SEX DIFFERENCES AND THE PROTECTIVE INFLUENCE OF ESTROGEN IN A MOUSE MODEL OF DEMYELINATING DISEASE

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ABSTRACT

LORELEI CARISSA TAYLOR: Sex Differences and the Protective Influence of
Estrogen in a Mouse Model of Demyelinating Disease
(Under the direction of Glenn K. Matsushima)

Demyelinating diseases such as multiple sclerosis may be influenced by gender dimorphisms and sex hormones. Here, the cuprizone model of toxin-induced oligodendrocyte death and demyelination was used to investigate sex differences in two mouse strains, SJL and C57BL/6. The results indicate that female SJL mice are partially protected from oligodendrocyte loss and demyelination compared to their male counterparts. No sex differences were exhibited in the C57BL/6 strain. Furthermore, SJL mice exhibited differences in demyelination pattern and severity compared to C57L/6 mice, suggesting a genetic influence on responses to cuprizone intoxication. In addition, the potential therapeutic benefit of estrogen was investigated by administration of 17β-estradiol (E2), at pregnancy levels, to mice during cuprizone intoxication. Interestingly, E2 was effective in partially reducing oligodendrocyte loss and demyelination. This protection was accompanied by a delay in microglia accumulation as well as expression of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) and the growth factor insulin-like growth factor -1 (IGF-1). These studies indicate that estrogen signaling represents an attractive avenue for future studies into therapy of demyelinating disease.

iii

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

3β-HSD-1	3β-hydroxysteroid dehydrogenase-1
AD	Alzheimer's disease
ALS	amylotrophic laterel sclerosis
APC	antigen presenting cell
AR	androgen receptor
B6	C57BL/6
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BMT	bone marrow transplant
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
cAMP	cyclic AMP
CCR5	CC-chemokine receptor 5
CD200R	CD200 receptor
CGT	ceramide galactosyltransferase
CNPase	2'3'-cyclic nucleotide 3'-phosphohydrolase
CNS	central nervous system
COX	cyclooxygenase
DAPI	4',6-diamidino-2-phenylindole
DPN	diarylpropionitrile
E1	estrone
E2	estradiol

E3	estriol
EAE	experimental autoimmune encephalitis
EE	17α-ethinylestradiol
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage-colony stimulating factor
GSTpi	glutathione-S transferase
HRP	horse radish peroxidase
I-A$_\beta$	major histocompatibility complex class II
ICAM	intercellular adhesion molecule
IFN	interferon
IGF-1	insulin-like growth factor-1
IgSF	immunoglobulin superfamily
IL	interleukin
IL-18R	interleukin-18 receptor
IL-1R	interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
KPBS	potassium-phosphate-buffered saline
LAG-3	lymphocyte activation gene-1

LFA-1	lymphocyte function-associated antigen-1
LFB-PAS	luxol fast blue-periodic acid Schiff's
LPS	lipopolysaccharide
LT	lymphotoxin
LTP	long term potentiation
LTβR	lymphotoxin-β receptor
MAG	myelin-associated glycoprotein
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MCP-1	macrophage chemotactic protein-1
MHC	major histocompatibility complex
MHV	mouse hepatitis virus
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NFκB	nuclear factor κ B
NGF	nerve growth factor
NGS	normal goat serum
NMSS	National Multiple Sclerosis Society
NK	natural killer
NO	nitric oxide
NT	neurotrophin
OPC	oligodendrocyte progenitor cell

PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PD	Parkinson's disease
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PFA	paraformaldehyde
PGE_2	prostaglandin E_2
PI3K	phosphoditidylinositol 3-kinase/protein kinase B
PLP	proteolipid protein
PNS	peripheral nervous system
PR	progesterone receptor
PrP	prion protein
RCA-1	*Ricinus communis agglutin*-1
sLAG-3	soluble lymphocyte activation gene-3
MAPK	mitogen-activated protein kinase
MRI	magnetic resonance imaging
RIA	radioimmunoassay
SERM	Selective estrogen receptor modulators
SOD1	superoxide dismutase
SVZ	subventricular zone
TCR	T cell receptor
TGFβ	transforming growth factor-β

Th1   T helper 1

Th2   T helper 2

TMEV   Theiler's murine encephalitis virus

TNFR   tumor necrosis factor receptor

TNF-α   tumor necrosis factor-α

Treg   regulatory T cell

TUNEL   terminal deoxynucleotidyl transferase dUTP nick end labeling

QTL   quantitative trait loci

VCAM   vascular cell adhesion molecule

Y^- deletion of *Sry* from the Y chromosome

CHAPTER 1

INTRODUCTION

1.1   Pathogenesis of Multiple Sclerosis

Overview of presenting symptoms.

Multiple Sclerosis (MS) is a degenerative disease of the Central Nervous System (CNS) characterized by multiple focal inflammatory lesions to the white matter of the brain and spinal cord [1]. The varied location and severity of lesions leads to clinical symptoms which differ from patient to patient [1]. Among the most common initial symptoms are: monocular visual loss (optic neuritis), double vision (diplopia), weakness or decreased dexterity in one or more limbs, sensory disturbance, gait instability, and ataxia. Later in disease, bladder dysfunction, heat sensitivity and fatigue also occur in many patients. In advanced cases of MS cognitive deficits such as memory loss, impaired attention, and difficulties with problem-solving are also common.

In order to standardize diagnosis and reporting, the Advisory Committee on Clinical Trials of New Agents in MS of the National Multiple Sclerosis Society (NMSS) undertook a survey of the international MS clinical research community in an effort to develop a consensus on definitions and terminology which describe clinical outcomes and course patterns of MS patients [2]. This survey resulted in 4 categories of the clinical course of MS being defined: relapsing-remitting, secondary progressive, primary progressive, and progressive relapsing. The most common form of MS is "relapsing-remitting" and is characterized by acute attacks of neurological symptoms (commonly referred to as "relapses") which resolve partially or completely and are separated by periods of remission during which there is lack of disease progression. Typically this pattern persists for many years, until the repair mechanisms diminish and the patient enters a stage of steady and

irreversible decline (with or without superimposed relapses) termed "secondary progressive".

A minority of patients (10-20%) initially present with a progressive decline that does not

resolve and this form of MS is termed "primary progressive" if no relapses occur, or

"progressive relapsing" if relapses are superimposed on the progressive decline.

Histopathology of MS lesions.

Inflammation and myelin destruction are the primary hallmarks of the MS lesion.

These plaques can occur anywhere in the white matter of the CNS, and may also occur in the

gray matter [3]. The classically defined active lesion contains myelin sheaths in the process

of dissolution and lymphocytes as well as infiltrating macrophages which contain myelin

degradation products [4, 5]. In comparison, inactive lesions are relatively devoid of myelin

and oligodendrocytes and contain only a few lymphocytes and macrophages at perivascular

sites. Demyelinated axons in the inactive lesions are embedded in astrocytic glial scar tissue.

Due to the long duration of MS, it is rare that newly formed lesions are examined for

pathological characterization. However, recently, a few reports describing the cellular

activity of early-stage lesions have been published. These reports indicate a role for primary

oligodendrocyte dysfunction in the etiology of MS lesions, thus challenging the idea that all

MS lesions are induced by autoimmune attack on myelin proteins.

In 2000, Lucchinetti and colleagues undertook an extensive examination of over 200

actively demyelinating lesions from autopsy and biopsy derived MS tissue [6]. They

concluded that MS lesions could be divided in to four types. Patterns I and II were the most

common and displayed the classical hallmarks of perivenous distribution, large demyelinated

plaques and a dominance of macrophage and T cell involvement