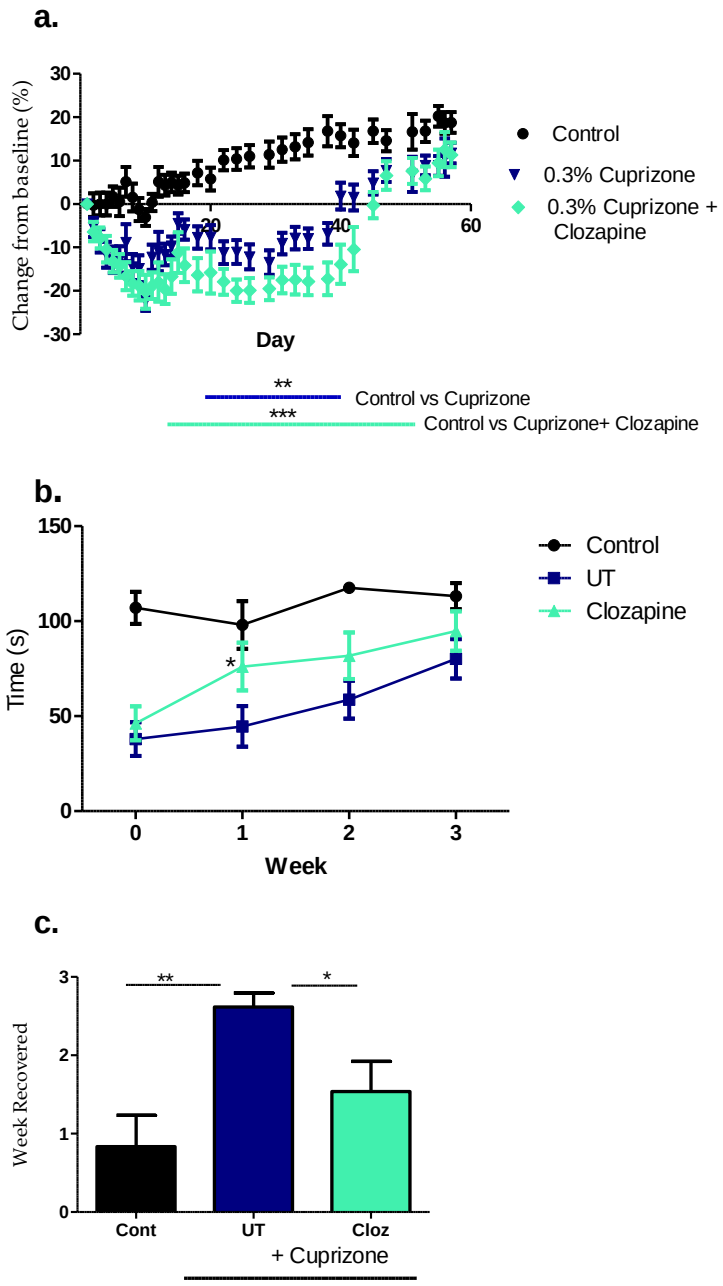

Figure 5.2: Clozapine treatment did not prevent cuprizone-induced weight loss but improved motor deficits. (a) Mice were fed 0.3% cuprizone and UT (untreated) or treated with clozapine or clozapine vehicle. Cuprizone led to a significant reduction in weight **p<0.01,*** p<0.001 by two way ANOVA with Bonferroni's multiple comparison post-test. Motor coordination was assessed weekly using the rota-rod at 28 rpm (b) and week recovery was assigned based on maximum performance reached; a score of 3 means animals did not 'recover'(c). *p<0.05 with one way ANOVA and Bonferroni's multiple comparison test. Shown are means with SEM for 3 individual experiments with n=6-15 per group.

5.2.3 Cuprizone impaired horizontal bar performance

In addition to the rota-rod assay, a second behavioural assay was introduced for these experiments. The horizontal bar assay is a rapid way to assess grip strength and coordination. Mice are placed in the middle of a suspended rod, using their front paws only to grip. The time taken for the mouse to grip onto the bar with its hind limbs/tail and reach either end of the suspended bar is recorded. A shorter time is therefore associated with an improved grip strength and coordination. A diagram of the experimental set up can be seen below (Figure 3).

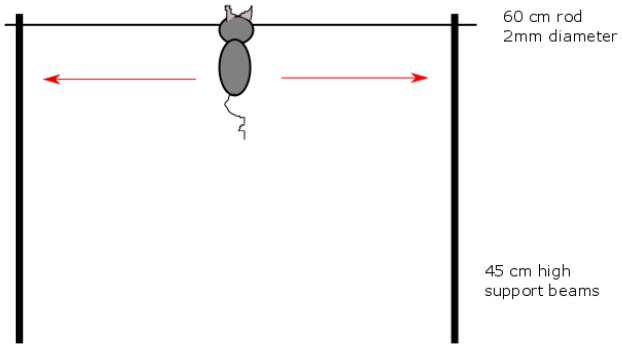

Figure 5.3: Diagram of horizontal bar set up.
Mice were placed on the middle of the raised bar suspended by their front paws. Time taken to reach either end was recorded.

At all time-points assessed the healthy animals performed best, with an average time of 11 seconds taken to reach either end of the set up. In all treatment groups receiving cuprizone, the time taken to complete the task was increased, with some animals failing to complete the task and being assigned a maximum score of 30 seconds. Over the course of the experiments the clozapine-treated groups showed a trend towards an increased performance. Significance was not reached

between any of the treatment groups at any of the time points, possibly due to low animal numbers. (Figure 5.4). When viewed in a graph over time the rate of improvement (Figure 5.5) is similar between all groups, with the control group showing the least improvement.

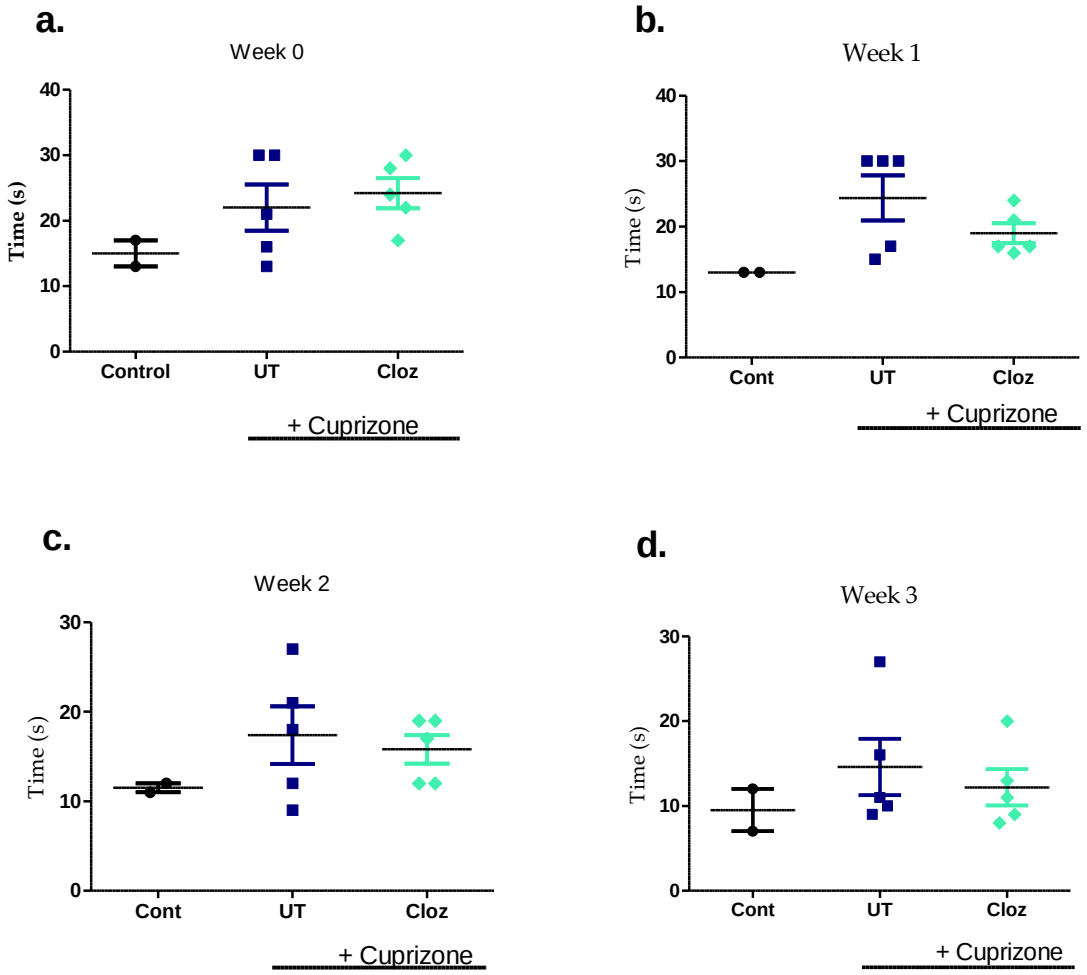

Figure 5.4: Clozapine did not improve performance on horizontal bar assay
Mice were placed on the horizontal bar and the time taken to reach either end was recorded. Performance for individual animals at each time point are shown. Shown are the means and SEM for an individual experiment with n=2-5. Significance was not reached.

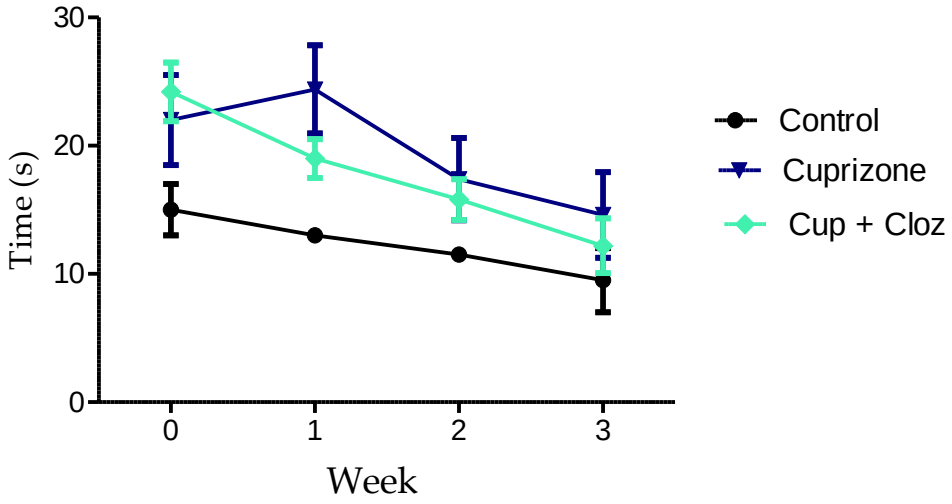

Figure 5.5: Horizontal bar progression over time.
Mice were placed on the horizontal bar and the time taken to reach either end was recorded. Shown are means with SEM of one experiment, n=2-5.

5.2.4 Clozapine treatment did not alter remyelination or astrocyte activation

As in previous experiments in chapter 3 and 4, 5-7 µm sections were collected and stained for myelin using luxol fast blue. Representative images of test groups can be seen in Figure 6 below.

These sections were randomised and presented to a group of individuals blinded to the treatment groups and were scored based on the level of myelin in the corpus callosum, with 1 being low levels of myelin and 3 being high levels of myelin present. Control sections were consistently scored as having a high level of myelin as expected and contained significantly more myelin in the corpus callosum than any other test group (Figure 5.6a). There was no difference in the level of myelin in the corpus callosum between the untreated, cuprizone-fed or the clozapine-treated, cuprizone-fed group with both receiving similar myelin scores (Figure 5.7a).

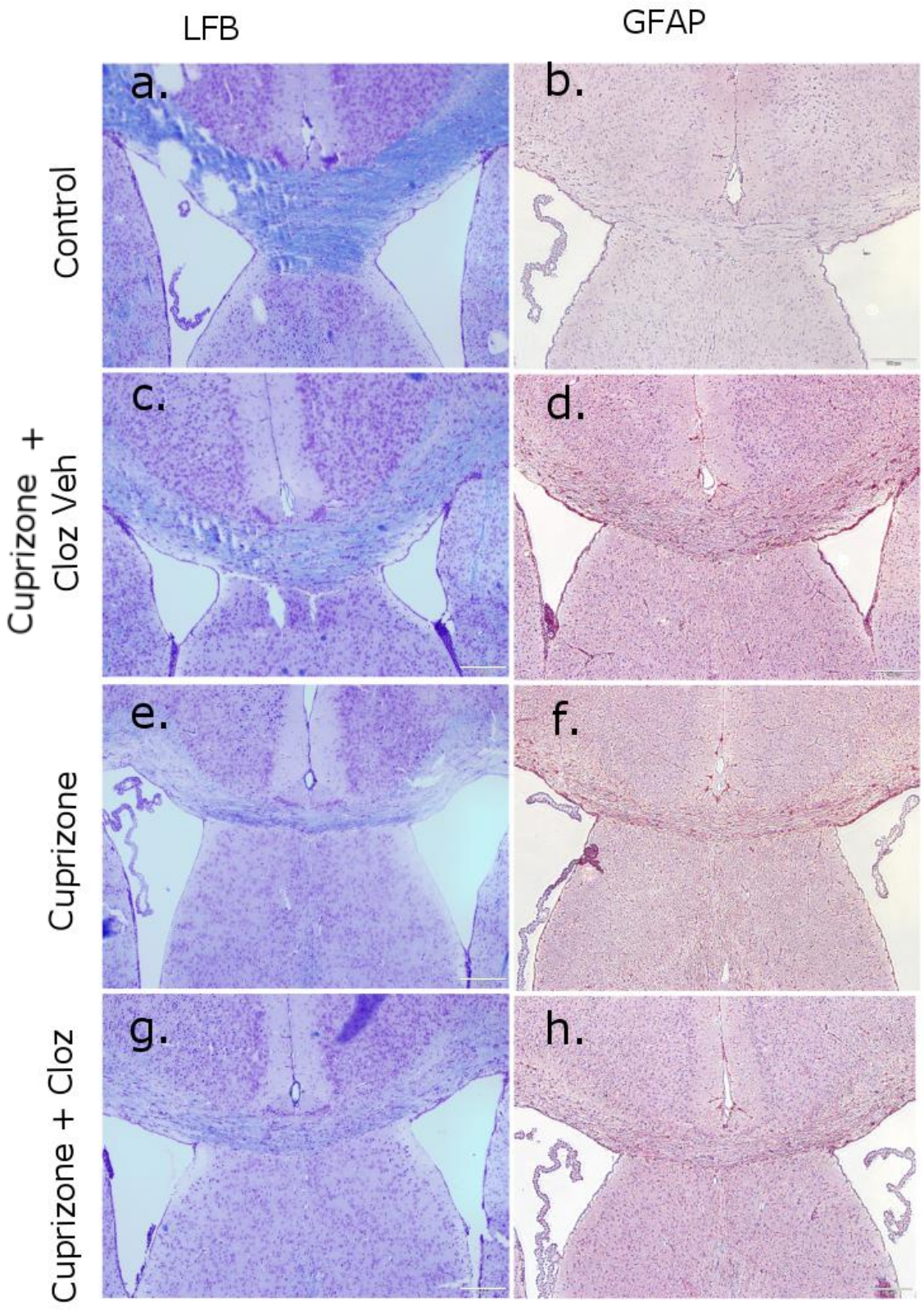

Figure 5.6: Representative images of remyelination after cuprizone intoxication and clozapine treatment. 5-7 µm sections were stained with luxol fast blue and cresyl violet to stain for myelin, or GFAP for astrocytes. Representative images are shown, scale bar = 100 µm. Clozapine vehicle group were also fed 0.3% cuprizone.

As well as staining for myelin, consecutive sections were stained for astrocytes, one of the most abundant cells in the CNS. Sections from control animals showed significantly lower numbers of astrocytes than untreated, cuprizone-fed animals, indicating control animals had a significantly lower level of astrocyte activation (Figure 5.6b and 5.7b), as GFAP is upregulated on activated astrocytes. Clozapine-treated, cuprizone-fed animals also showed significantly increased astrocyte activation compared to control animals (Figure 5.7b), but there was no significant difference detected between the untreated, cuprizone-fed animals and the clozapine-treated, cuprizone-fed animals (Figure 5.7b).

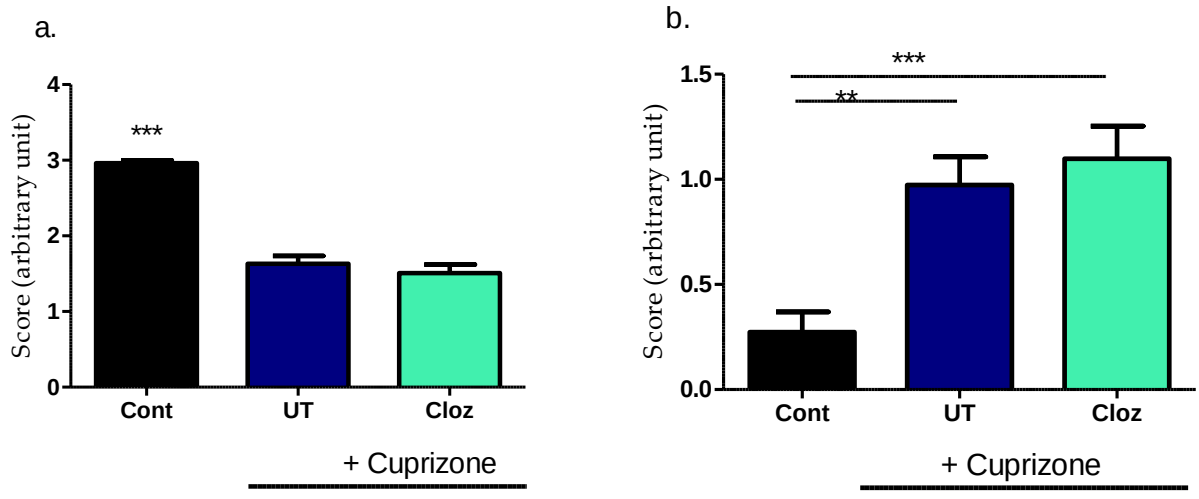

Figure 5.7: Sections stained for myelin and astrocytes were scored by individuals blinded to treatments. Sections were scored by individuals blinded to their treatment groups using a scale from 1 (low myelin/low astrocyte reactivity) to 3 (high levels of myelin/high astrocyte reactivity) in the corpus callosum with (a) showing myelin and (b) showing astrocyte scores. n=4-12, one way ANOVA **p<0.01, ***p<0.0001 with Bonferroni's post-test.

Taken together these results suggest that although clozapine improved performance in behavioural assays, it does not lead to an enhancement of remyelination in the corpus callosum or reduction in astrocyte activation following 6 weeks of cuprizone induced demyelination and 2 weeks of 'recovery'.

5.2.5 MIS416 did not prevent cuprizone induced weight loss or behavioural deficits

To determine the effect of MIS416 administration on cuprizone-fed animals, animals were injected with 100 μg or 200 μg of MIS416 i.v weekly. Animals were weighed and observed daily to monitor any signs of distress. All animals receiving cuprizone in their feed lost weight at the onset of the experiment. MIS416-treated, cuprizone-fed animals lost a similar percentage of body weight as untreated, cuprizone-fed animals (Figure 5.8a), with MIS416 treatment alone having no effect on body weight.

Mice were tested on the rota-rod at weekly time points, on the day prior to injection. This was assessed as a percent improvement from initial performance and over time (Figure 5.8b). Animals in the control group and MIS416 alone group did not show improvement as they performed optimally from week 1. All cuprizone-fed groups showed increased performance over time, however no difference was seen between the 100 μg MIS416-treated, cuprizone-fed mice and the untreated, cuprizone-fed mice. Mice treated with 200 μg MIS416 showed a slightly greater improvement at week 2; however this difference did not reach significance. The week which mice achieved their maximum performance time on the rotarod was recorded as the week recovered (Figure 5.8 c). Both control and MIS416 alone groups recovered between week 0 and 1 as they achieved their maximum performance at baseline or after the first week. This was significantly different from all the cuprizone-fed groups, which did not recover until after week 2 or 3. There was no difference in week recovery seen between MIS416-treated, cuprizone-fed animals and untreated, cuprizone-fed animals.

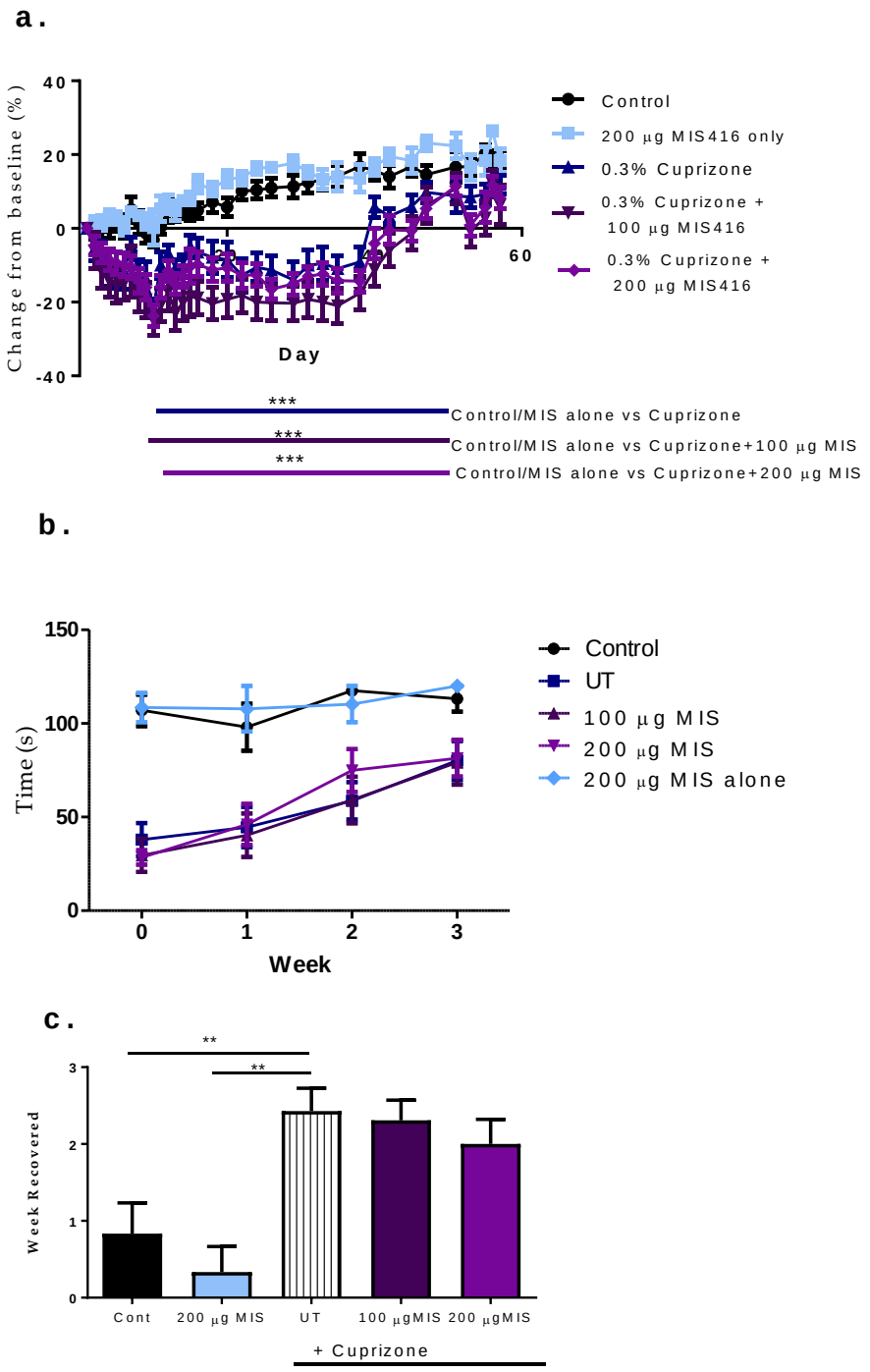

Figure 5.8: MIS416 did not prevent cuprizone induced weight loss or motor deficits.
Mice were fed a diet containing 0.3% cuprizone and treated with MIS416 i.v during cuprizone feeding. Weights were recorded (a) and a rota-rod assay was conducted weekly to assess coordination (b). Week of maximum rota-rod performance was recorded as week recovered (c). Data shown are mean and SEM for 3 individual experiments with n=4-10. ***p<0.0001, **p<0.01 with one way ANOVA and Bonferroni's post-test.

The horizontal bar assay was used alongside the rota-rod assay to assess grip strength and fine motor control (Figure 5.9). Control animals and animals receiving MIS416 alone consistently had the shortest times, indicating the best performance of the task. All groups receiving cuprizone in the feed had an increased performance time, with some animals failing to complete the task in the 30 second time limit. There was no significant difference between MIS416-treated, cuprizone-fed animals or untreated, cuprizone-fed animals. At the end of week 2 and 3 there was a slight increase in MIS416-treated, cuprizone-fed animals (Figure 5.9c and d) relative to untreated cuprizone animals, which was the same pattern seen in the rota-rod assay.

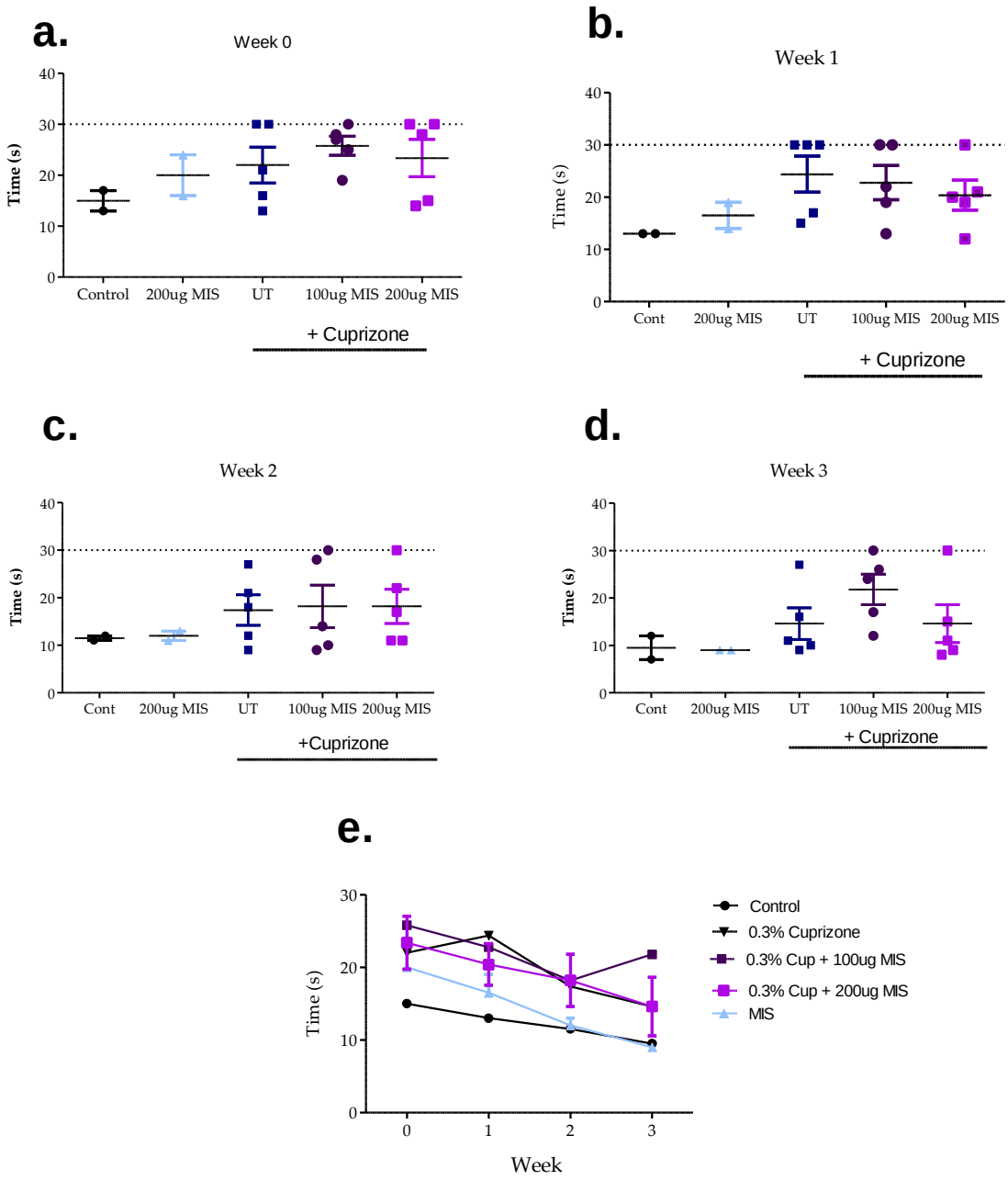

Figure 5.9: Cuprizone administration impaired performance on the horizontal bar assay.
Grip strength and fine motor control was measured weekly using the horizontal bar assay. Time taken to reach either end of the set up was recorded, with a maximum performance time of 30 seconds. (a)-(d) are means and SEM n= 2-5. (e) shows performance over time.

5.2.6 MIS416 treatment did not improve remyelination rates or cuprizone-induced astrogliosis

To assess the effect of MIS416 administration on cuprizone-fed animals, sections were stained for myelin with luxol fast blue and for astrocytes with an anti-GFAP antibody. Representative images are shown in Figure 5.10, with scores from blinded individuals in Figure 5.11.

Control animals and MIS416 alone-treated animals received maximum scores for the level of myelin in the corpus callosum as no demyelination was induced (Figure 5.11). MIS416-treated, cuprizone-fed animals and untreated, cuprizone-fed animals received significantly lower remyelination scores than control animals, indicating that remyelination had not occurred to the extent of a healthy control. There was no difference between MIS416-treated, cuprizone-fed animals and untreated, cuprizone-fed animals indicating that MIS416 did not enhance the rate of remyelination after cuprizone-induced demyelination. Cuprizone caused an increase in astrogliosis, which MIS416 treatment was unable to alter significantly (Figure 5.11 b).

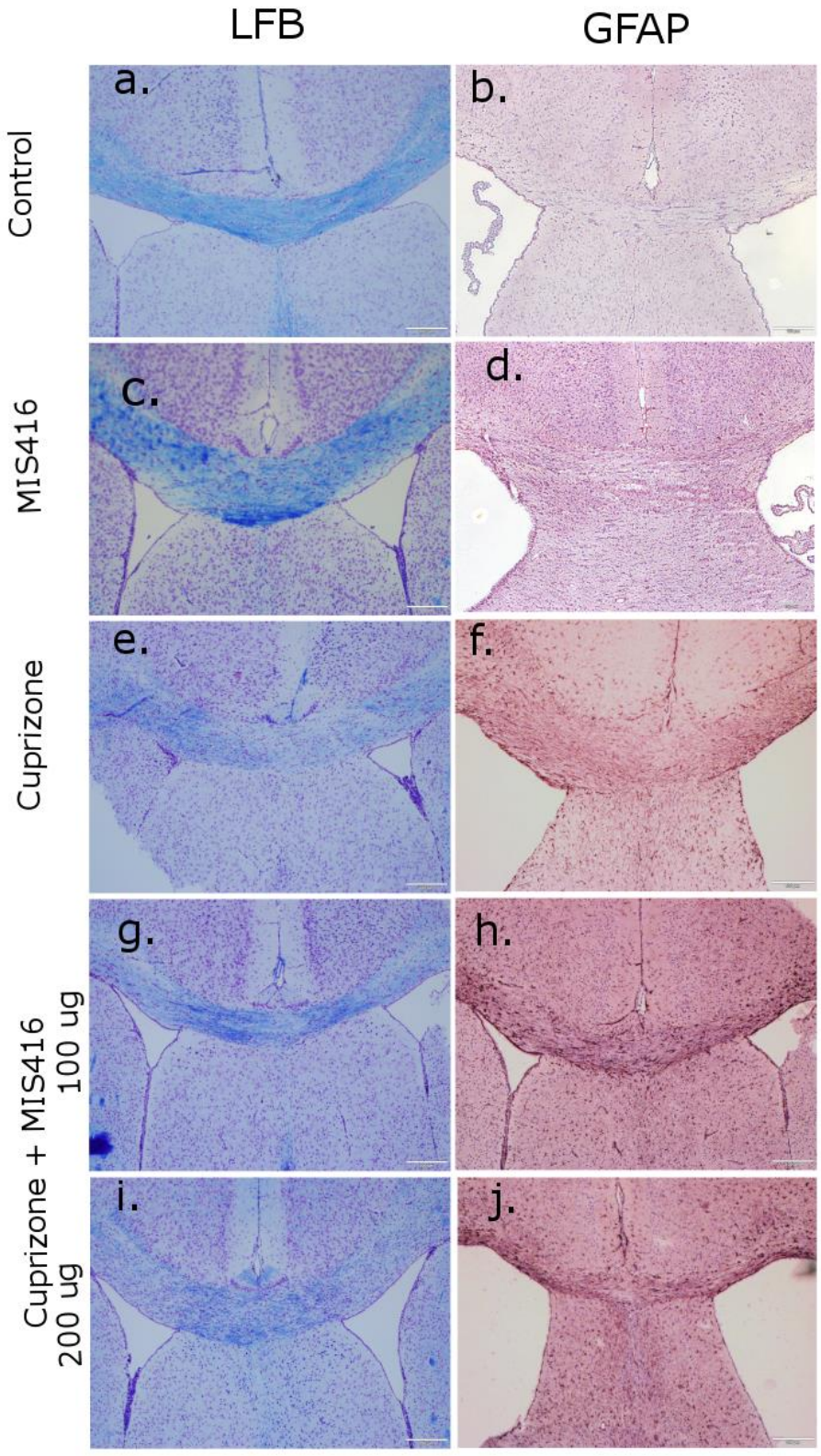

Figure 5.10: Representative image of myelin stained sections.
5-7 µm sections were stained with luxol fast blue and cresyl violet to stain for myelin, or GFAP for astrocytes. Representative images are shown, scale bar = 100 µm.

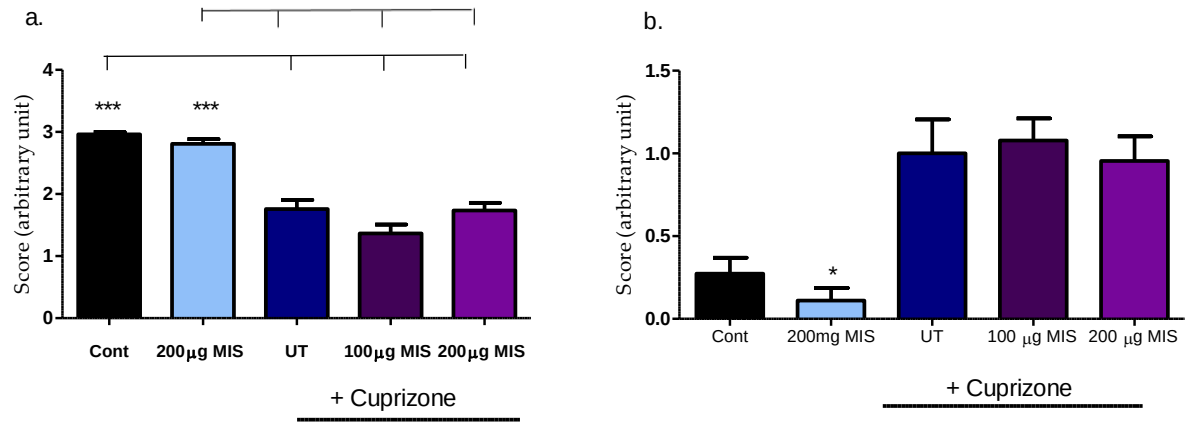

Figure 5.11: MIS416 treatment did not cause significant remyelination or alter astrogliosis.
Sections were scored by individuals blinded to their treatment groups using a scale from 1 (low myelin/low astrocyte reactivity) to 3 (high levels of myelin/high astrocyte reactivity) in the corpus callosum with (a) showing myelin scores and (b) showing astrocyte scores. Data shown are means and SEM from 3 individual experiments with n=4-12, one way ANOVA ***p<0.001with Bonferroni's post-test *p<0.05.

5.2.7 MIS416 but not clozapine increased splenocyte populations.

As seen previously (chapter 4), treatment with MIS416 led to a trend towards an increase in splenocyte populations (Figure 5.12 a). Treatment with clozapine did not significantly alter splenocyte counts as expected (Figure 5.12 b). Spleen enlargement was visible to the naked eye after organ removal. Figure 5.13 shows example spleens from different treatment groups to give an indication of the scale of enlargement seen with MIS416, an effect which has been previously detected.

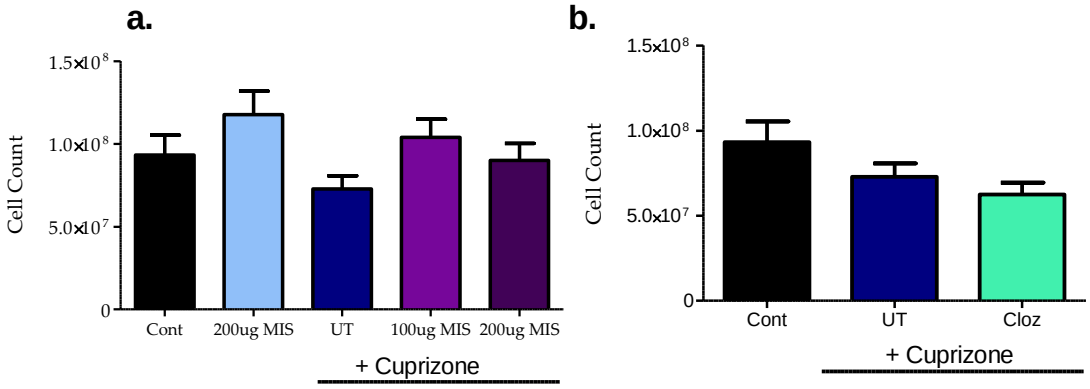

Figure 5.12: Treatment with MIS416 caused an increase in splenocyte populations
Single cell suspensions were created from isolated spleens before being counted using the trypan blue exclusion method. Data shown are means and SEM from 3 individual experiments with n=4-12. Same control and UT counts were used for both graphs.

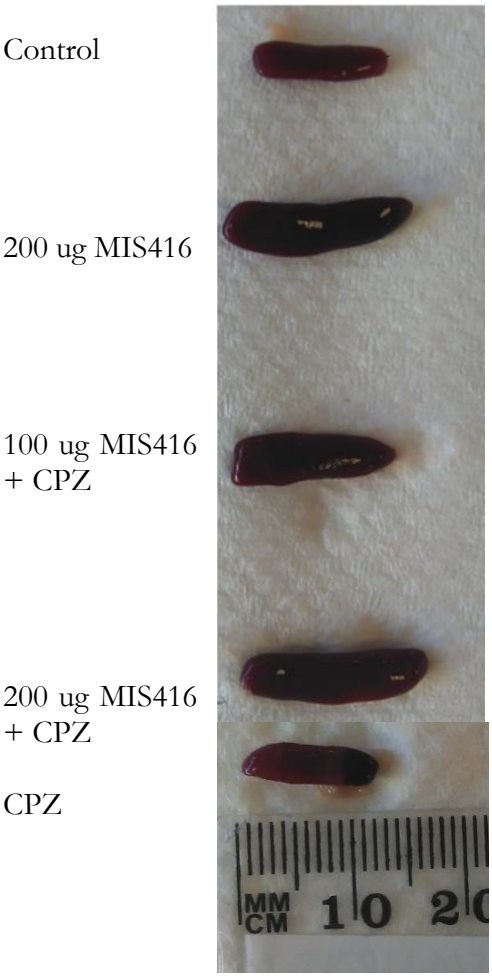

Figure 5.13: Spleen enlargement after MIS416 treatment.
Prior to passing through a single cell strainer, examples of spleens were photographed to show their increased size.

Splenocyte populations were separated based on the gating strategy shown in Figure 5.14. Treatment with MIS416 had no significant effect on the number of CD4+, CD8+ or Treg (CD4+CD25+) cells relative to healthy control or untreated, cuprizone-fed mice (Figure 5.15 a,b,c). Treatment with MIS416 alone led to a significant reduction in NK1.1+ cells compared to healthy controls. In both 100 μg and 200 μg MIS416-treated, cuprizone-fed animals, there was a significant reduction in NK1.1+ cells relative to untreated, cuprizone-fed animals indicating MIS416 treatment led to a reduction of NK1.1+ cells, and this reduction is not altered by the presence of cuprizone.

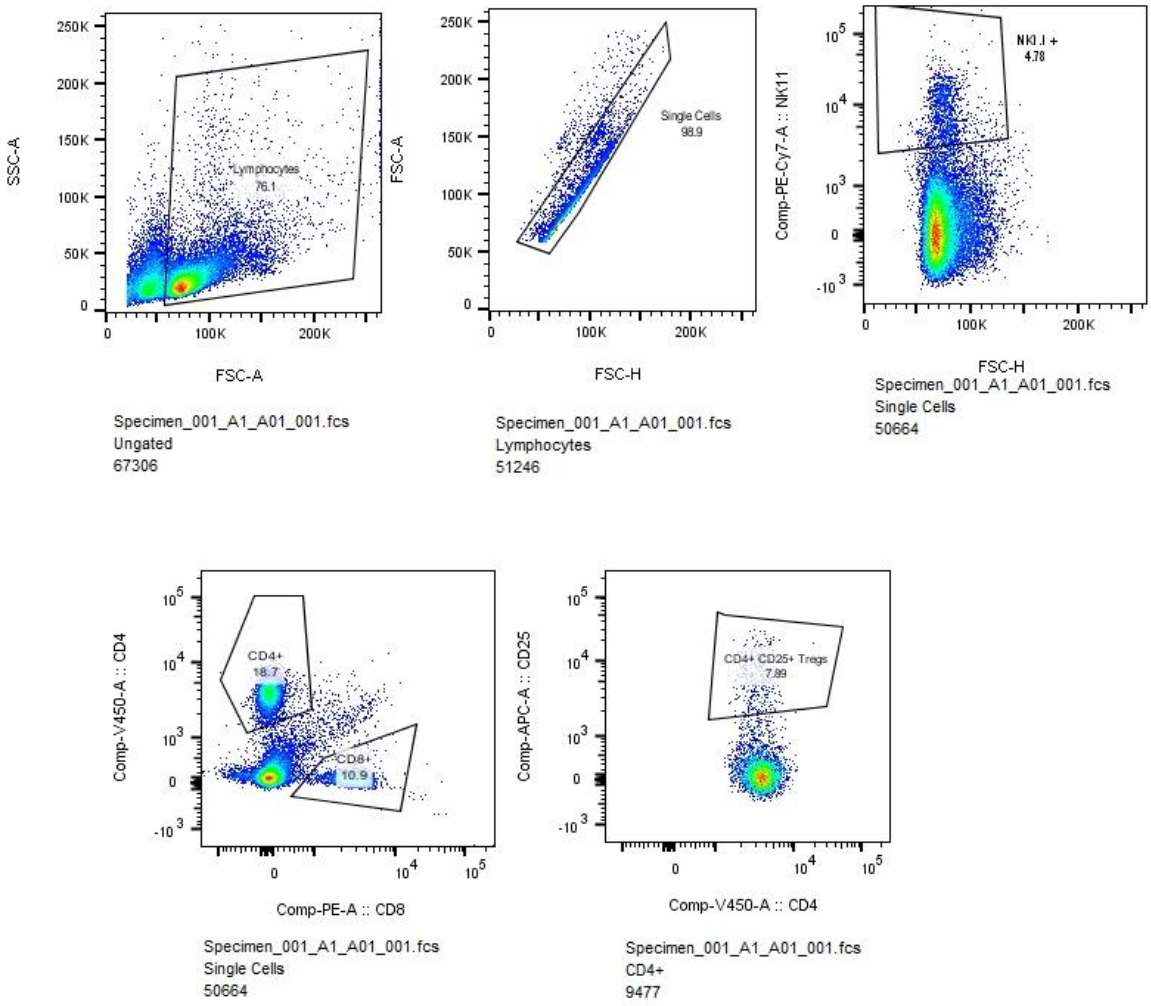

Figure 5.14: Example gating strategy for T cell populations.
Live single cells were isolated based on their SSC and FSC properties. NK cells were identified based on staining for NK1.1. CD4 cells were identified as CD4+CD8- with CD8 cells as CD8+CD4-. Tregs were identified as CD4+ CD25+ cells.

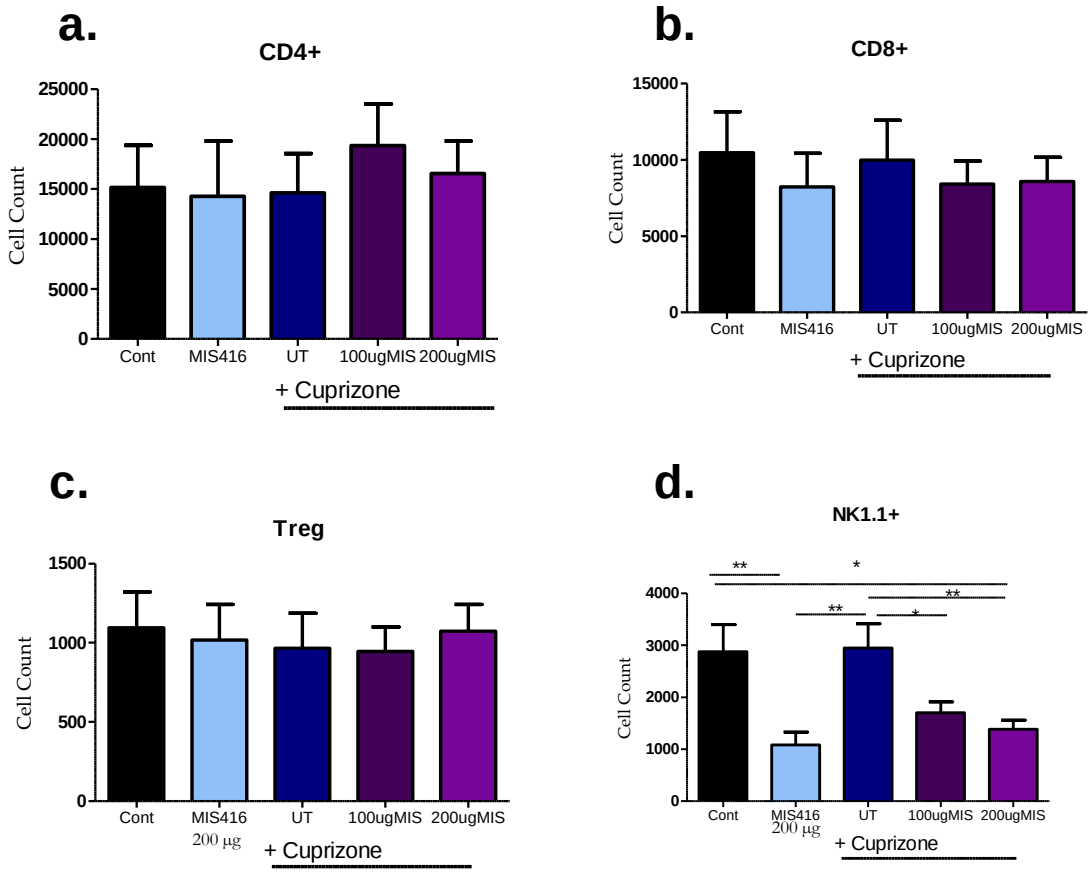

Figure 5.15: Treatment with MIS416 alters NK1.1+ splenocyte populations.
Splenocytes were identified as CD4, CD8, Treg or NK cells based on cell surface markers. Treatment with MIS416 significantly altered the number of NK cells *p<0.05, **p<0.01 with one way ANOVA and Bonferroni's post-test. Data shown are means and SEM from 3 individual experiments with n=4-12.

After treatment with MIS416, an increase in the number of neutrophils (Gr-1^{high}, Ly6G^{high}) and monocytes (CD11b+ Ly6C+) could be observed (Figure 5.16b & c). This increase was significant for all MIS416-treated groups relative to control healthy animals and untreated, cuprizone-fed animals. Moreover, treatment with 200 µg MIS416 trended towards an increased number of neutrophils and monocytes relative to 100 µg MIS416-treated animals. Treatment with cuprizone alone (UT) did not cause any changes to these populations relative to healthy control animals. Treatment with 200 µg MIS416 also caused a significant increase in the number of dendritic cells (Ly6C^{low}, CD11c+) relative to cuprizone-fed, untreated animals. Cuprizone itself did not affect the number of dendritic cells when compared to control (Figure 5.16 d). Finally, treatment with 200

μg MIS416 led to a significant increase in the number of red pulp macrophages (CD11b-, F4/80+) compared to untreated cuprizone-fed animals (Figure 5.16 a). In all other groups receiving MIS416, there was a trend towards an increase in red pulp macrophages, however significance was not reached. Cuprizone itself had no effect on the number of red pulp macrophages. Together, these results indicate that MIS416 causes distinct changes to myeloid and NK cells without significantly altering splenic T cell populations.

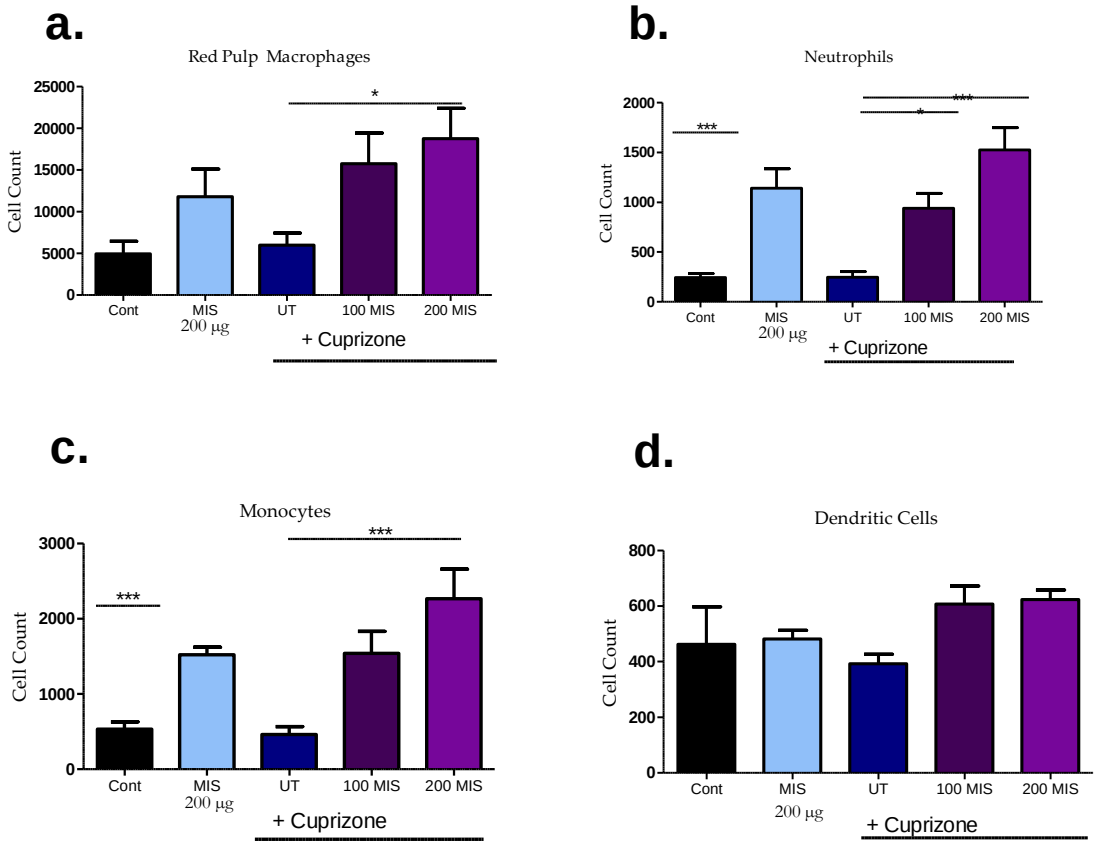

Figure 5.16: MIS416 caused significant changes to splenocyte populations
Single cell suspensions were prepared from spleens and stained with the appropriate antibodies. Treatment with MIS416 caused an expansion of the red pulp macrophage, neutrophil, monocyte and dendritic cell populations. *p<0.05, ***p<0.001 with one way ANOVA and Bonferroni's post-test. Data shown in a-c are means and SEM from 3 individual experiments with n=4-12. d is one experiment, n =2-5.

5.2.8 Clozapine but not MIS416 improved cuprizone-induced behavioural deficits at an earlier time point

As no significant changes were detected when comparing clozapine-treated or MIS416-treated, cuprizone-fed animals with untreated, cuprizone-fed animals in regards to astrogliosis or remyelination, it was decided to investigate an earlier time point. Based on the behavioural data it was decided to sacrifice animals after 2 weeks of treatment as this is where clozapine-treated and MIS416-treated animals appeared to have an improved performance.

As in the previous experiment animals were monitored daily and subjected to behavioural assays at weekly time points. The first test, termed pre-treatment was carried out after 5 weeks of a cuprizone diet, with cuprizone being removed from the feed after 6 weeks. This experimental set-up meant animals received treatment with clozapine or MIS416 for 2 weeks, the first week of treatment was simultaneous with cuprizone in the diet whereas in the second week cuprizone was removed from the feed.

Mice followed the same pattern in weight loss as previous experiments (Figure 5.17a & d) with all cuprizone-fed groups losing a significant amount of weight when compared to healthy control animals. Performance on the rota-rod was also similar to previous experiments. At the 2 week time point, clozapine-treated, cuprizone-fed mice had a significantly increased performance on the rota-rod when compared to untreated cuprizone-fed animals (Figure 5.17b) as well as an increased performance on the horizontal bars at this same time point (Figure 5.17e). Horizontal bar performance was similar to previous experiments, with healthy control animals recording the shortest performance time, while all animals receiving cuprizone in their feed showed a reduced performance. Treatment with MIS416 did not improve performance speed relative to untreated, cuprizone-fed mice (Figure 5.17c and f).

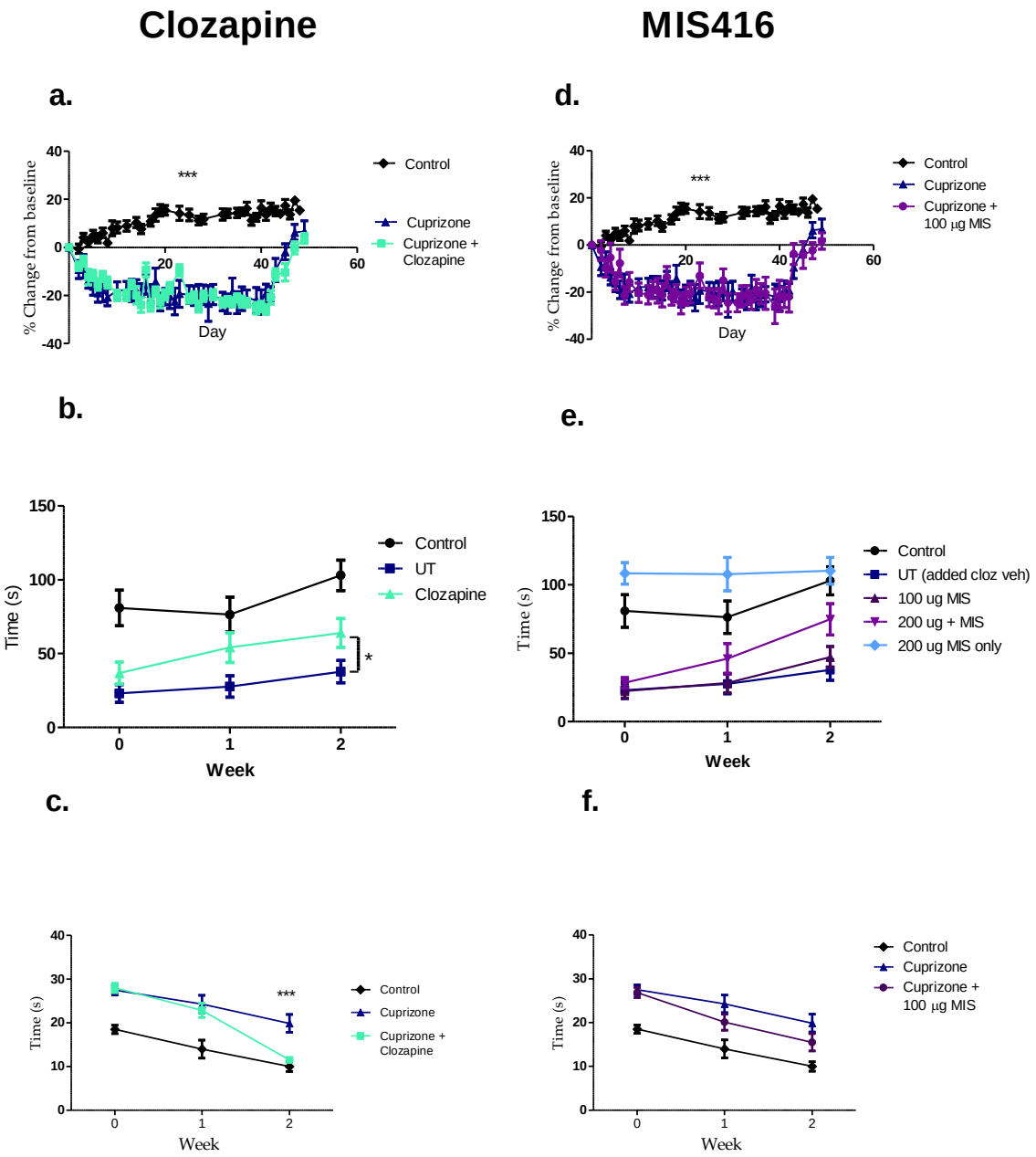

Figure 5.17: Clozapine significantly improved cuprizone-induced behavioural deficits.
Mice were weighed throughout the duration of the experiment (a & d) as well as performing the rota rod behavioural assay (b & e) and horizontal bar assay (c & f). Data shown are means and SEM from 2 individual experiments with n=4-8. p *<0.05, ***p<0.001 one way ANOVA with Bonferroni's multiple comparison test.

5.2.9 No significant effects were seen on remyelination after 7 weeks

To assess whether MIS416 or clozapine alter earlier stages of remyelination, animals were sacrificed one week after removal of cuprizone from the diet. As described previously sections were assessed for myelin using luxol fast blue and astrocytes using GFAP. Healthy control animals consistently scored higher levels of myelin present, with a maximum score of 3. Both clozapine-treated, cuprizone-fed and MIS416-treated, cuprizone-fed animals had significantly lower levels of myelin present in the corpus callosum when compared to the healthy controls. There was no significant difference between untreated, cuprizone-fed animals and MIS416-treated or clozapine-treated, cuprizone-fed animals, indicating that neither compound was significantly altering the rate of remyelination at this earlier time point (Figure 5.18b).

When assessing astrogliosis, healthy control animals had a significantly reduced level of astrocyte activation compared to all groups receiving cuprizone. There was no significant difference in the level of astrocyte activation between untreated, cuprizone-fed animals and MIS416-treated, cuprizone-fed animals, with both groups having strong levels of astrogliosis (Figure 5.18d). Clozapine-treated, cuprizone-fed animals had a significantly lower level of astrocyte activation when compared to untreated, cuprizone-fed animals (Figure 5.18b), indicating that at this earlier time point, clozapine may be reducing astrocyte activation. However, for these experiments a difference was detected in the level of GFAP activation in cuprizone-fed animals receiving clozapine vehicle compared to untreated, cuprizone-fed animals (Figure 5.19c). No difference was observed for any other parameter measured, and all previous work revealed no functional or phenotypic difference between these control groups. Thus, it is likely that the difference detected with GFAP between the untreated and vehicle-treated, cuprizone-fed groups is an artefact of the staining process, but will need to be investigated further.

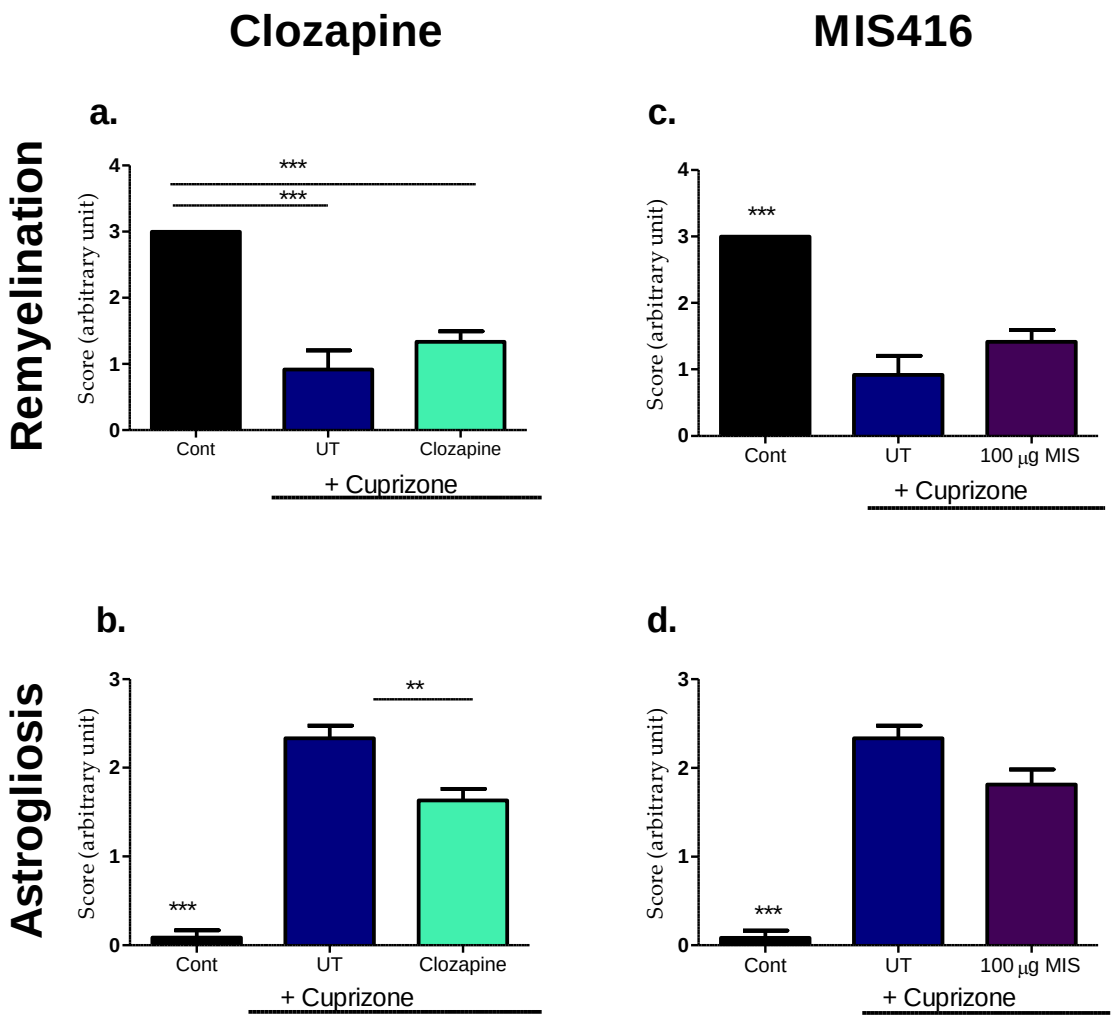

Figure 5.18: Clozapine and MIS416 did not enhance remyelination or alter astrogliosis after 7 weeks. Sections stained with luxol fast blue for myelin or GFAP for astrocytes were scored by observers blinded to treatment groups. 1= low remyelination/astrogliosis and 3= high remyelination/astrogliosis. Data shown are means and SEM from 2 individual experiments with n=2-8. **p<0.01, ***p<0.001 with one way ANOVA and Bonferroni's multiple comparison post-test.

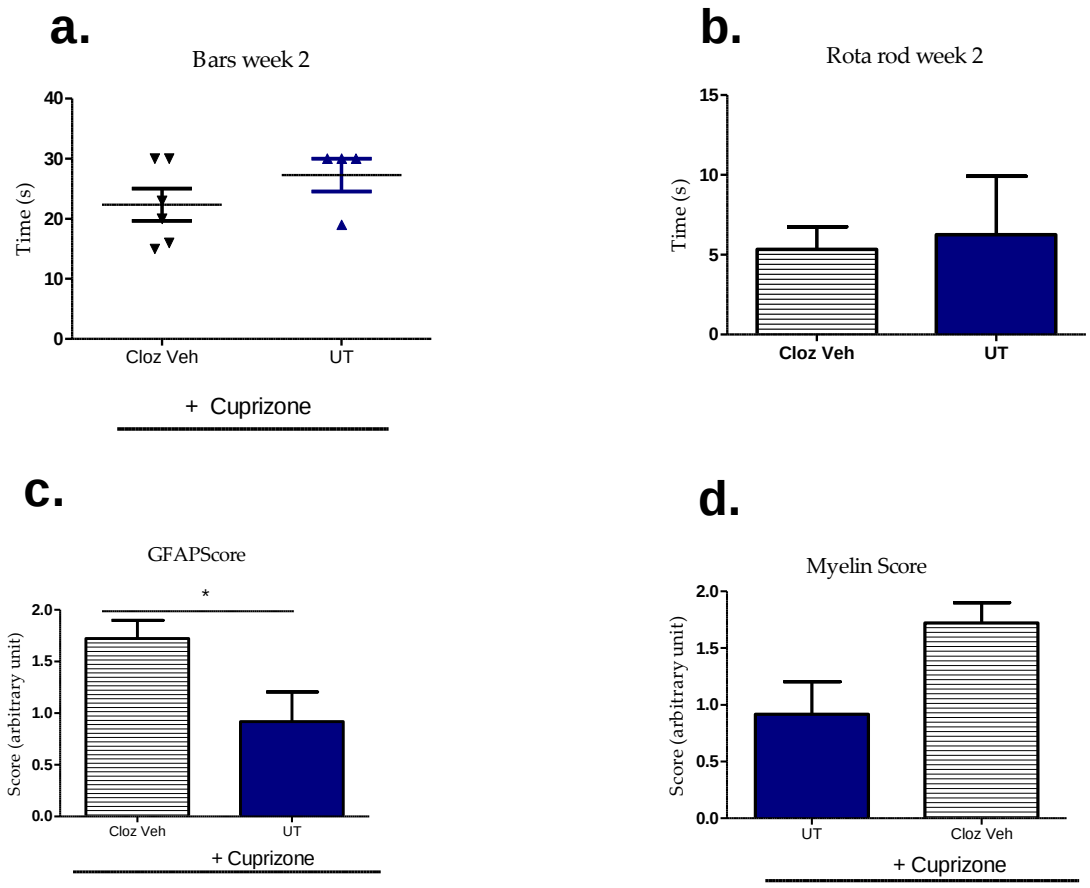

Figure 5.19: Clozapine vehicle altered levels of GFAP.
Treatment with clozapine vehicle in these two independent experiments led to a significant increase in GFAP score compared to untreated cuprizone fed animals (c) which was not seen in previous experiments. In all other parameters assessed there was no difference caused by clozapine vehicle, indicating it had no functional effect. * $p < 0.05$ with an unpaired t-test.

5.2.10 Oligodendrocytes

Mature oligodendrocytes are destroyed during cuprizone intoxication, therefore the ability to protect these cells could provide a therapeutic approach to targeting demyelination. To assess whether MIS416 or clozapine treatment was having an effect on the mature oligodendrocyte population, sections were stained using an anti-GSTpi transferase antibody. Control animals had a significantly higher number of mature oligodendrocytes (Figure 5.20) when compared to all groups receiving cuprizone, indicating that cuprizone is targeting this population. MIS416 or clozapine treatment had no protective effect on mature oligodendrocytes as no difference was seen between these groups when compared to the untreated, cuprizone-fed animals.

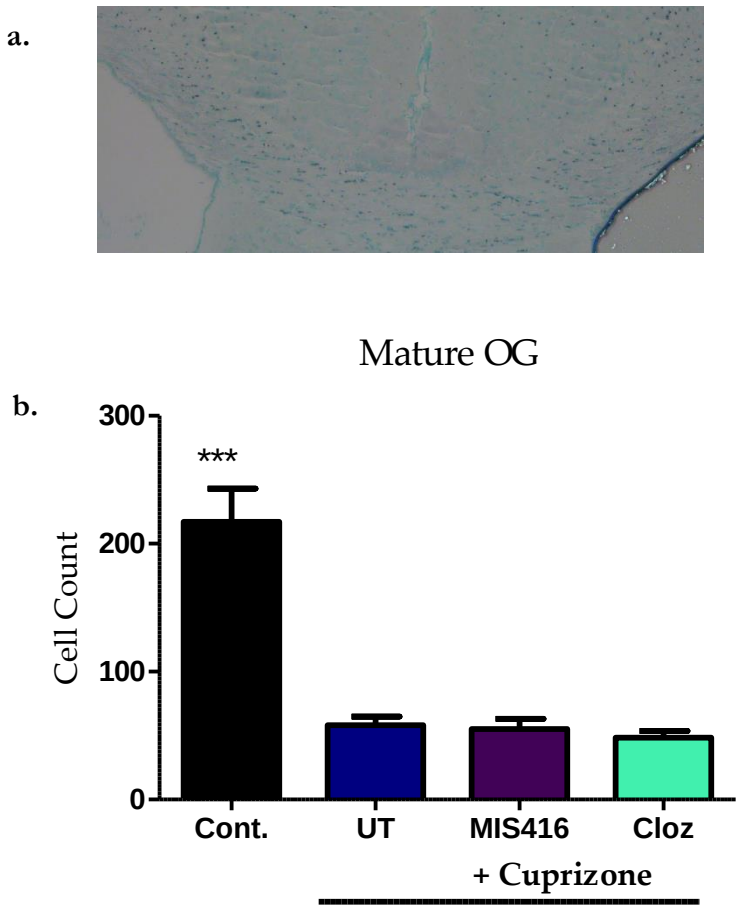

Figure 5.20: Clozapine or MIS416 did not alter the number of mature oligodendrocytes in the corpus callosum.
Sections were stained for mature oligodendrocytes using an anti-GST pi transferase antibody (a) and counterstained using methyl green. Sections showing the corpus callosum were photographed and cells were counted using imageJ. ***p<0.001 with one way ANOVA and Bonferroni's post-test. . Data shown (b) are means and SEM from 2 individual experiments with n=4-10.

5.2.11 MIS416 caused significant alterations to splenocyte populations at the early time point

To assess the peripheral effects of MIS416 administration, spleens were removed and single cell suspensions were prepared for flow cytometry. Treatment with MIS416 lead to a significant increase in splenocyte count (Figure 5.21) when MIS416-treated, cuprizone-fed animals are compared to untreated, cuprizone-fed animals.

Using the gating strategy shown in Figure 5.14, T cells were again identified based on surface markers CD4. CD8 and CD25. At this earlier time point of remyelination, no difference was seen in CD8 or Treg populations (as seen previously in Figure 5.15). A significant reduction was seen in the number of CD4+ cells in MIS416-treated cuprizone mice when compared to control animals, however this difference was small, and no difference was seen when comparing MIS416-treated, cuprizone-fed animals to untreated, cuprizone-fed animals indicating this reduction in CD4+ cells is likely a result of experimental variation rather than a true difference (Figure 5.22a). Treatment of cuprizone-fed animals with MIS416 lead to a significant reduction in NK cells at this earlier time point when compared to untreated, cuprizone-fed animals.

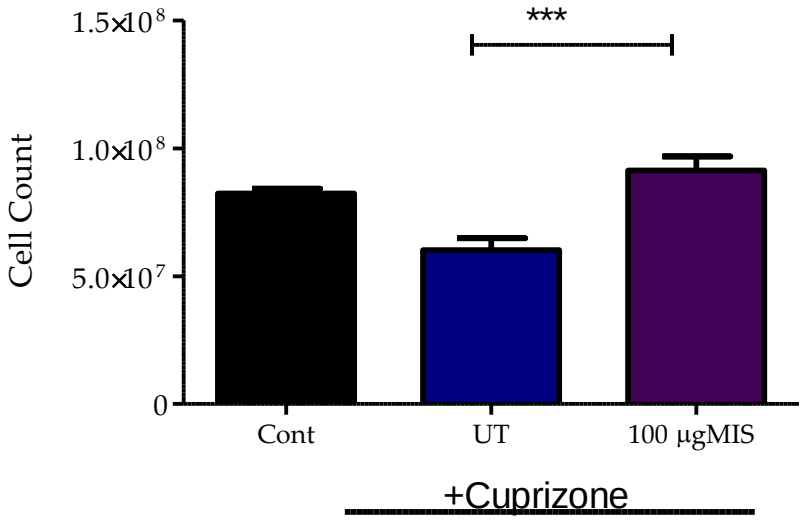

Figure 5.21: Treatment with MIS416 altered splenocyte counts
Single cell suspensions were created from isolated spleens before being counted using the trypan blue exclusion method. Significance was not reached. . Data shown are means and SEM from 3 individual experiments with n=4-10. ***p<0.001 with one way ANOVA and Bonferroni's post-test.

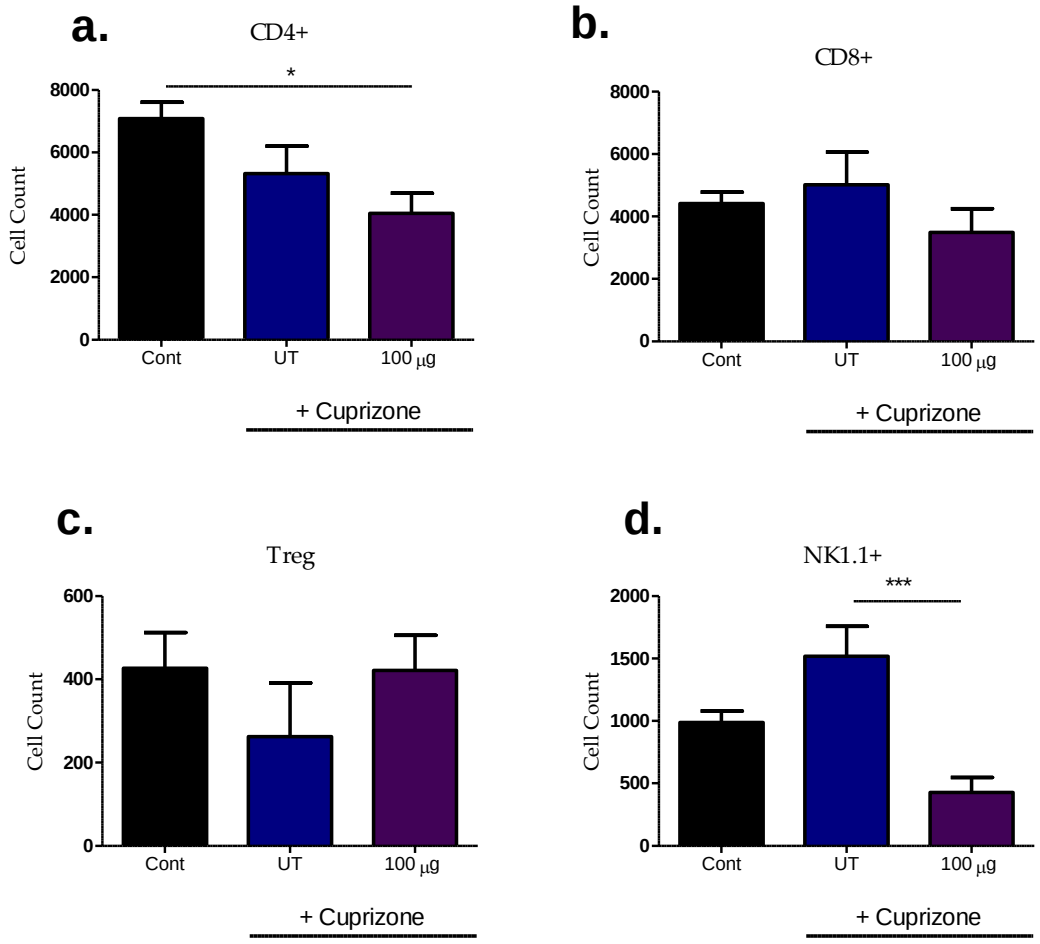

Figure 5.22: MIS416 caused a significant reduction in NK1.1+ cells.
Single cell suspensions were prepared for flow cytometry and stained with the appropriate cocktail of antibodies. Cells were identified as CD4 T cells (CD4+), CD8 T cells (CD8+), Treg (CD4+ CD25+) and NK cells (NK1.1+). One way ANOVA with Bonferroni's post-test *$p<0.05$, ***$p<0.001$. Shown are means and SEM from 2 individual experiments, n=4-10 per group.

A second antibody panel, different to that used previously was used to identify red pulp macrophages, dendritic cells (DC), granulocytes and white pulp macrophages. F4/80+CD11b- cells were identified as red pulp macrophages (Figure 5.22) with CD11b+ cells being characterised further. CD11b+Gr-1^{hi}SSC $^{med/hi}$ were classed as granulocytes with CD11b+Gr-1- cells classed as white pulp macrophages. Dendritic cells were identified as CD11b+CD11c+ cells. As shown in (Figure 5.24) MIS416-treated, cuprizone-fed mice had a significant increase in both their red pulp macrophage and granulocyte population when compared to untreated, cuprizone-fed mice and healthy controls. MIS416-treated, cuprizone-fed mice had a decrease in the dendritic cell population relative to untreated, cuprizone-fed animals, however this was not significantly different to healthy controls, indicating that the difference in DC cell count may be due to an increase in DC's as a result of cuprizone treatment which is reduced by the MIS416 treatment to 'normal' levels. A significant decrease in MDSC's was also seen in MIS416-treated, cuprizone-fed animals when compared to untreated, cuprizone-fed animals and healthy controls. Untreated, cuprizone-fed animals had a significantly higher level of MDSC's when compared to healthy controls, indicating that cuprizone treatment leads to an expansion of this population, which is then reduced to the levels of a healthy control following treatment with MIS416 (Figure 5.24).

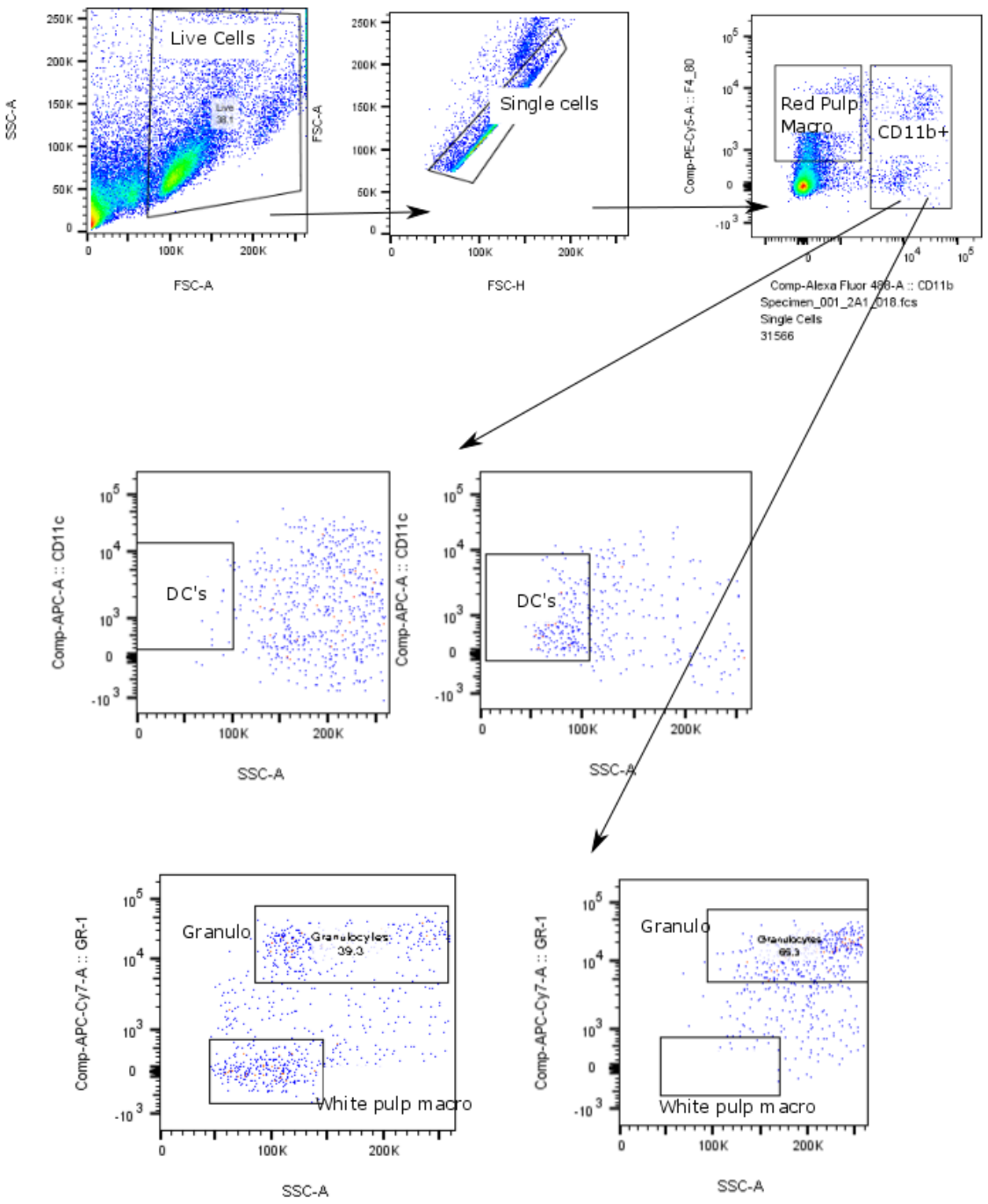

Figure 5.23: Example gating strategy for red pulp macrophages and CD11b+ splenocyte populations.
Single cell suspensions of splenocytes were prepared and stained with the appropriate mixture of antibodies before flow cytometry to identify granulocytes (granulo), dendritic cells (DCs) and myeloid derived suppressor cells (MDSCs). Data was analysed using FlowJo v.10.

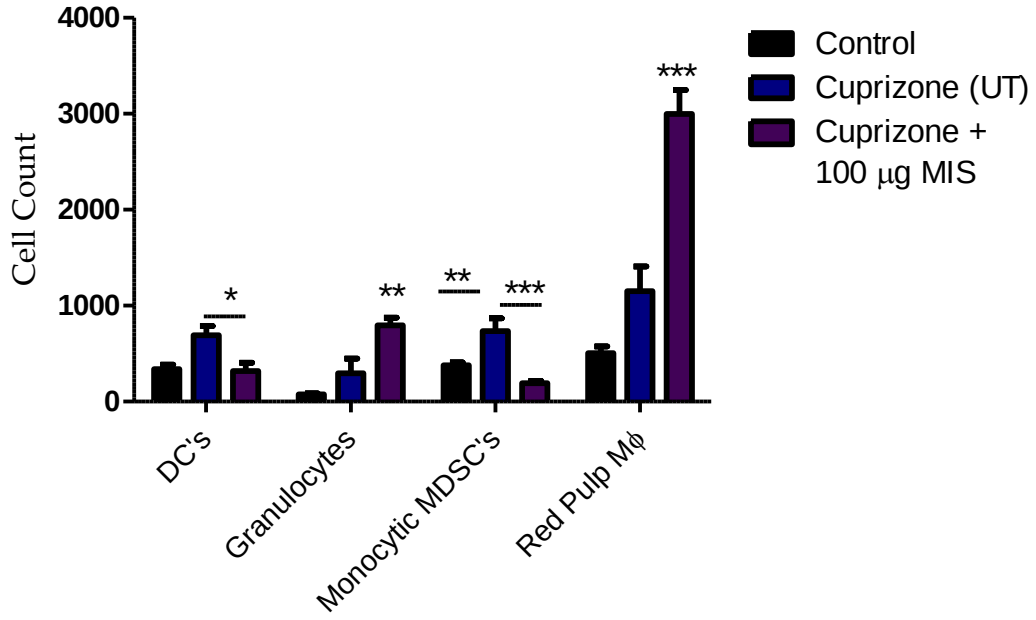

Figure 5.24: MIS416 treatment caused a significant increase in red pulp macrophages and granulocytes, and a reduction in white pulp macrophages.
Single cell suspensions of splenocytes were prepared and stained with the appropriate mixture of antibodies before flow cytometry. Data were analysed using FlowJo v.10. *p<0.05, **p<0.01, ***p<0.001. Data shown are the mean and SEM from two individual experiments with n=4-10 per group.

Previous work in our laboratory has shown treatment with MIS416 leans to an increase in levels of PDL-1 (programmed death ligand 1) and I-A/I-E in the EAE model and in healthy animals. To determine if these effects are maintained in the cuprizone model of remyelination the level of PDL-1 and I-A/I-E expression on different splenocyte populations was measured. As shown in Figure 5.25**,** treatment with MIS416 caused an increase in the levels of PDL-1 and I-A/I-E relative to untreated, cuprizone-fed animals. When assessing which population of cells were upregulating these markers, the majority of these cells were red pulp macrophages (CD11b-, F4/80+). PDL-1 expression was significantly increased in all cell types assayed compared to healthy controls and untreated, cuprizone-fed mice (Figure 5.26a). When looking at I-A/I-E expression (Figure 5.26b) there was a significant increase on red pulp macrophages relative to untreated, cuprizone-fed

animals and healthy controls. Cuprizone treatment alone had no effect on PDL-1 or I-A/I-E expression, so the increase seen is a result of MIS416 treatment.

Taken together these results indicate that MIS416 increases PDL-1 and I-A/I-E expression when administered during the cuprizone model. MIS416 administration also leads to a significant reduction in the number of NK1.1+ cells at this earlier time point.

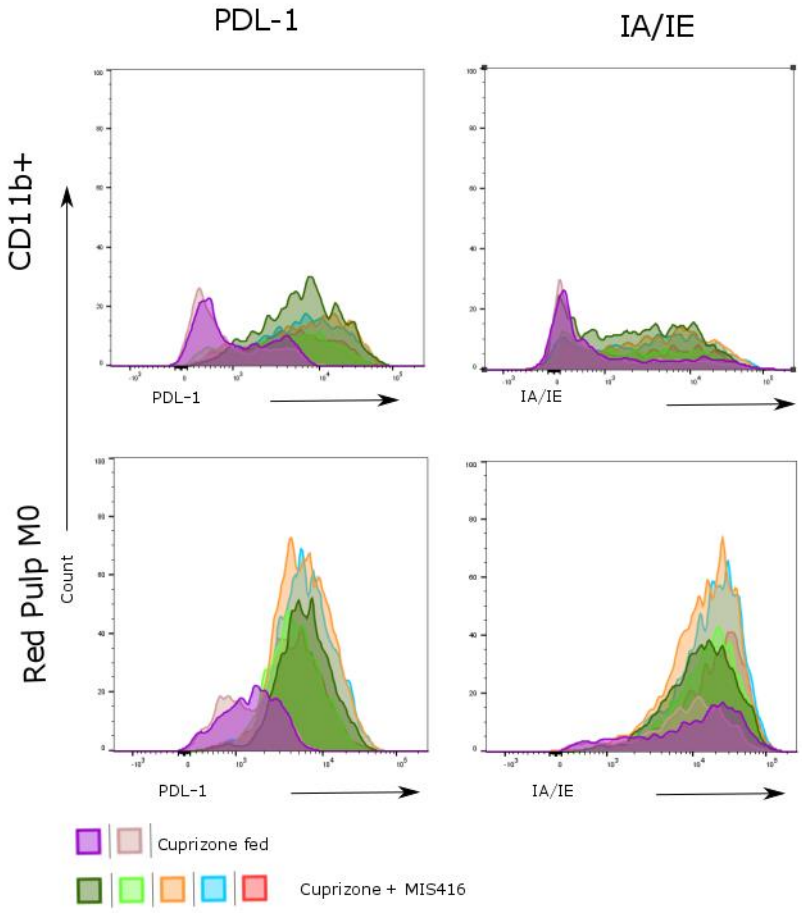

Figure 5.25: Histogram showing effect of MIS416 treatment on levels of PDL-1 and I-A/I-E expression. Single cell suspensions of splenocytes were prepared and stained with the appropriate mixture of antibodies before flow cytometry. Data were analysed using FlowJo v.10.

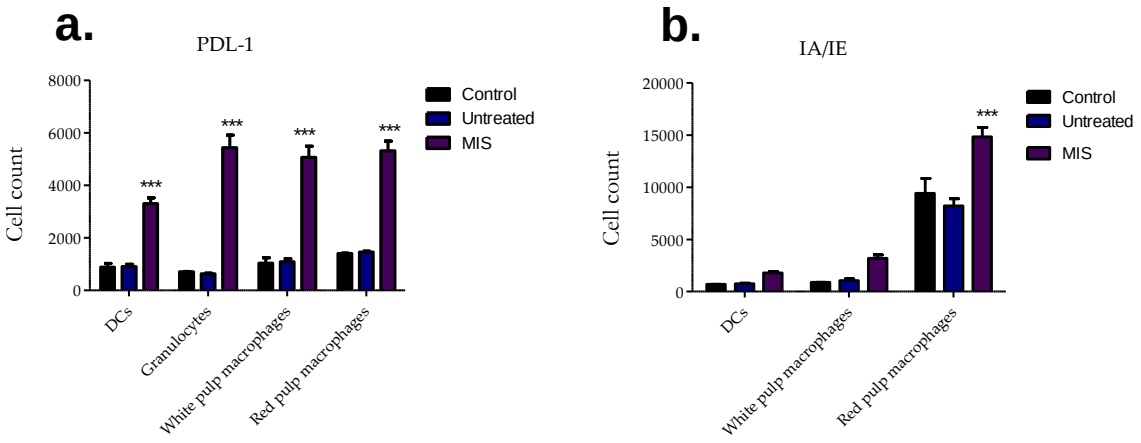

Figure 5.26: MIS416 causes increases in PDL-1 and I-A/I-E expression in different splenocyte populations. Cells were characterised based on their surface markers and the level of (a) PDL-1 or (b) I-A/I-E expression was assessed. ***p<0.001 with two way ANOVA and Bonferroni's post-test. Data shown are the mean and SEM from two individual experiments with n=4-10 per group.

5.3 Discussion

The aim of the experiments in this chapter was to determine whether remyelination could be accelerated, or improved by treatment with clozapine or MIS416. The cuprizone model is an especially appropriate model, because both remyelination and demyelination can occur at the same time, as occurs in MS (Bruce, Zhao, & Franklin, 2010; Franklin, 2002). No difference in rate of remyelination was detected in the corpus callosum following treatment with MIS416 or clozapine, however clozapine treatment led to a significant increase in performance on the rota-rod and horizontal bar assay and a reduction in astrogliosis.

As the behavioural experiments progressed, all cuprizone treated animals showed improvement from their starting point. This would be expected regardless of any therapeutic intervention, as once cuprizone is removed from the diet, remyelination has been shown to occur spontaneously (Benetti et al., 2010; Xiang Gao et al., 2000). The improved performance seen in the horizontal bar assay could be attributed to an improvement in grip and coordination or could be down to learning. This effect of improvement however was not seen in the control groups so is most likely due to an improvement in grip strength and coordination. This is not surprising because as the experiment continues, cuprizone is removed from the diet so some spontaneous remyelination is expected to be occurring. Histological data support this conclusion, with patches of remyelination occurring following removal of cuprizone.

During MS, the ability to remyelinate becomes lost, as myelin sheaths are destroyed and oligodendrocytes die during the inflammatory phases of disease (Chamberlain et al., 2016). The mechanism of remyelination failure is still not clear; however, a leading hypothesis is that OPC's become stalled and are unable to differentiate into mature OG's capable of forming myelin. In MS, OPCs continue to be recruited to sites of demyelination but fail to develop into mature myelin producing cells leading to an increased severity of disease.

Shao et al. (2015) showed that following exposure to 0.2% cuprizone for 1 week, mice had a reduced number of oligodendrocytes, more increased astrocyte and microglia immunoreactivity in the corpus callosum, caudate putamen and the hippocampus. They also found higher levels of TNF-α and IL-6 in these brain regions. All of these changes, however, were prevented in the cuprizone group treated with the atypical antipsychotic quetiapine. The results of their study showed quetiapine had an anti-inflammatory effect in cuprizone-fed mice, and that the protection of the oligodendrocytes may have been responsible for the improvement in behaviour seen in the quetiapine-treated group. Clozapine is another FDA-approved atypical antipsychotic used for the treatment of schizophrenia. In the current study, clozapine was unable to significantly alter astrogliosis at the later time point or protect the oligodendrocyte population, as quetiapine has been previously shown to do. This is possibly due to the length of cuprizone exposure, it is possible that after only one week of cuprizone, clozapine may have shown similar effects to quetiapine, yet these effects are not maintained after longer periods.

Previous studies have shown clozapine can reduce inflammation, which was discovered as a result of it being used as a treatment for schizophrenia. More recent studies by Xu et al. (Haiyun Xu et al., 2014) have assessed the ability of typical and atypical antipsychotics (including clozapine) to support oligodendrocyte (OG) differentiation into a mature myelinating oligodendrocytes. In the process of OG differentiation, NG2+ cells first develop into O4+ cells. Rat NG2+ cells exposed to cuprizone *in vitro* and then treated with different antipsychotics were assessed to determine the percentage of O4+ and NG2+ cells. It was found that both clozapine and quetiapine, another atypical antipsychotic, significantly reduced the cuprizone-induced attenuation of NG2+ cell differentiation into O4+ cells. The ability of antipsychotics to affect oligodendrocyte maturation was assessed using CNP and MBP as markers for mature oligodendrocytes. Again, in this cell population, treatment with cuprizone reduced the number of CNP+ cells compared to O4+ cells. Clozapine treatment protected from the cuprizone-induced decrease in CNP+ cells and a similar effect was seen when looking at percentage of MBP positive cells. Taken together these results

indicate that clozapine can protect from cuprizone induced prevention of oligodendrocyte differentiation and maturation in an *in vitro* culture system.

Like the study by Shao et al., Xu and colleagues showed that these effects were ameliorated by treatment with quetiapine or clozapine. To our knowledge the effect of *in vivo* clozapine treatment in the cuprizone model has not previously been investigated. Therefore, we hypothesised that like quetiapine, clozapine may have a protective effect on the oligodendrocyte population. Like previously reported results (Cammer, 1999; Glenn K. Matsushima & Pierre Morell, 2001), cuprizone treatment lead to a reduction in the mature oligodendrocyte population (Figure 5.20), with neither clozapine nor MIS416 able to protect the mature oligodendrocyte population (GST-pi transferase+ cells). Although there was a clear difference between healthy animals and those fed cuprizone, sample size for this experiment was low, n of 2-5 per group, so repeats would be required to be sensitive enough to detect a small difference between cuprizone-fed treatment groups However, the effect of clozapine on oligodendrocyte precursor cells was not able to be evaluated due to difficulties with finding a primary antibody suitable for detecting this population in our paraffin embedded tissues and time constraints. Further useful information could be gained about the effect of clozapine on mature oligodendrocytes *in vivo* by an experiment more closely resembling that of Shao et al. in which mice were only exposed to cuprizone for one week and treated with clozapine throughout to determine if the presence of clozapine throughout the demyelination period is able to provide protection like that seen with quetiapine.

Furthermore, a study looking at the role of quetiapine in remyelination after cuprizone induced demyelination (Y. Zhang et al., 2012) showed that quetiapine treatment during the recovery period (after cuprizone was withdrawn) significantly improved behavioural performance and myelin restoration. Quetiapine was shown to increase the number of mature oligodendrocytes present at the demyelinated lesion sites, possibly by the recruitment of mature OGs from other regions or by enhancing the differentiation of OPCs.

Traditional antipsychotics (e.g. haloperidol) exert their effects primarily through blocking dopamine receptors, and some studies have reported links between myelin deficits and dopamine dysfunction (Lindholm & Jazin, 2007). Quetiapine however, shows low dopamine receptor occupancy, indicating that it is providing its protective effects via a non-dopamine mediated pathway. Clozapine, like quetiapine is an atypical antipsychotic and ongoing work in our laboratory is looking at the role of dopamine and serotonin receptors in clozapine treatment during EAE.

Based on these early studies it is possible that quetiapine, and potentially clozapine, could be used alongside current MS therapies to protect mature OGs and recruit OPCs to sites of lesions to aid in the remyelination process.

A recent study by Du (Du et al., 2016) used the cuprizone and EAE models to study the effect of the kappa opioid receptor antagonist, U50-488, on myelin levels in the corpus callosum. A kappa opioid receptor (KOR) knockout mouse exacerbated EAE, whilst activation of this receptor reduces EAE symptoms. Interestingly a reduction of immune cell infiltration was not identified as the therapeutic mechanism, instead, activation of the KOR led to oligodendrocyte differentiation and myelin formation both *in vitro* and *in vivo*. This study provides further weight to the idea of targeting MS not only through altering immune cell infiltration, but by targeting the process of myelin formation directly.

MIS416 has been shown to reduce disease severity in EAE (White et al., 2014) an immune-mediated model of demyelination, however its mechanisms of action are not fully understood. MIS416 activates the TLR-9 and NOD2 pathways, mediating the balance of Th cell subsets. Activation of the TLR-9 and NOD2 receptors can lead to the induction of interferons, including IFN-γ, which is of particular interest. In EAE, IFN-γ plays a crucial protective role, with disease protection by MIS416 being lost in IFN-γ knockout animals (White et al., 2014). Gao et al. (Xiang Gao et al., 2000) showed that during cuprizone intoxication, transgenic mice expressing a low level of IFN-γ were resistant to the demyelination, astrogliosis and microgliosis that was present in wild

type cuprizone-fed mice. These and other studies indicate a complicated and not fully understood role for IFN-γ in the process of demyelination and remyelination (White et al., 2014; Wood & Sawitzki, 2006). By including MIS416 in these experiments we were able to assess any effect of MIS416 on cells of the central nervous system and myelination without the involvement of peripheral immune cell invasion, a process which occurs in EAE due to breakdown of the blood brain barrier (Pham et al., 2011). Invading immune cells are able to release cytokines altering the CNS environment (Komiyama et al., 2006) and so excluding them by using the cuprizone model allows for investigation of a direct impact of MIS416 on cells in the CNS. MIS416 treatment did not prevent any of the cuprizone-induced behavioural deficits, indicating that it is unlikely to be directly altering the rate of remyelination. Looking into the level of IFN-γ in the CNS would provide information on how MIS416-induced IFN-γ production previously seen in EAE (White et al., 2014) impacts the level of de/remyelination occurring in the cuprizone model and whether this is associated with a high or low level of the cytokine.

Astrocytes are the most abundant cell population in the CNS and have been implicated in many diseases, as well as playing a role in normal healthy brain function (Dong & Benveniste, 2001; Nair et al., 2008). Astrocytes are responsible for the formation of glial scars around lesions, which limits damage to neighbouring tissue. Scar formation in MS, however, can also be damaging as it restricts the entry of OGs to produce myelin and can lead to chronic inflammation (Chamberlain et al., 2016). Astrocytes have been shown to interact directly with OGs and also indirectly by acting on microglia and invading T cells. While invading T cells are not a substantial component of the cuprizone model, controlling the activation of microglia, potentially through the activation of astrocytes, could provide a promising therapeutic approach. As seen in experiments carried out in this chapter, cuprizone led to an increase in the level of astrocyte activation as measured by upregulation of GFAP and morphological changes. Quetiapine, another atypical antipsychotic, has been reported to decrease this cuprizone induced astrogliosis (Y. Zhang et al., 2008). Treatment with MIS416 was unable to alter the level of astrocyte activation significantly, so it is unlikely that

this compound can provide a therapeutic effect via modulation of astrocyte activity or an indirect effect on microglia/OGs through factors secreted by astrocytes.

One of the key changes observed in this chapter was the increase in splenocyte populations following MIS416 administration (Figure 5.12 & 5.13). Previous work in our lab has shown that treatment with MIS416 significantly increased the leukocyte count in the spleen, and the populations primarily responsible for this increase were red pulp macrophages, dendritic cells and granulocytes (White, 2015). As MIS416 acts through TLR9 and NOD2 receptors, it is unsurprising that phagocytic cells of the spleen are responding to MIS416. Early work from our lab showed that MIS416 was able to be phagocytosed and formed a depot in the liver and spleen, with macrophages, granulocytes and red pulp macrophages taking up fluorescently-labelled MIS416. As EAE is an immune-mediated disease, the immune system is highly active, with many immune cells being recruited to the CNS and exposed to MIS416. Unlike EAE, the cuprizone model is non-immune mediated so it was of interest to determine whether these previously reported immune effects are still present and to what extent. In the absence of an immune-modulated disease, MIS416 is still able to cause similar alterations to leukocytes in the spleen, namely a significant increase in neutrophils and monocytes after 3 weeks of recovery from cuprizone and weekly MIS416 treatments. MIS416 treatment also lead to a decrease in NK1.1 cells in this model, an effect which was not seen previously in the EAE model (White, PhD thesis 2015). This reduction in NK1.1+ cells was not seen with cuprizone alone, indicating that it was as a result of MIS416 and not a side effect of the cuprizone model.

No significant changes were seen in the splenocyte populations after treatment with clozapine, which is in line with available data suggesting that antipsychotics are able to normalize immune responses including the level of proliferation of lymphocytes (Basta-Kaim et al., 2006), however little effect would be expected in this model as there is minimal immune involvement.

In the experiments where animals were culled at an earlier time-point, the expression of PDL-1 was investigated. PDL-1 is an anti-proliferative ligand present on the surface of APCs and can alter the response of T cells by reacting with the PD-1 receptor on the T cell surface, preventing T cell proliferation (Yao, King, Praither, Yin, & Moorman, 2007). In this non-immune-mediated model of demyelination, an increase in levels of PDL-1 was seen, similar to those observed in the EAE model (White, PhD thesis 2015). As this increase is also seen in the cuprizone model where T cell activity is not a contributing factor, it indicates that this upregulation of PDL-1 is a direct result of MIS416 administration and is not model specific. In untreated mice, approximately 1000 cells were PDL-1positive, a level seen in control healthy animals. Treatment of animals with MIS416 led to a 6-fold increase. Again, no changes in the level of PDL-1 expression was seen with clozapine treatment.

As well as looking at PDL-1 expression, the expression of IA\IE (MHC class II) was assessed. MHC class II is involved in presenting exogenous antigens to T cells during both normal immune surveillance and during disease (Murphy et al., 2008). As seen with PDL-1 expression, IA\IE was upregulated on both CD11b+ and F4/80+ cells following treatment with MIS416, however this effect was more pronounced in the red pulp macrophage population (F4/80+CD11b-). This population will be a key responder during an infection/inflammatory response as it is one of the main APC populations, so an upregulation of I-A\I-E enables these cells to phagocytose more efficiently/rapidly. Because there is no underlying inflammatory response or infection during the cuprizone model, but there is a clear upregulation of I-A\I-E expressing cells, this upregulation is likely a direct and key process in the mechanism for MIS416 action.

Experiments in this chapter were unable to identify any impact of MIS416 or clozapine treatment on oligodendrocyte precursor cells, which would be a key step before undertaking further experiments. Studies have demonstrated (Tognatta & Miller, 2016) (Rassul, Neely, & Fulton, 2016) that OPCs at the sites of lesions develop into oligodendrocytes capable of remyelination (Chamberlain et al., 2016; Purger, Gibson, & Monje, 2016), and that transplantation of OPCs to

lesion sites can also remyelinate with high efficiency. For these reasons, any therapeutic intervention able to recruit OPCs to lesion sites during remyelination or to speed up the differentiation of OPCs to mature OGs could provide a useful therapy in diseases such as MS. Future experiments in this area would include the optimisation of a protocol for detecting and quantifying oligodendrocyte precursor cells in response to treatment with either MIS416 or clozapine in the cuprizone model during both early and later stages of remyelination.

5.4 Summary

Neither clozapine nor MIS416 treatment was able to prevent cuprizone-induced weight loss, however clozapine was able to enhance recovery from cuprizone-induced behavioural deficits. After 6 weeks demyelination followed by a 1 or 3 week recovery period, remyelination rates were not improved by either compound. Cuprizone lead to astrogliosis, which was not significantly altered by MIS416 or clozapine after 3 weeks recovery; however, treatment with clozapine did reduce astrogliosis at an earlier time point, which is in line with the observed enhancement in recovery from behavioural deficits during this period. MIS416 treatment caused an increase in leukocyte populations in the spleen as well as causing an upregulation of PDL-1 and I-A\I-E. These results indicate that although MIS416 is unable to alter remyelination in the corpus callosum it is altering the peripheral immune response, which may confer a protective effect through a mechanism other than remyelination.

CHAPTER 6: Effect of clozapine and MIS416 on cytokine production and growth factors *in vitro* and *in vivo*

6 Introduction

Microglia are key responders to CNS inflammation and also contribute to maintenance of the CNS environment during normal conditions, and it is hypothesised that microglia are useful targets for drugs acting in the CNS. Debate exists as to whether this population of cells plays a protective or detrimental role during CNS inflammation (Benveniste, 1997; Napoli & Neumann, 2010). Neumann and colleagues (Neumann et al., 2009) have shown that microglial activation is essential for the clearance of debris following demyelination, which is critical for remyelination to occur. Studies by Napoli also suggest microglia are involved in neuroprotection as they secrete soluble factors triggering the migration of cells involved in neural regeneration and repair (Napoli & Neumann, 2010). However, microglia are also involved in the differentiation of T cells, which can lead to tissue destruction and inflammation at lesion sites (Sanders & De Keyser, 2007). In one of the first studies describing the cuprizone model in C57BL/6 mice, microglial activation was reported to precede astrogliosis and it was therefore suggested that microglial activation actively contributed to demyelination (Hiremath et al., 1998). It is likely that the microglial response to an inflammatory environment cannot be described as 'good' or 'bad', but rather the effect the microglia produce on the surrounding cells depends on the time at which they are responding to the inflammation and the other cellular events occurring in their local environment.

From previous experiments a functional recovery was seen in early remyelination experiments following treatment with clozapine. While no change was seen in the astrocyte activation at the later time point, or oligodendrocyte population, it is a possible that the microglial responses were altered in response to administration of clozapine or MIS416. From previous studies we know that MIS416 targets myeloid cells, and that clozapine is able to have an effect on microglia. It is therefore highly likely that both of these compounds will have a direct impact on primary microglial cells. No previous work looking at the direct effect of cuprizone on microglial cells *in vitro* has been found, indicating that this is a novel aspect of the current study and is the first time

the direct effect of cuprizone on microglial cells *in vitro* has been assessed. Therefore, this chapter will look at the direct effects of these agents on microglia when they are exposed to cuprizone.

In addition to altering myeloid cell activation and neuroinflammation in the EAE model, MIS416 has previously been reported to alter serum cytokine levels in healthy mice, mice with EAE, and in MS patients (White et al., 2014). Because this elevation in serum cytokine production may contribute to the ability of MIS416 to modify neuroinflammation driven by microglia and other myeloid cells, the serum from mice receiving MIS416 and cuprizone were analysed for cytokines known to be associated with MIS416 treatment such as IFN-γ, IL-6 and IL-10, as well as other soluble factors, to determine if a similar pattern of cytokine production was induced by MIS416 in the cuprizone model and thus potentially promote immune modulation of CNS resident myeloid cells like microglia.

Primary microglia cultures have been previously used as an expedient method for identifying the response of microglia to certain stimuli (Jeohn, Kong, Wilson, Hudson, & Hong, 1998; Stansley, Post, & Hensley, 2012). One of the key benefits to using primary cells over an immortalised cell line is that microglia derived from mouse brain will retain functional characteristics such as the cytokine secretion profile and surface markers, and they more closely resemble microglia in their CNS environment (Henn et al., 2009; Stansley et al., 2012). While their direct response to a stimulus can be measured, the key drawback to this monoculture approach is the absence of signals from other cells in the CNS with which they will normally be in close communication. Despite this limitation, the use of primary microglial cultures is a valuable tool for addressing specific, targeted research questions.

6.1 Aims

The aims for this chapter were to characterise the cytokine response of primary microglial cells *in vitro* in response to stimulation with MIS416 and clozapine in the presence of cuprizone and to identify changes to serum cytokines as a result of MIS416 treatment *in vivo*, which could contribute to the immunomodulatory effects of MIS416 in the cuprizone model.

6.1.1 Specific Aims

1. Measure LPS-stimulated NO, IL-12 and TNF-α cytokine production by microglia in the presence of MIS416, clozapine and/or cuprizone.

2. Identify whether MIS416 modulates serum cytokine levels in the cuprizone model.

6.2 Results

6.2.1 Isolation and identification of Microglia

Single cell suspensions were prepared from mouse brains and a small sample of cells was stained for microglial markers $CD45^{int}$ and $CD11b^{+}$ (Figure 6.1). The purity of the single cells isolated using the percoll gradient was then assessed based on the number of CD45+CD11b+ cells. Individual isolations ranged in purity from 70-85% following this method (Figure 6.1) after which microglia were expanded for 4 weeks in M-CSF prior to being stimulated with cuprizone, clozapine or MIS416.

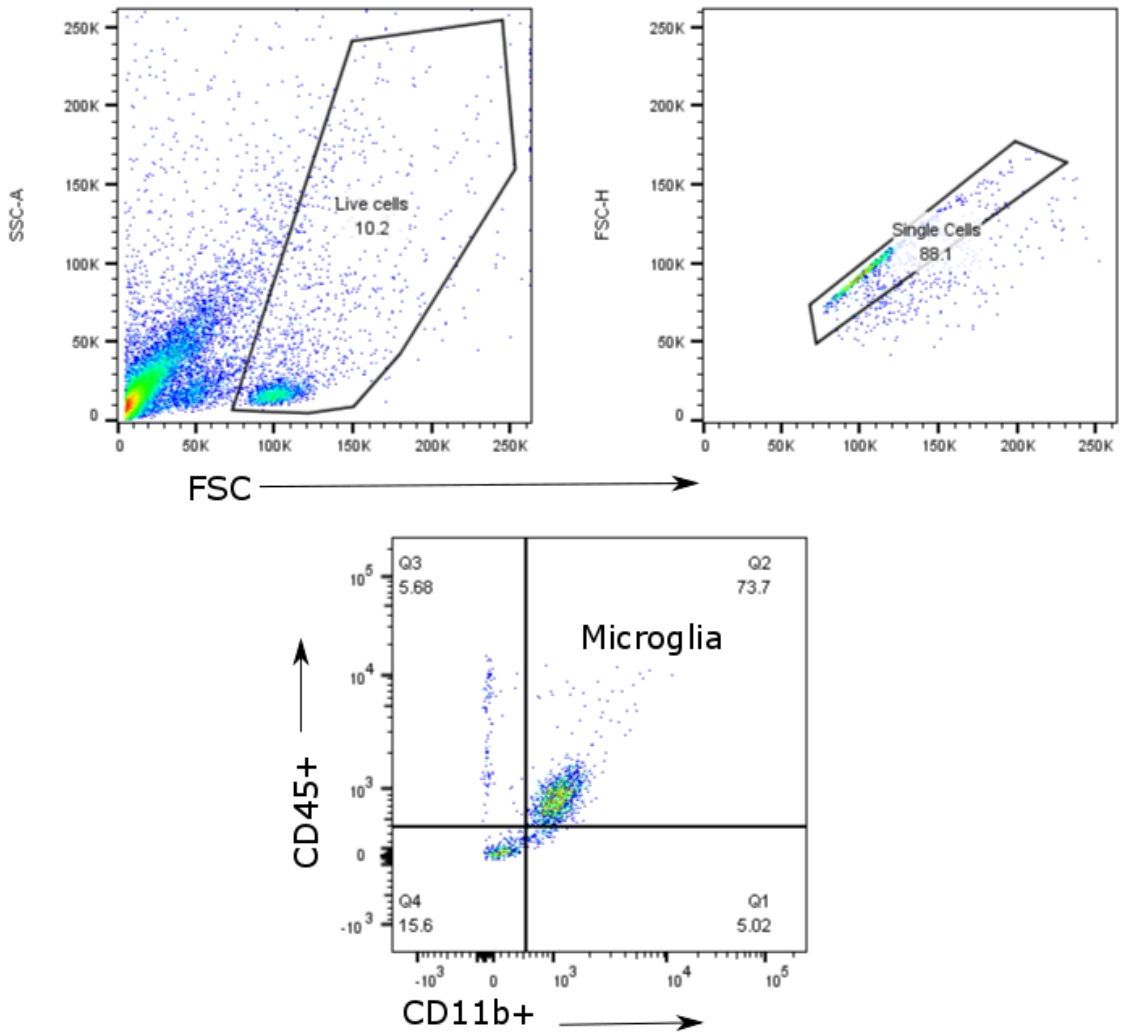

Figure 6.1: Microglia were identified based on CD45+ CD11b+ through flow cytometry.
For one culture cells from 5 mice were pooled following isolation using a percoll gradient. Prior to plating for culture, purity of the microglial isolate was determined using flow cytometry. Purity ranged from 70-85% for individual isolations.

6.2.2 Microglial viability was not altered by treatment with test compounds at lower cuprizone concentrations

Following stimulation with compounds an MTT assay was performed to assess viability of the microglial cells. All values were assessed as a percent of the media only control wells. None of the treatment conditions had a significant effect on the viability of the microglial cells (Figure 6.2), however cell viability appeared to be reduced at higher cuprizone concentrations especially in the presence of clozapine. Therefore, experiments were carried out at lower (100 μM- 200 μM) cuprizone concentrations where no effect on viability has observed.

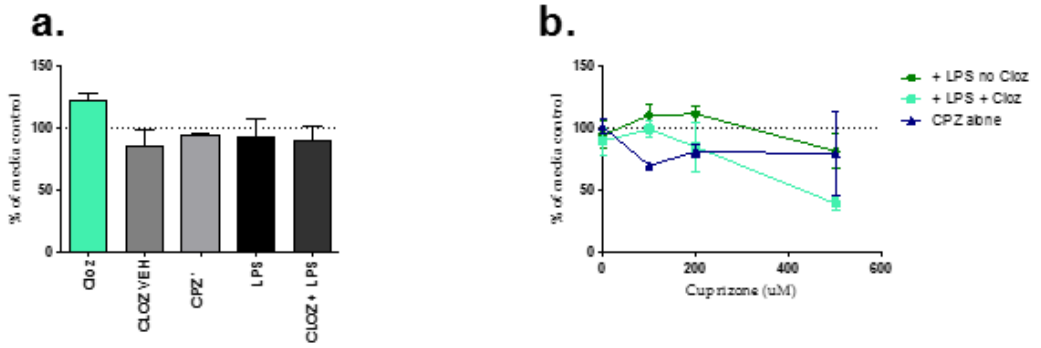

Figure 6.2: Microglial viability following treatment.
Primary microglial cells were stimulated for 24 hours with clozapine (10 μM), LPS (200ng/mL) and cuprizone (0, 100 μM, 200 μM, 300 μM & 500 μM. Following stimulation, supernatant was removed and an MTT assay was performed to assess cell viability (a) over a range of cuprizone concentrations (b). Treatment with all compounds had no significant effect on cell viability. Data are presented as mean and SEM, dotted line at 100 represents the media alone control. n=3-9.

6.2.3 Clozapine altered levels of IL-12, TNF- α and NO

After four weeks of culture, microglia were stimulated with test compounds, and the effect on microglial viability and LPS-mediated cytokine production assessed. As expected, LPS stimulation alone led to a significant increase in the levels of IL-12 produced compared to media alone (Figure 6.3a) while 200 μM of cuprizone had no effect on IL-12 production. Additionally, clozapine alone had no significant effect on levels of IL-12 (clozapine vs media). When microglia were stimulated with clozapine in the presence of 100 μM cuprizone there was a significant increase in IL-12 levels

compared cuprizone without clozapine. At higher concentrations of cuprizone this effect was lost, indicating that at the lower cuprizone concentration clozapine had a different and unexpected effect on IL-12 production.

Clozapine treatment also led to a significant increase in the level of LPS-induced TNF- α at both 100 μM and 200 μM of cuprizone. As the concentration of cuprizone increased the total level of TNF-α gradually decreased, indicating that cuprizone concentration is also having an effect on TNF- α. LPS alone strongly stimulated TNF-α production, however when clozapine was added alongside LPS the level of TNF-α produced was significantly reduced (Figure 6.4 c).

LPS alone significantly increased levels of NO production, which was reduced significantly when clozapine was added (Figure 6.3 f), indicating that clozapine attenuated the LPS-induced production of NO. In the presence of cuprizone (Figure 6.3 f), the level of NO produced was not significantly altered by the addition of clozapine at 200 μM, 400 μM or 500 μM, indicating that cuprizone had a suppressive effect on the clozapine-mediated suppression of NO produced in response to LPS. At a lower cuprizone concentration (100 μM) there was a slight increase in the level of NO in clozapine-treated cells, however this increase was not significant.

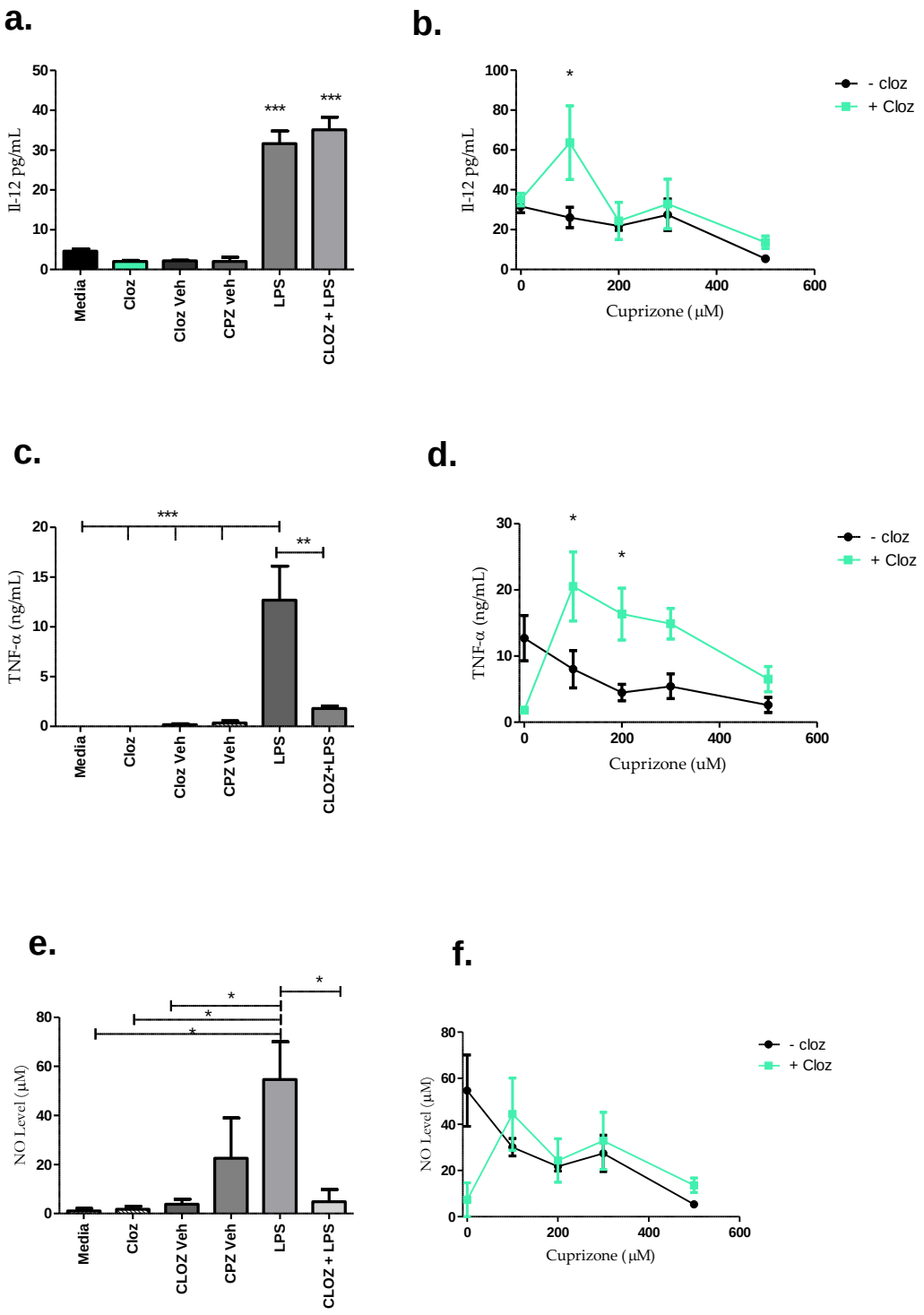

Figure 6.3: Microglial IL-12. TNF-α and NO production was significantly altered.
Shown are data from 2 individual experiments. Supernatant from microglial cultures was collected 24 hours post stimulation with the selected compounds and then supernatant IL-12 levels (a & b) or TNF- α (c & d) and NO production (e & f) were measured. (a, c & e). One way ANOVA *p<0.05, **p<0.01, *** p<0.0001 with Bonferroni's post-test (b,d & f) two- way ANOVA with Bonferroni's post-test *p<0.05.

6.2.4 MIS416 altered IL-12 and NO production *in vitro*

Cell viability tests (MTT) were unable to be carried out on MIS416-treated cells. However, based on previous experiments showing no reduction in microglial viability at 200 μM cuprizone nor in viability of bone marrow-derived macrophages exposed to 10-20 μg of MIS416, we expect that the cuprizone concentrations used in the following experiments should not impact cell viability. Thus, for the following experiments, a single cuprizone concentration of 200 μM was used rather than a range, allowing concentration-dependent MIS416 effects to be tested given that microglia numbers were limited. This cuprizone concentration was chosen based on the findings of the previous experiments with clozapine where cell viability was not impacted and there were detectable differences in cytokine production.

Treatment with 10 μg or 20 μg MIS416 alone did not significantly increase IL-12 relative to media alone whereas LPS strongly induced IL-12 production by microglia (Figure 6.4a). The addition of cuprizone abrogated LPS-stimulated IL-12 production but the addition of MIS416 to cultures with LPS and cuprizone removed this inhibition.

The addition of cuprizone to MIS416-treated cells did not significantly alter the level of TNF-α detected. When LPS was present, treatment with MIS416 led to a significant increase in the production of TNF-α by microglial cells (Figure 6.4 d). Increasing the level of MIS416 trended towards an increase in TNF-α levels (purple line; Figure 6.4 d, which was reduced by the presence of cuprizone (Figure 6.4 b). Addition of LPS to MIS416-containing wells increased the total amount of TNF-α being produced, which was reduced in the presence of cuprizone. No NO was detected for MIS416 treated wells, however it is likely that due to low cell numbers for this assay, NO production was below detectable levels. Together these results suggest that cuprizone can reduce LPS-stimulated microglial production of IL-12, TNF-α, and NO and that clozapine and MIS416 have distinct and direct effects on microglia and can reverse the effects of cuprizone alone.

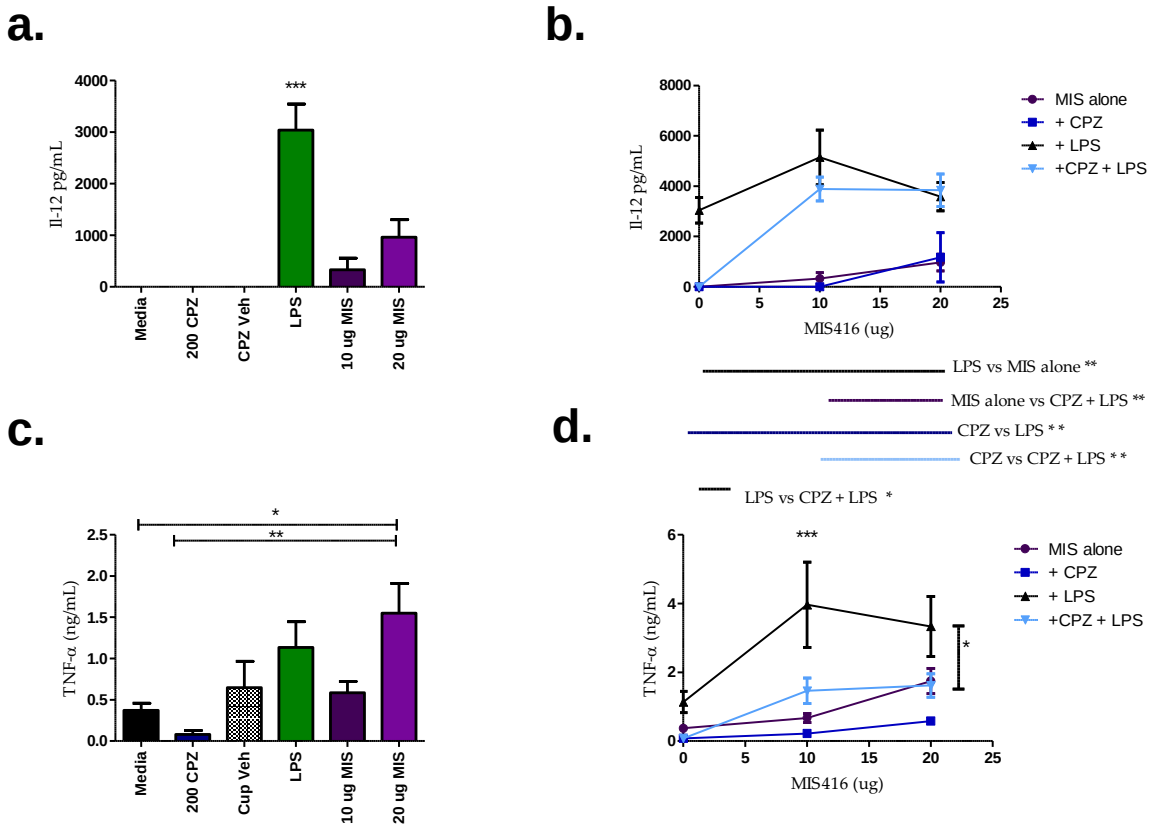

Figure 6.4: MIS416 caused significant changes to microglial IL-12 and TNF- α production.
Shown are data from 2 individual experiments. Cells were treated with LPS (200 ng/mL, MIS416 (10 μg, 20 μg) or cuprizone (200 μM). Supernatant was collected 24 hours post stimulation with the selected compounds and then supernatant TNF- α (a & b) and IL-12 levels (c & d) were measured. (a,& c) one way ANOVA *** p<0.0001 **p<0.01, *p<0.05 with Bonferroni's post-test. (b & d) two- way ANOVA with Bonferroni's post-test *** p<0.0001 **p<0.01, *p<0.05.

6.2.5 MIS416 significantly alters serum cytokines

Prior to collection of tissues for histology, a cardiac puncture was performed to collect blood for cytokine analysis, to determine whether MIS416 induced any changes in circulating cytokine levels. Based on previous work (White et al., 2014) we know MIS416 induces certain serum cytokines in healthy mice, however the effect of cuprizone on these cytokines is unknown. Given the mild effects of MIS416 on cuprizone-mediated pathology, it is possible that cuprizone is inhibiting this cytokine release and thus suppressing any beneficial effect of MIS416.

Treatment with cuprizone had no effect on serum concentrations of IFN-γ, IL-10, CXCL10 or CCL5, however, cuprizone led to a significant decrease in serum IL-6 (Figure 6.5 d) compared to healthy mice. As observed previously, levels of IFN-γ were increased after administration of MIS416 in both healthy and cuprizone-fed animals. Levels increased approximately 300-fold, from being undetectable in control animals and reaching between 200-300 pg/mL with MIS416 administration (Figure 6.5a). Another cytokine which showed a similar pattern was CXCL10/IP 10, which was undetectable in control animals and increased significantly with MIS416 treatment (unpaired t-test, $p<0.05$) (Figure 6.5c). Treatment with MIS416 led to a trend towards increased levels of IL-10 and IL-6, however these differences were not significant when compared to untreated-cuprizone fed control animals, probably due to the low number of serum samples tested. CCL5/RANTES was also found at significantly increased levels in the serum of MIS416-treated animals when compared to cuprizone-fed controls and healthy controls. The level of CCL5/RANTES was increased further in MIS416 alone treated animals when compared to MIS416 treated-cuprizone fed animals, indicating that cuprizone could be acting to reduce the MIS416-induced increase in CCL5/RANTES.

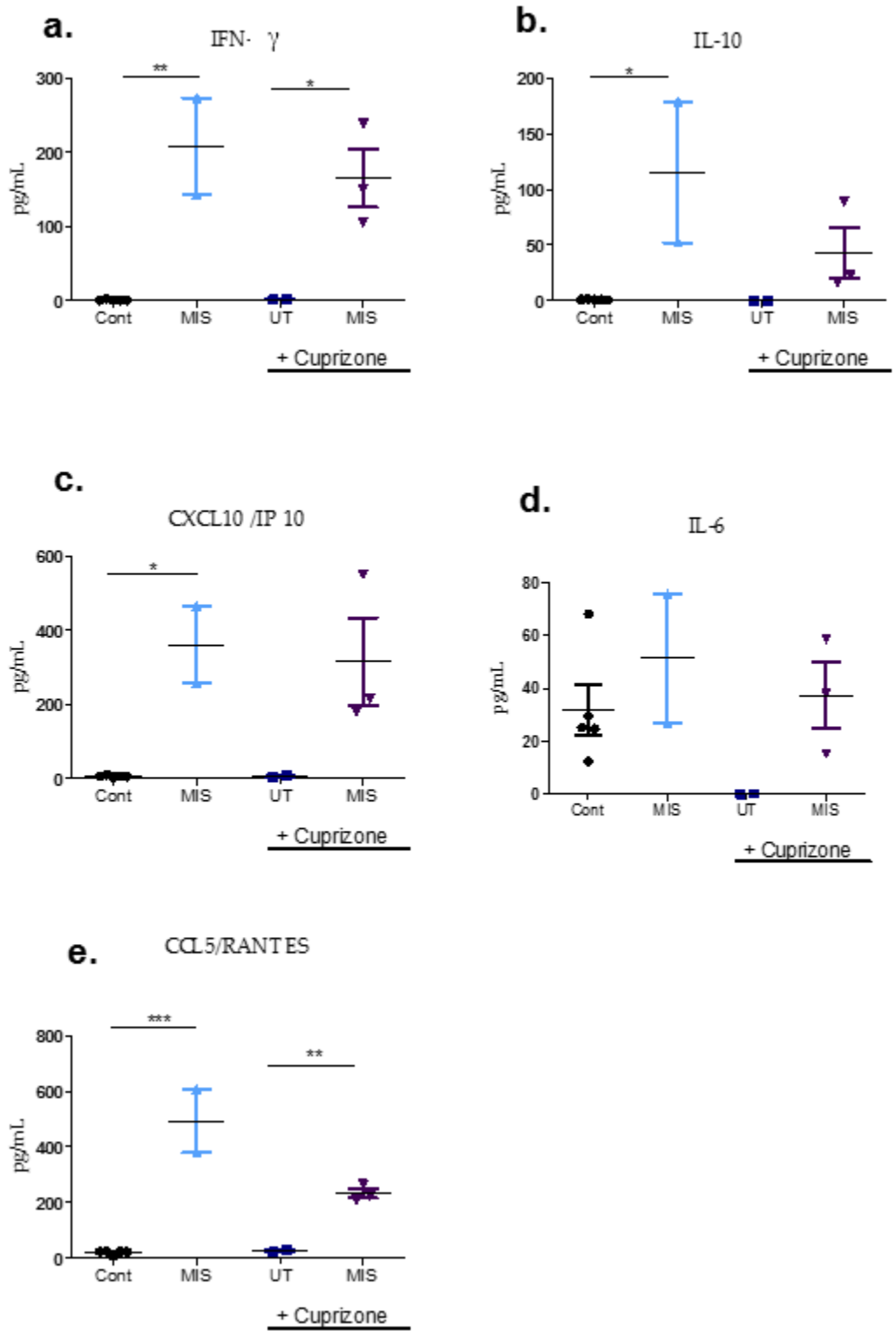

Figure 6.5: MIS416 caused significant changes to serum cytokines.
Following administration of 100 µg MIS416 i.v weekly, serum was isolated from blood after cardiac puncture and cytokines were detected via CBA according to manufacturer's instructions. Due to small sample size normality was assumed and an unpaired t-test was used *$p<0.05$ **$p<0.01$ ***$p<0.005$.

6.2.6 MIS416 significantly altered growth factors and inflammatory markers

To explore other markers of inflammation, the serum levels of ICAM, VEGF, and EPO were determined. Cuprizone had only a minor effect on serum levels of ICAM whereas it led to a significant decrease in both EPO and VEGF. ICAM was found to be significantly increased in the serum of MIS416-treated mice when compared to healthy controls and cuprizone-fed, untreated animals, increasing from approximately 200 pg/mL to 1500 pg/mL in cuprizone fed animals (Figure 6.6 a). This increase was even larger in animals not receiving cuprizone.

While treatment with cuprizone had no significant effect on ICAM, cuprizone caused a significant decrease in the growth factors EPO and VEGF (Figure 6.6). Treatment of cuprizone-fed animals with MIS416 rescued the levels of VEGF to those of healthy animals. Interestingly, treatment with MIS416 alone appeared to merit further investigation.

When animals were fed cuprizone, there was a significant decrease in the level of EPO in the serum of animals. In contrast, EPO were not significantly altered by treatment with MIS416 alone (Figure 6.6c). However, as with VEGF, treatment of cuprizone-fed animals with MIS416 restored levels of EPO to that of healthy, control animals. Together, these results indicate that cuprizone treatment has a negative effect on the level of circulating growth factors and that MIS416 treatment can abrogate this effect.

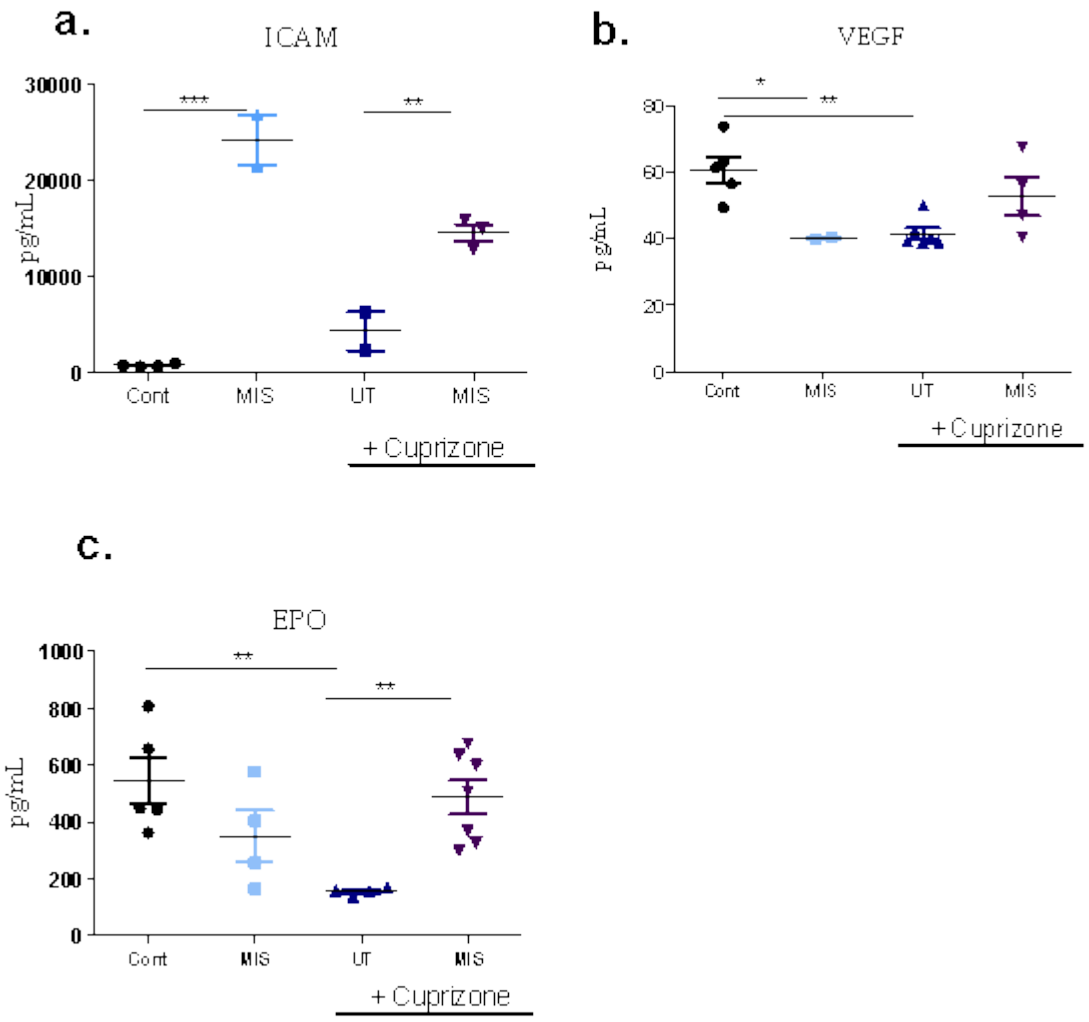

Figure 6.6: Treatment with MIS416 significantly altered growth factors and inflammatory markers. Following 0.3% cuprizone diet and weekly i.v with 100 µg MIS416, serum was isolated from blood after cardiac puncture and cytokines were detected. Due to small sample size normality was assumed and an unpaired t-test was used. *p<0.05 **p<0.01 ***p<0.001.

6.3 Discussion

As the resident immune cells of the CNS, microglia play a crucial role in responding to CNS injury, acting as the first defence against invading pathogens and maintaining homeostasis by coordinating other CNS populations through the release of soluble factors such as cytokines (Merson, Binder, & Kilpatrick, 2010). Microglia are the primary immunocompetent cells, which release NO and ROS to regulate inflammatory processes. Activation of microglia can also cause damage to neighbouring cells, an effect known as bystander damage. For this reason, controlling microglial activation during MS could provide a therapeutic target. Post mortem studies have indicated the microglia are often seen closely associated with oligodendrocytes (H. Lassmann, Bruck, Lucchinetti, & Rodriguez, 1997), and that in one case-study, the density of HLA-DR+ microglial cells was positively correlated with the level of remyelination seen in lesions (Pasquini, Calatayud, Bertone Una, et al., 2007).

Following treatment with clozapine, no significant alterations were detected in the production of IL-12 in the presence of cuprizone or clozapine, indicating that clozapine is not acting directly through microglia to alter the levels of the pro-inflammatory IL-12. Clozapine, however, was able to significantly reduce levels of TNF-α and NO when cuprizone was not present. Bian (Bian et al., 2008) assessed the effect of quetiapine on microglial cells and found that quetiapine, another atypical antipsychotic, was able to significantly inhibit NO and TNF-α release from IFN-γ activated microglia. This finding is in line with the current study, where LPS-induced NO and TNF-α production was significantly reduced by clozapine. This finding could provide key evidence for the mechanism of protection provided by atypical antipsychotics, as by inhibiting the release of pro-inflammatory cytokines from microglia the damage to neurons and other CNS cells could be limited.

This finding that clozapine is able to reduce these pro-inflammatory cytokines in inflammatory conditions (i.e. in the presence of LPS) is also in line with literature indicating that *in vivo,* clozapine is able to lead to a reduction in inflammation in schizophrenic patients who are treated with

clozapine (Hu et al., 2012; Kato et al., 2011; Meltzer & McGurk, 1999). The current finding, that in the presence of clozapine, TNF-α is suppressed provides promising preliminary data, however the addition of cuprizone inhibited this effect, so further research is needed as to whether clozapine may provide a protective effect in progressive MS where lesions more closely resemble those seen in the cuprizone model (Lucchinetti et al., 2000). The level of LPS induced NO production was also found to be decreased with the addition of clozapine *in vitro*, further supporting its ability to reduce LPS-induced inflammation in the absence but not presence of cuprizone. The finding that clozapine was able to significantly reduce LPS-induced NO is in direct contrast to data in a review by Monji et al (Monji, Kato, & Kanba, 2009) which showed no inhibitory effect of clozapine on LPS induced NO production (Hou et al., 2006). This discrepancy could be due to multiple factors including the cells used (M9 vs primary microglial culture), the length of stimulation by clozapine and the concentration of clozapine to which the cells were exposed.

These results presented in this chapter, in combination with published literature indicate that clozapine may provide an anti-inflammatory effect through the inhibition or alteration of microglial responses. This could provide protection to neurons and immature oligodendrocytes as well an many other CNS cell populations which are negatively affected during inflammation associated with MS and play a significant role in disease pathology.

In an inflammatory setting (i.e. LPS exposure), levels of the pro-inflammatory cytokine IL-12 were increased with MIS416 treatment, however these levels were lower than with LPS. Once LPS was present, MIS416 did not alter IL-12 levels. In contrast to this, MIS416 was able to remove the cuprizone-mediated suppression of IL-12 in the presence of LPS. No NO production was detected in response to MIS416 administration.

MIS416 is able to induce enhanced levels of TNF-α, and is able to do this in the presence of cuprizone, indicating that MIS416 is directly stimulating microglia to be pro-inflammatory. Further

investigation is required to determine whether driving microglia into a pro-inflammatory state provides a beneficial effect on the cuprizone model.

This discrepancy in the role of activated microglia being both protective and detrimental can be explained, in part by the time at which they are activated. It is clear that early microglial activation is required for the removal of myelin fragments (Neumann et al., 2009), however, if microglial activation continues past this point through to the remyelination phase it could be detrimental by producing a sustained inflammatory response and continuing to recruit pro-inflammatory microglia to the lesion.

Glatiramer acetate, a current therapy for MS administered to mice prior to a cuprizone diet also improved remyelination and promoted oligodendrocyte maturation (Rosato Siri, Badaracco, & Pasquini, 2013) and led to microglial activation. Further *in vitro* MG assays showed that GA primed MG had a significant increase in IL-10 and IL-4 production. Since GA was shown to provide protection and promote effective remyelination, potentially mediated through increasing IL-10 levels, the MIS416 induced significant increase in serum IL-10 seen in the current study could provide a mechanism through which MIS416 is acting.

Feeding with cuprizone led to a significant reduction in levels of erythropoietin (EPO) relative to healthy control levels in the current study, which is a novel finding. When treated with MIS416, EPO levels were increased to a level not significantly different to healthy control animals, but significantly higher than cuprizone-fed animals. This change indicates that cuprizone acts to lower EPO levels in the serum, and treatment with MIS416 is able to return these levels to within a 'normal' range. Hagemeyer (Hagemeyer et al., 2012) showed that injecting EPO on alternate days throughout a 6 week cuprizone diet led to EPO-treated animals performing significantly better on motor tasks than placebo controls. These EPO animals also had less cuprizone-induced ventricle enlargement when compared to placebo controls and EPO attenuated the microgliosis normally seen with cuprizone intoxication. Again, no behavioural improvement was seen in the current

study with MIS416 treatment, however if MIS416 is able to increase EPO levels sufficiently it is possible a protective effect could be seen during remyelination. A further area for investigation would be looking at changes to ventricle enlargement or microgliosis.

Vascular endothelial growth factor (VEGF) is another serum growth factor that was significantly reduced during cuprizone feeding. As seen with EPO, MIS416 treatment is able to restore VEGF levels in cuprizone fed mice to a level not significantly different to levels in healthy mice. A survey of current literature was unable to identify any studies where the role of VEGF in the cuprizone model or EAE was investigated, indicating that this reduction in VEGF in the cuprizone model is a novel finding. Furthermore the increase of VEGF in response to MIS416 treatment is also new. This increase in VEGF could be allowing for the angiogenesis to occur in the CNS, allowing the tissue remodelling and lesion repair to occur successfully after a demyelinating injury, which could have occluded blood supply and caused cell apoptosis in a localised environment. This increase in VEGF, combined with the increase in EPO seen following MIS416 treatment indicates that MIS416 could be targeting the bone marrow to increase the production of red blood cells, a process which would be mediated by the new vessel formation triggered by increasing levels of VEGF. This novel finding is exciting and provides an avenue for further experimental work, potentially investigating the relationship between MIS416 and its direct effect on bone marrow cells *in vitro* as well as the dose response relationship between MIS416 and EPO levels. Repeating demyelination experiments with higher, or more frequent doses of MIS416 could show a sufficient increase in EPO levels to provide protection, if EPO production is a direct result of MIS416 treatment, which, based on serum EPO data from this chapter, is possible.

Identification of EPO as a potential therapy for MS, especially progressive MS, is not a new idea, with studies showing EPO to be anti-apoptotic, anti- inflammatory and neurotrophic in the nervous system in both animal models of MS (Diem et al., 2005; Ehrenreich et al., 2007; Saettler et al., 2004) and in humans (Ehrenreich et al., 2007). A review published by Bartels et al. (Bartels, Spate, Krampe, & Ehrenreich, 2008) extensively summarised the protective effects of EPO

administration reported in EAE as well as summaries of human MS EPO trials. In a phase IIa trial (Ehrenreich et al., 2007) it was found that with EPO treatment, patients had improved motor function and reduction in the expanded disability status scale, a questionnaire commonly used to assess MS patients. This finding, combined with the low occurrence of adverse events points towards EPO being a potential therapeutic for MS, so any link between MIS416 and EPO levels is worth investigating fully.

IFN-γ has been shown to be crucial for the protective effects provided by MIS416 in the EAE model (White et al., 2011). Treatment with MIS416 has also been shown to significantly increase levels of IFN-γ in the serum of healthy and EAE mice and MS patients. Again this same increase in IFN-γ levels was seen in both healthy, MIS416-treated and cuprizone-fed, MIS416-treated animals, with levels increasing significantly from zero to approximately 150 pg/mL (Figure 6.6). The role of IFN-γ, a pro-inflammatory cytokine secreted by activated T cells, in demyelinating disease is still contended, with some papers reporting a deleterious effect of IFN-γ during MS and EAE (Lin et al., 2006). However, some mouse strains normally resistant to the development of EAE will become susceptible to EAE when the IFN-γ receptor or gene is knocked out, indicating that in these mice, IFN-γ is conferring protection (Gao et al., 2000). In a study by Gao (Gao et al., 2000) the effect of IFN-γ in the cuprizone model was examined. It was found that transgenic mice expressing low levels of IFN-γ in the CNS were protected from demyelination, oligodendrocyte death, astrogliosis and microgliosis seen in the non-transgenic, cuprizone-fed controls. This finding indicates that in the cuprizone model, an increase in the level of serum IFN-γ is protective during periods of demyelination. The experiments in this chapter measured a significant increase in serum IFN-γ, which is expected with MIS416 treatment (White et al., 2014), and although no protective effect was seen in remyelination, it is possible that at an earlier time point, such as during the demyelination period of disease an MIS416 induced increase in IFN-γ could have provided a protective effect. Serum cytokines were not assayed during demyelination experiments and so this could prove a useful future experiment to determine whether the MIS416 induced increase in IFN-

γ will be sufficient to prevent demyelination as seen in previous studies where animals had increased IFN-γ secretion (Gao et al., 2000). Gao et al. also reported an increase in levels of insulin-like growth factor in the CNS of animals with elevated IFN-γ, which has previously shown a protective effect against demyelination induced by cuprizone (Ye & D'Ercole, 1999) so the protective effect seen in their studies could be due to an interaction of these cytokines rather than solely a result of IFN-γ, explaining why a similar prevention of demyelination was not seen in our early demyelination experiments (chapter 4).

Lin and colleagues (Lin et al., 2006) also studied the effect IFN-γ expression in the CNS and found that in both the cuprizone model and EAE there was a severe reduction in the level of remyelination with IFN-γ. This failure to remyelinate was also correlated with a reduction in the number of oligodendrocytes at lesion sites. This study used a similar experimental set up to ours, with mice receiving a 0.2% cuprizone diet before being allowed to 'recover' for 3 weeks. It is possible that if MIS416 treatment had raised the IFN-γ levels sufficiently in these animals a negative impact on remyelination could have occurred. Conversely, had MIS416 raised the levels of IFN-γ even higher, a protective effect could have been seen, similar to that reported by Gao (Gao et al., 2000). These opposing roles of IFN-γ could be due to the time at which the cytokine levels are rising, for instance early increases in IFN-γ could prevent demyelination, and later on in cuprizone-mediated disease, higher levels of IFN-γ could play a detrimental role to the remyelination process. To investigate this further, and assess any potential therapeutic role for MIS416 in regulating IFN-γ levels, serum cytokines would need to be assayed at multiple different time points and the level of IFN-γ required to have an effect in the CNS would need to be determined, as would the dose of MIS416 required to achieve this response. Repeats with a higher number of samples will also improve the significance and help interpret the importance of these data.

CXCL10 is a cytokine involved in recruiting microglia, and is IFN-γ inducible. In mice treated with MIS416, we saw a significant increase in levels of CXCL10 (Figure 6), indicating that any reduction in CXCL10 levels caused as a result of cuprizone could be returned to 'normal' levels. Clarner (Clarner et al., 2015) used genome wide expression studies to investigate the effect of cytokines during cuprizone mediated demyelination and found that *Cxcl10* knock out mice had significantly reduced levels of microglial activation, and that this was associated with an improvement in cuprizone-induced toxicity at later time points. Further *in vitro* studies also showed that CXCL10 induced migration and a pro-inflammatory phenotype in cultured microglia, and that *Cxcl10* mRNA was mainly expressed by astrocytes, providing further information supporting the idea that astrocytes are the primary recruiters/activators of microglia during inflammation. Previous work from our lab group has shown that MIS416 treatment leads to an increase in CXCL10 and IFN-γ in EAE. As CXCL10 is an IFN-γ-induced protein, the increase in serum levels of both these cytokines fits with what we already know about MIS416 treatment in the EAE model. Because we have observed the same increase in the cuprizone model, it provides evidence that this alteration is a direct result of MIS416, is part of its specific activity/mechanism and is not model specific.

One of the findings from this chapter was a trend towards an increase in levels of IL-6 with MIS416 treatment in cuprizone-fed animals (Figure 6) as well as an MIS416-driven increase in IL-6 to 'normal' levels in cuprizone fed animals. The role of IL-6 is multi-functional and although it is often found at increased levels in serum and CSF of patients with MS, the role of this cytokine in disease is still debated (Maimone, Guazzi, & Annunziata, 1997). In MS lesion histology, a protective role for IL-6 was reported, (Schönrock, Gawlowski, & Brück, 2000) with IL-6 levels positively correlating to the number of oligodendrocytes at the lesion. *In vitro* studies backed up this protective role showing that IL-6 could promote oligodendrocyte differentiation and survival (Pizzi et al., 2004). Using the cuprizone model, Petkovic et al. (Petkovic et al., 2016) made transgenic mice to produce IL-6 from astrocytes to determine the effect of IL-6 levels on demyelination. These mice showed a significant reduction in microglial accumulation and

astrogliosis in the corpus callosum when compared to normal mice receiving cuprizone. As no significant effect on astrogliosis was seen in previous chapters, MIS416 is unlikely to alter the IL-6 levels enough to directly impact these cells, however experimental groups were small (n 4-12). The lack of microglial activation seen by Petkovic and colleagues was associated with a lack of efficient myelin removal after demyelination, and so was associated with a detrimental outcome.

6.4 Summary

Clozapine and MIS416 are both able to alter the cytokine profile of primary microglia *in vitro.* With clozapine causing alterations toward a less pro-inflammatory phenotype, characterised by a significant reduction in LPS-induced NO and TNF-α. Treatment with MIS416 produces similar serum cytokine changes in the cuprizone model to those previously documented in EAE, as well as reverses cuprizone induced reduction of EPO and VEGF, a finding novel to this study. Additionally, in the presence of cuprizone, both clozapine and MIS416 enhance cytokine production. Taken together the results presented in this study indicate that clozapine and MIS416 are able to alter the cytokine profile *in vitro* and *in vivo*, respectively.

CHAPTER 7: General Discussion

7 General Discussion

Overall the results of this study have highlighted that clozapine is able to improve cuprizone-induced behavioural deficits following demyelination and while it may have a beneficial effect on astrocyte activation in the early recovery stage, clozapine does not appear to enhance remyelination significantly. Additionally, this study found that MIS416 did not inhibit demyelination, enhance remyelination, or enhance recovery from behavioural deficits induced by cuprizone administration despite maintaining its previously documented immunomodulatory properties in the cuprizone model. Finally, it was demonstrated that cuprizone administration caused a significant reduction in serum growth factors and this deficit was rescued by MIS416 treatment. Together this work shows that two known immunomodulatory agents, currently being investigated as potential therapies for SPMS, have distinct and beneficial effects in a non-immune model of demyelination.

Previous work from our lab group has identified the immunomodulatory agent MIS416 as being able to reduce disease severity in EAE (White et al., 2011), an immune mediated progressive model of MS. MIS416 acts primarily on the innate myeloid cells through activation of TLR-9 and NOD-2 receptors. Clozapine, an atypical antipsychotic, has also been shown to reduce disease severity in the EAE model by our lab group (O'Sullivan et al., 2014). As the mechanisms by which these two immunomodulatory compounds provide protection are not fully understood, further work is needed to characterise beneficial effects, as well as assess their ability to provide protection in other disease models. The use of another model for multiple sclerosis, the cuprizone model, allows the assessment of any therapeutic properties of MIS416 or clozapine with the exclusion of peripheral immune components.

In the current study it was found that neither MIS416 nor clozapine were able to reduce the rate of demyelination in the corpus callosum, or prevent the cuprizone induced motor deficits seen in these experiments. Since no improvement in behaviour was seen throughout the experimental phase, it was not surprising to see no protection from demyelination of the corpus callosum. Once the myelin around neurons has been degraded signal transduction is altered (Bruce et al., 2010; Franklin, 2002), leading to motor impairments as well as a recruitment of cells for debris clearance, promoting an inflammatory environment. When astrocytes were assessed for activation in the corpus callosum there was strong activation (astrogliosis) induced by cuprizone, which was not reduced significantly by MIS416 or clozapine (chapter 4). This astrogliosis is likely in response to the demyelination occurring, as the astrocytes are involved in the clearance of myelin debris and secretion of cytokines involved in the recruitment of other phagocytic cells to the site, such as CXCL10 to recruit macrophages/microglia (Clarner et al., 2015).

As one of the key features of SP-MS is the loss of the ability to remyelinate, the effects of MIS416 and clozapine on remyelination was examined using the cuprizone model. The current study was unable to detect any improvement in behaviour with MIS416 during the remyelination period of disease, however a significant behavioural improvement was seen with clozapine administration during this remyelination period (chapter 5). This behavioural improvement was not associated with an increase in myelin levels in the corpus callosum as assessed by luxol fast blue staining, nor was in associated with an alteration in astrocyte activation. In MS patients, remyelination at lesion sites is associated with remission periods of disease and an improvement in motor coordination (Franklin, 2002), so this improvement seen in behaviour in the current study is an indication that clozapine could be altering myelin levels.

Previous work from our group (White, 2015) has shown that MIS416 alters the splenocyte populations as well as their cytokine profile in EAE. As seen previously, treatment with MIS416

led to a visible spleen enlargement (chapter 5), as well as a significant reduction in the number of NK1.1 cells. Interestingly in the cuprizone model we saw a reduction in the number of Treg cells following MIS416 administration, which were increased following MIS416 treatment in EAE (White, 2015). In EAE, an immune mediated MS model, the upregulation of Tregs could provide a protective effect by suppressing immune cell activity, whereas the cuprizone model does not require this immune suppression. Additionally, in the cuprizone model, MIS416 administration caused similar serum cytokine changes to those seen in EAE, indicating that the increase in serum IFN-γ, CXCL10 and IL-6 are induced by MIS416 and are independent of the disease model. One of the novel findings from this chapter (chapter 6) was the finding that MIS416 led to restoration of the cuprizone induced reduction in VEGF and erythropoietin, two factors involved in red blood cell production and vasculogenesis.

Microglia have long been identified as playing a key role in demyelinating disorders (Benveniste, 1997; Hendriks et al., 2005). The direct effect of MIS416 and clozapine on these cell populations provides novel and key information on how these treatments could be altering the CNS in periods of demyelination or remyelination.

7.1 Cuprizone as a model for Multiple Sclerosis

The cuprizone model for demyelination is a model of toxic demyelination, where the peripheral immune system is excluded from the CNS, allowing the formation of lesions that more closely mimic those seen in patients with SP-MS. Although EAE is commonly used to assess therapies for MS, the cuprizone model has recently become more widely used as an MS model. Use of the cuprizone model allows the oligodendrocyte population to be more closely studied *in vivo*. Cuprizone selectively targets the mature oligodendrocyte population, leading to demyelination and motor impairments. Since the blood brain barrier remains intact in this model, the effects of the peripheral immune system can be excluded to produce lesions more closely resembling those in

progressive MS. Using this model can allow investigators to determine whether a compound of interest can protect the mature oligodendrocyte population, or whether it can alter the rate of OPC maturation or increase mature oligodendrocyte migration to lesions sites. All of these effects could be beneficial in preventing demyelination or improving rates of remyelination and could be harnessed as a therapy targeted towards those patients with progressive MS. The cuprizone model is becoming increasingly useful, as investigators are looking towards targeting remyelination as a therapy to provide in combination with the current immunomodulatory therapies (Chamberlain et al., 2016; Tognatta & Miller, 2016) such as GA or IFN- β.

7.2 Implications for therapy

Although the current study identified changes and improvements to a variety of parameters following MIS416 or clozapine treatment, it is unclear yet as to how these changes would benefit MS patients. A study which would provide useful information would be assessing any benefit provided by combining either MIS416 or clozapine with a currently approved MS therapy such as GA IFN-β, to determine if any synergistic effects can be produced by combining treatments which work through different pathways. This idea is becoming more prevalent in the literature, with laboratories aiming to find ways to target remyelination through the oligodendrocyte population as an alternative or supplement to current therapies (Chamberlain et al., 2016; Chew & DeBoy, 2016).

Another point to consider when assessing therapeutic potential of the test compounds is the method and ease of administration for the patients who would rely on them. For the current experiments clozapine was administered orally, and based on its current use to manage schizophrenia we know it can be absorbed through the gut and that the dose required is tolerated by humans. Additionally, the use of clozapine as a treatment for MS is advantageous as it is already a well- known and prescribed medication. This would mean doctors would be more comfortable prescribing it, and the side effect profile is well characterised, meaning adverse reactions could be spotted immediately. Clozapine being a well-known drug and being used clinically for decades

would also lead to greater patient confidence in the medication which could also improve compliance.

A trial for clozapine has just begun recruiting for a phase 2 trial to measure safety and acceptability in patients with SP-MS. The trial is a randomised, placebo controlled and blinded, designed to determine any effects on MS severity or adverse events. The findings from the current study showing a significant beneficial effect of clozapine on behaviour, as well as the protection clozapine provides in EAE (O'Sullivan et al., 2014) indicate that clozapine has the potential to reduce inflammation and disease in these patients.

MIS416 has been shown to be most effective during EAE when administered with weekly intravenous injection (White, 2015). Due to the motor impairments present in those with MS, and the difficulty in performing intravenous injections, treatments would need to be performed in a medical setting or by a nurse able to travel to patients' homes. This could place a large stress on patients with limited mobility, as accessing this care would not be easy during disease episodes, as well as coming with an associated cost. MIS416 is currently undergoing a phase 2b randomized double blind placebo controlled clinical trial based in New Zealand and Australia to assess the efficacy and safety in treating patients with SP-MS.

7.3 Future Directions

The work in this thesis investigated whether clozapine or MIS416 were able to provide protection in the cuprizone model of demyelination. Results from this study showed that clozapine was able to improve some of the cuprizone-induced behavioural deficits, however the mechanism through which this protection was conferred has yet to be identified.

Although the corpus callosum was the region of interest for the current study, several other areas of the brain are subject to demyelination in the cuprizone model, including the hippocampus, cerebellum and the striatum (Groebe et al., 2009; Gudi et al., 2009). A key next step would be assessing demyelination in other areas of the brain, especially the striatum and the cerebellum. These are areas targeted during cuprizone-induced demyelination (Gudi et al., 2009; Koutsoudaki et al., 2009) and are both areas involved in motor co-ordination. Identifying the effect of clozapine treatment on demyelination and remyelination in these areas could provide answers as to how clozapine is improving the cuprizone-induced behavioural deficits seen in this study.

MIS416 was shown to alter splenocyte population and cytokine profiles in a similar way to that seen in EAE, indicating that these alterations are key to its mechanism of action. In addition to cytokine changes already investigated in other disease models, two novel changes were noted as a result of MIS416 treatment; changes to EPO and VEGF levels.

EPO has previously been trialled as a therapy for MS, including progressive MS, with results from animal and human studies indicating that EPO administration is anti-apoptotic, anti-inflammatory and neurotrophic in the CNS (Diem et al., 2005; Ehrenreich et al., 2007; Saettler et al., 2004). A phase IIa trial (Ehrenreich et al., 2007) found EPO treatment improved patients motor function and reduced patient scores on the extended disability status scale. These results, combined with preliminary data from the current study indicate MIS416 could have a therapeutic potential through EPO, so this link between MIS416 and EPO merits investigation.

The serum cytokine and growth factor results presented in chapter 6 showed novel cuprizone-induced changes that were reversed by administration of MIS416 suggesting that MIS416 administration may provide unexpected benefits to MS patients. Administration of EPO has shown to provide beneficial effects when administered to patients with MS (chapter 6). Although the results from this chapter are preliminary and exploratory, the effect of MIS416 induced changes to EPO levels is a study which once verified could provide another therapeutic benefit of MIS416

administration. Another cell that could provide some protective effect are microglia. Due to inability to get an antibody to consistently stain these cells, this was not achieved in the current study, however because of the close relationship between microglia and astrocytes during demyelination (Skripuletz et al., 2013) this cell population could be exerting a significant effect. Previous work from our lab has also shown that clozapine was able to reduce Iba-1 in the EAE model.

Another experiment which would provide important information when identifying the effect of clozapine and MIS416 in the cuprizone model is the direct effect of these drugs on oligodendrocytes *in vitro* and *in vivo.* Oligodendrocyte death is a key feature of lesions in SP-MS (Lucchinetti et al., 2000) and so the ability to protect these cells provides a promising therapeutic avenue. *In vivo* studies using the cuprizone model have previously shown that administration of quetiapine could significantly increase remyelination and enhance the number of mature oligodendrocytes present in lesions (Zhang et al., 2012), as well as improving spatial working memory. Although no increase in the number of mature OG's was seen in the current study, it is possible that if assessed at a later time point, as in the study by Zhang (2012) there would be an increase in mature OG's. Further supporting the protection provided to OG's by quetiapine was the finding by Shao (Shao et al., 2015). Their study showed that administration of quetiapine *in vivo* reversed the cuprizone-induced increase in TNF-α and IL-6 in certain brain regions, as well as a reduction in astrogliosis, microglial activation and an increase in the number of OG's at lesion sites.

Little work has been carried out to examine the effect of antipsychotics on oligodendrocytes *in vitro* in the presence of cuprizone. One study by Xu (Xu et al., 2014) showed that cuprizone inhibited or reduce different stages of oligodendrocyte development, resulting in a decrease in the number of OG's capable of producing myelin. All of the cuprizone-induced inhibitory effects were ameliorated with clozapine and quetiapine. Further study into the mechanism of quetiapine showed that quetiapine directly prevented the attenuation of development from O4+

oligodendrocytes to CNP and MBP positive oligodendrocytes capable of producing myelin. By replicating these experiments with the use of primary mouse oligodendrocytes a direct effect of clozapine on the oligodendrocyte population could be characterised. Based on current literature it would be expected that clozapine would reduce or inhibit any detrimental effect of cuprizone on the oligodendrocyte development. The ability to show a direct effect of clozapine on oligodendrocytes would yield exiting results, supporting the use of clozapine as a therapy in MS for the protection of oligodendrocytes and the potential to remyelinate old lesions.

In conclusion, the results from this thesis have outlined several clozapine and MIS416-mediated changes in the cuprizone animal model. Although the direct benefit some of these changes could provide in patients with MS is yet to be determined, preliminary results show some novel and exciting avenues for further investigation. Determining the mechanism by which clozapine provides improved performance on behavioural assays will be key in determining whether it is having a direct effect on myelin or whether it is acting more widely to alter the CNS environment. MIS416-induced changes in serum cytokines EPO and VEGF also indicate another avenue in which MIS416 may be providing the protection and disease symptom improvement in MS patients, however further work will be needed to confirm this possibility.

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Appendices

9 Appendices

9.1 Appendix: Effect of Clozapine on Splenocyte populations

Clozapine or clozapine vehicle had no significant effect on the cell count of the spleen. This was expected based on the appearance of the spleens during organ removal, as spleens from clozapine treated mice were not visibly different to control or untreated-cuprizone fed animals.

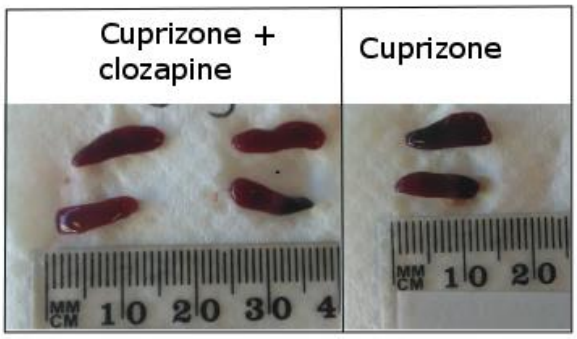

Figure A.1: Clozapine did not visibly alter spleen size.
Following 6 weeks of a cuprizone diet and 3 weeks recovery, spleens were isolated and photographed to identify any visible enlargement.

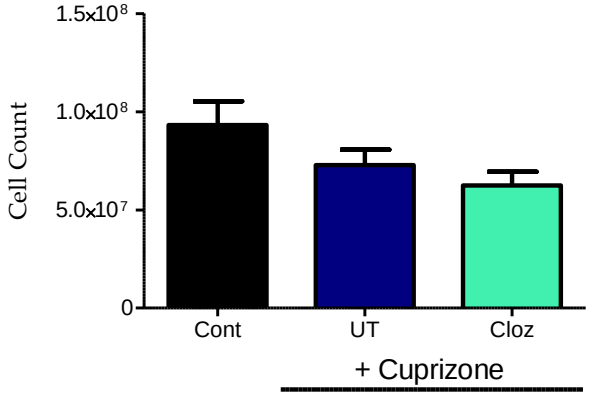

Figure A.2: Clozapine did not significantly alter the total cell count of the spleen following 6 weeks demyelination + 3 weeks recovery.
Following 6 weeks of a cuprizone diet and 3 weeks recovery, spleens were isolated and a single cell suspension was prepared prior to a cell count using trypan blue.

As in chapter 5, splenocytes from clozapine treated-cuprizone fed animals were processed into a single cell suspension for flow cytometry. Cells were identified as CD4 cells (CD4+), CD8 cells (CD8+), Tregs (CD4+ CD25+) and NK cells (Nk1.1+) cells. Cuprizone alone (UT) did not significantly alter the cell count for any of these cell populations. Clozapine treatment did not alter the total count of any of these cell populations, with clozapine vehicle also having no effect.

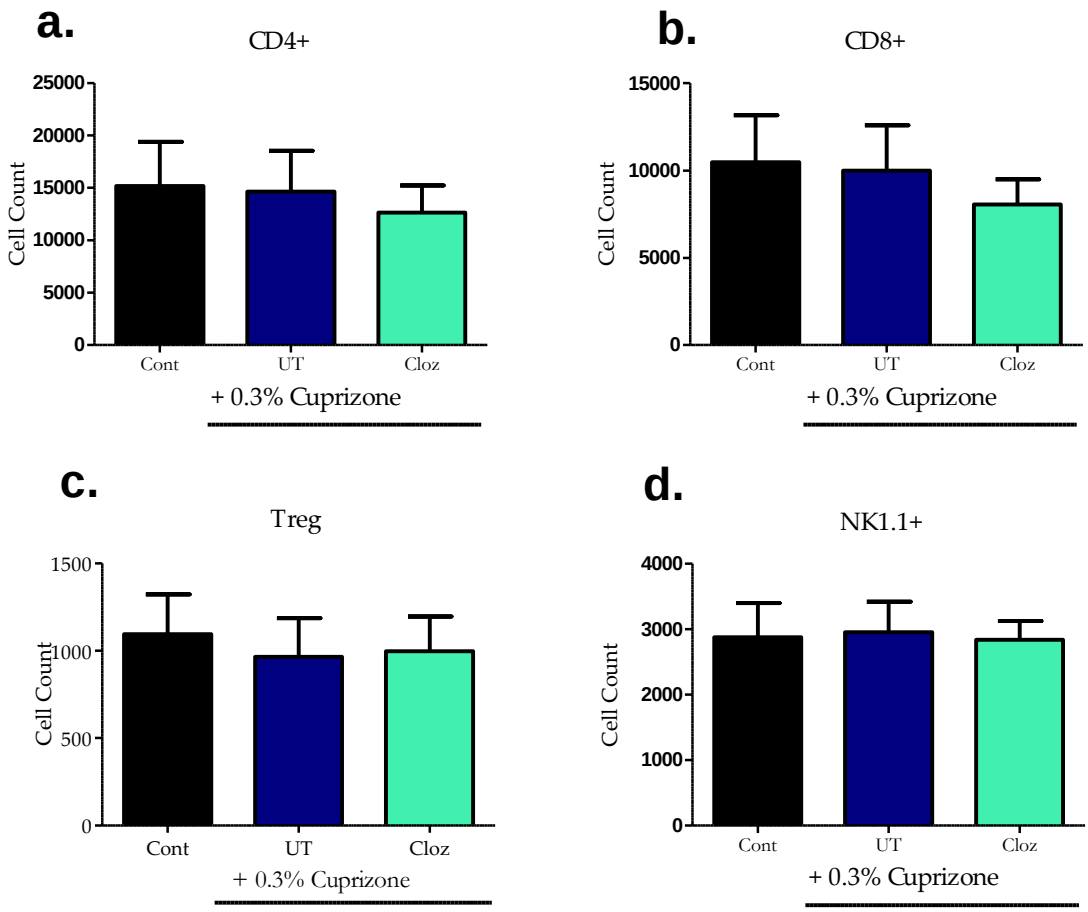

Figure A.3: Clozapine does not alter total cell counts in the spleen.
Following isolation of spleens and preparation of a single cell suspension, cells were stained with the appropriate cocktail of antibodies for flow cytometry. Clozapine did not significantly alter the number of CD4+, CD8+, Treg or NK1.1+ cells.

A second antibody panel was used to identify myeloid cells. F4/80+CD11b- cells were identified as red pulp macrophages with neutrophils being Gr-1^{high}, Ly6G^{high}, monocytes CD11b+ Ly6C+

and dendritic cells Ly6C^{low}, CD11c+. Again, clozapine had no significant effect on the numbers of cells in these populations, with total counts being not significantly different to cuprizone alone (UT) or control animals. Clozapine vehicle had no effect.

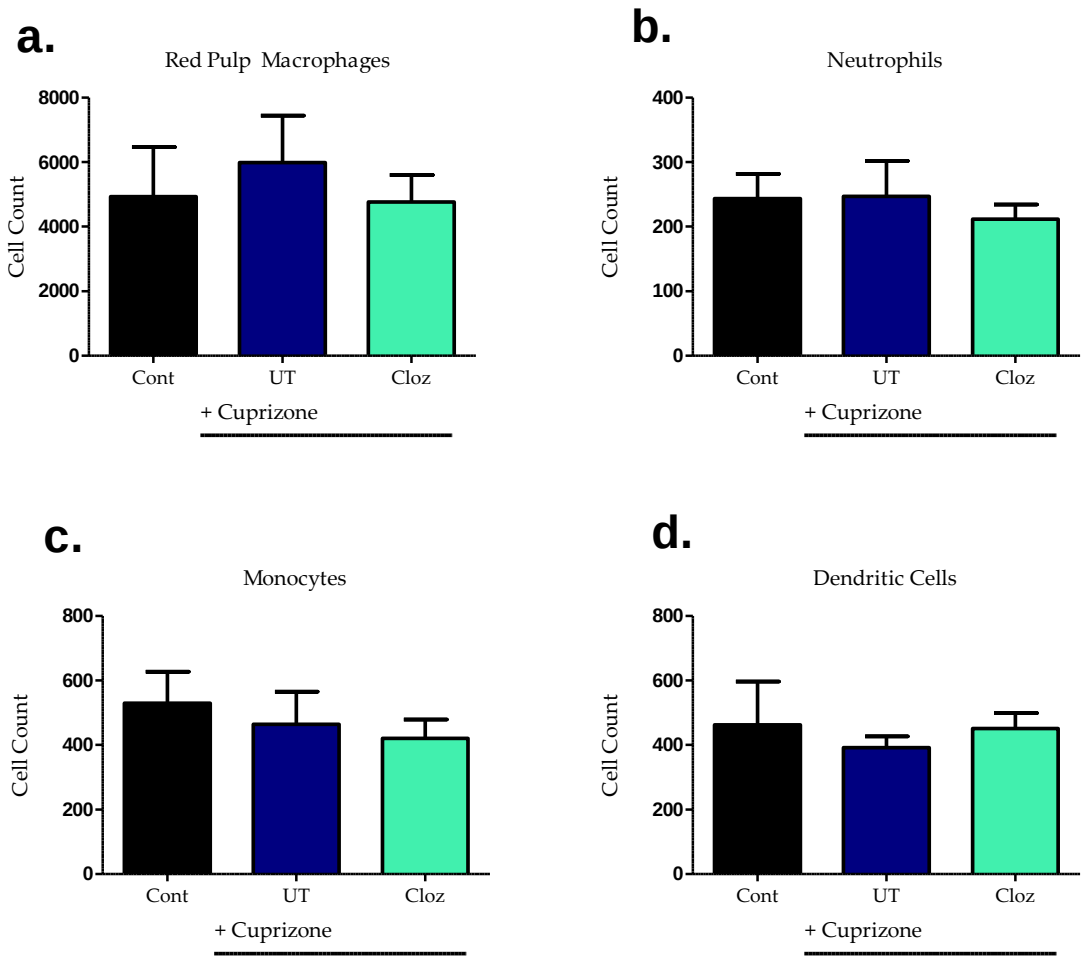

Figure A.4: Clozapine did not alter macrophage, neutrophil, monocyte or dendritic cell numbers.
Following isolation of spleens and preparation of a single cell suspension, cells were stained with the appropriate cocktail of antibodies for flow cytometry. Clozapine did not significantly alter the number of red pulp macrophages, neutrophils, monocytes or DC's.

As in chapter 5, remyelination experiments were repeated with an earlier end point, with only 1 week of 'recovery'. After 6 weeks of cuprizone diet and 1 week of no cuprizone (recovery) the effect of clozapine on splenocyte populations was again assessed. Treatment with clozapine started

in the final week of the cuprizone diet and continued until the end of the experiment (2 weeks total). At this earlier time point there was a significant decrease in clozapine treated-cuprizone fed animals when compared to control, however there was no difference between untreated-cuprizone fed animals and clozapine treated- cuprizone fed animals.

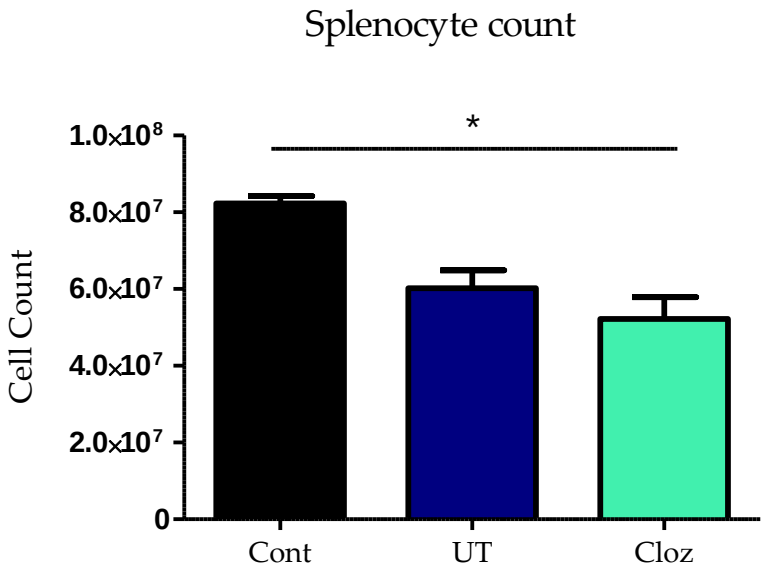

Figure A.5: Splenocyte count at earlier time point.
Following 6 weeks of a cuprizone diet and 3 weeks recovery, spleens were isolated and a single cell suspension was prepared prior to a cell count using trypan blue. Clozapine-treated cuprizone fed animals had a significant decrease in total splenocyte count compared to control animals. One way ANOVA with Bonferroni's post-test * $p<0.05$.

The myeloid populations of the spleen were again assessed with a different antibody panel to identify red pulp macrophages, dendritic cells (DC), granulocytes and white pulp macrophages. F4/80+CD11b- cells were identified as red pulp macrophages (Figure 22) with CD11b+ cells being characterised further. CD11b+Gr-1^{hi}SSC $^{med/hi}$ were classed as granulocytes with CD11b+Gr-1- cells classed as white pulp macrophages. Dendritic cells were identified as CD11b+CD11c+ cells. For gating strategy refer to chapter 5.

Cuprizone alone (UT) led to an increase in white pulp macrophages when compared to control animals. Clozapine treatment led to a significant reduction in white pulp macrophages compared to untreated-cuprizone fed animals. This clozapine induced reduction of white pulp macrophages

was to a level not significantly different to control animals, indicating that clozapine is able to reduce this cuprizone induced increase in white pulp macrophages. A similar effect was seen with dendritic cells.

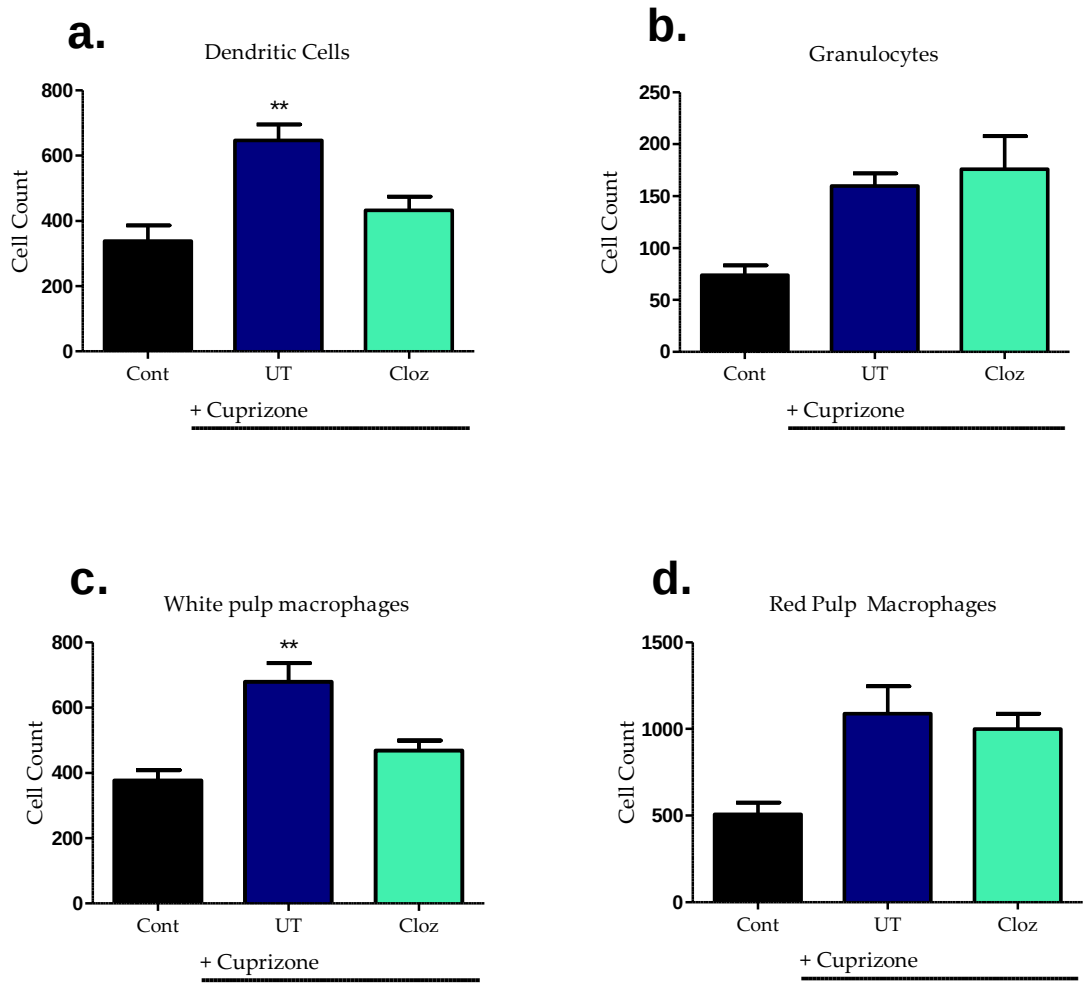

Figure A.6: Clozapine significantly altered the number of red pulp macrophages and white pulp macrophages.
Following isolation of spleens and preparation of a single cell suspension, cells were stained with the appropriate cocktail of antibodies for flow cytometry. Clozapine significantly altered the number of white pulp macrophages, one way ANOVA followed by Bonferroni's post-test *$p<0.05$.

Expression levels of PDL-1 and I/A-I/E were also assessed on splenocyte populations from animals treated with clozapine. Clozapine was found to have no impact on the expression of these markers (Figure 7).

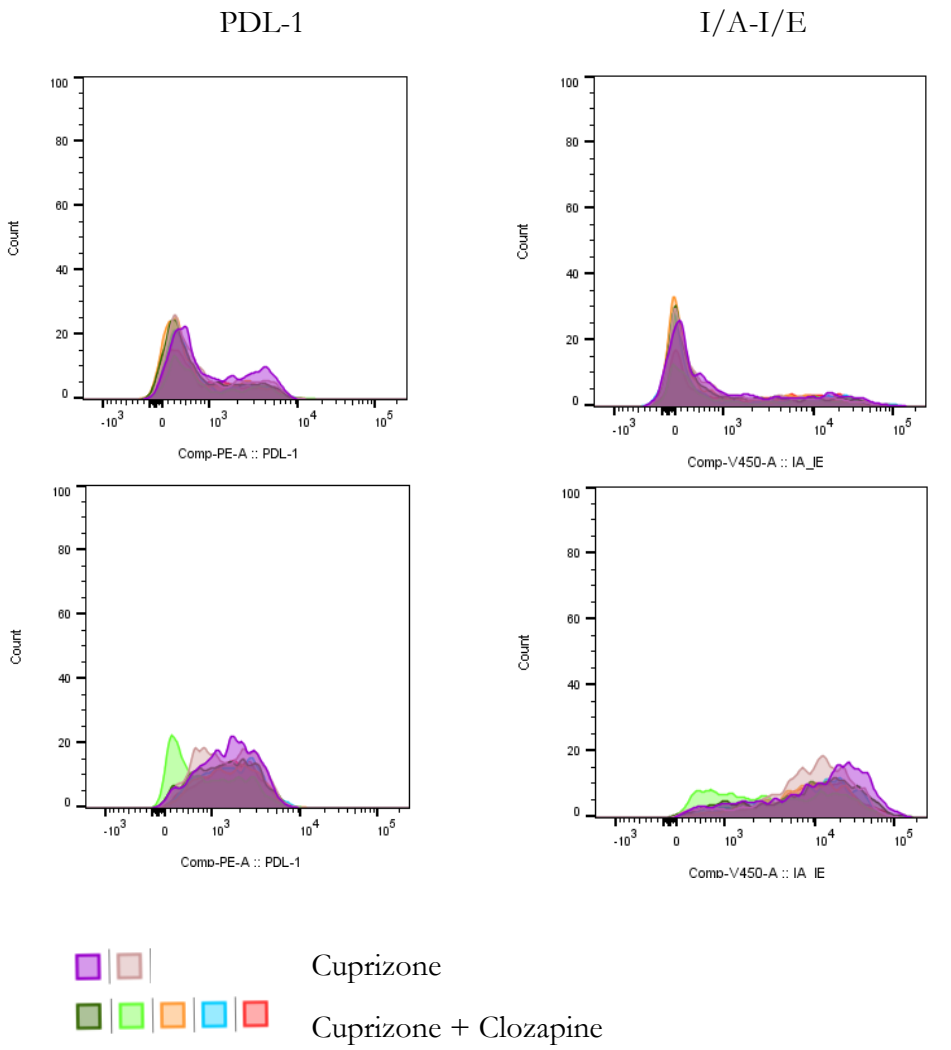

Figure A.7: Clozapine did not alter IA/-I/E or PDL-1.
Single cells were identified as either red pulp macrophages (F4/80+, CD11b-) or CD11b+ populations. Levels of PDL-1 and I/A-I/E on these populations was recorded using flow cytometry. Treatment with clozapine did not alter levels of PDL-1 or I/A-I/E.

Summary

Clozapine treatment in the cuprizone model caused no visual enlargement of the spleen, which was reflected with splenocyte counts showing no significant differences. There was a small decrease in cell count when healthy animals were compared to clozapine-treated cuprizone-fed mice, however this is likely a result of animal variation rather than an effect of clozapine, as n was small for these experiments (2-4). Number of Tregs, CD4, CD8 or NK1.1 cells were also not affected by clozapine treatment, with clozapine also having no significant impact on I/A-I/E or PDL-1 expression.

9.2 Appendix: Flow Cytometry Antibodies

Specificity	**Label**	**Species + Isotype**	**Manufacturer**	**Optimal Dilution**
CD4	BV421	Rat IgG2b	Biolegend	1:400
CD45	V450	Rat IgG2b	BD pharmingen	1:400
CD45	PE-Cy5	Rat IgG2a	BD pharmingen	1:500
CD45	BV510	Rat IgG2b	Biolegend	1:400
CD11b	AF488	Rat IgG1	Biolegend	1:400
CD11b	BB515	Rat IgG2b		1:600
CD8α	PE	Rat IgG2a	BD pharmingen	1:1000
Ly6C	PE	Rat IgG2c	Biolegend	1:800
F4/80	PerCP Cy5.5	Rat IgG2a	eBioscience	1:400
CD11c	PerCP Cy5.5	Rat IgG2a	eBioscience	1:400
NK1.1	PE-Cy7	Rat IgG2a	Biolegend	1:300
CD25	APC	Rat IgG1	Biolegend	1:400
Ly6G	APC	Rat IgG2a	Biolegend	1:500
Gr-1	APC-Cy7	Rat IgG2b	BD pharmingen	1:400

B220	V500	Rat IgG2a		1:400
CD3	APC-Cy7	Rat IgG2b	Biolegend	1:200
CD11c	APC	Hamster IgG	Biolegend	1:400
IA/IE	BV421	Rat IgG2a	BD pharmingen	1:400
PDL-1	PE	Rat IgG2a	eBioscience	1:400
Isotype Controls				
Isotype control	PE	Rat IgG2a	BD pharmingen	1:400
Isotype control	APC	Rat IgG1	Biolegend	1:400
Isotype control	APC	Rat IgG2a	BD pharmingen	1:800
Isotype Control	APC-Cy7	Rat IgG2b	BD pharmingen	1:800
Isotype Control	V450	Rat IgG2b	BD pharmingen	1:800
Isotype control	BV421	Rat IgG2b k	Biolegend	1:400
Isotype Control	PE-Cy7	Rat IgG2a, k	Biolegend	1:250
Isotype Control	PerCP-Cy5.5	Rat IgG2a, k	Biolegend	1:400
Isotype Control	AF488	Rat IgG1, k	Biolegend	1:400
Isotype Control	PE-Cy5	Rat IgG2a	BD Pharmingen	1:500
	BV510			
	BB515			

9.3 Appendix: ELISA Antibodies

All standard curves were produced using 2-fold serial dilutions in duplicate, with the final well in each series being blank.

IL-12p40 (BD Biosciences)

Reagent	**Dilution/Concentration**	**Diluent**
Capture	1:1000	0.1 M Na_2HPO_4, pH=9
Block Solution	10% FCS	1 x PBS pH=7.4
Top Standard	4ng/mL	5% FCS in 1 x PBS pH=7.4
Detection	1:1000	5% FCS in 1 x PBS pH=7.4
Strepdavidin horseradish peroxidase	1:2000	5% FCS in 1 x PBS pH=7.4

IL-10 (BD Biosciences)

Reagent	Dilution/Concentration	Diluent
Capture	1:200	0.1 M Na_2HPO_4, pH=6
Block Solution	10% FCS	1 x PBS pH=7.4
Top Standard	25ng/mL	10% FCS in 1 x PBS pH=7.4
Detection	1:2500	10% FCS in 1 x PBS pH=7.4
Strepdavidin horseradish peroxidase	1:1000	10% FCS in 1 x PBS pH=7.4

TNF-α (BD Biosciences)

Reagent	Dilution/Concentration	Diluent
Capture	4 μg/mL	0.1 M Na_2HPO_4, pH=6
Block Solution	5% FCS	1 x PBS pH=7.4
Top Standard	4ng/mL	5% FCS in 1 x PBS pH=7.4
Detection	1:1000	5% FCS in 1 x PBS pH=7.4
Strepdavidin horseradish peroxidase	1:2000	5% FCS in 1 x PBS pH=7.4

9.4 Appendix: Buffers and Solutions

10 X PBS (1L)

NaCl 170 g
Na2HPO4.12H2O 62.32 g
NaH2PO4.2H2O 4.04 g
2 litres ddH2O

1 X PBS

Dilute 10:1 ddH2O for working solution of 1X PBS.

PBST

1 X PBS

0.2% Triton-X

Pertussis toxin buffer

Tris 15mM
Sodium chloride 0.5M
Triton X-100 0.017% (v/v)
In ddH2O, filter sterilised using 0.22µm syringe filter

Wash Buffer

Dulbecco's Modified Eagle Medium 96%
HEPES buffer (1M) 3%
Penicillin/Streptomycin (100 U/ml/10 mg/ml) 1%

4% Paraformaldehyde Solution

Paraformaldehyde 4%
1xPBS 96%
ph adjusted to 7.4

Clozapine Drinking water

0.1M Acetic acid

Clozapine at 60 mg/kg

Percoll Diluent

10X PBS 45 ml
0.6M HCl 3 ml
ddH2O 132ml
Sterilise with a syringe filter

70% Percoll for CNS

63% percoll

37% Percoll diluent

37% Percoll for CNS

53% of 70% Percoll

47% 1 X PBS

30% Percoll for CNS

43% of 70% percoll

57% 1 X PBS

CTCM

Dulbecco's Modified Eagle Medium 85.9%
FCS 10%
β-Mecaptoethanol (55 mM) 0.1%
Non-essential amino acids (10nM) 1%
L-glutamate (200 mM) 1%
HEPES buffer (1 M) 1%
Penicillin/Streptomycin (100 U/ml/10 mg/ml) 1%

Microglia Media

DMEM 43.955 mL

45 μL2-mercaptoethanol

500 μL L-glut
500 μL PenStrep
5 mL FCS

Griess solution A
Sulphanilamide 1%
In 2.5% Phosphoric acid

Griess solution B
N-(1-napthyl) ethylenediamine 1%
In 2.5% Phosphoric acid

FACs Buffer

FCS 2%
Sodium azide (1 M) 0.1%
1xPBS 97.9%

MTT Solution (50 mL)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 250 mg

1 X PBS to 50 mL

Sterile filter and protect from exposure to light.

MTT Solubilizer (stop) solution (500 mL)

10% SDS 80 g

45% N, N-DMF 225 mL

Dilute to 500 mL with Milli-Q ddH_2O

pH adjusted to 4.5 using glacial acetic acid

Luxol fast blue solution

400 mL 95 % EtOH

4 g Luxol fast blue powder

0.1% Lithium Carbonate

Lithium Carbonate 0.1%
ddH2O 99.9%

Cresyl echt violet solution

2.0 g cresyl violet

300 mL ddH2O

30 mL NaAcetate, 1M

Glacial acetic acid =17.4M [11.5mL/188.5mLH2O]

pH to 3.7 - 3.9

Acid Alcohol

HCl 0.3%
Ethanol 70%
ddH2O 29.7%

Sodium Citrate Buffer

Tri-sodium citrate (dehydrate) 2.94 g

Distilled water 1000 mL

Adjust pH to 6.0 with 1N HCl

Add 0.5 mL Tween 20 and mix well

ELISA capture buffer
Na2HPO4 0.1M
In ddH2O, adjusted to pH 9.

ELISA stop solution
H2SO4 0.18M
In ddH2O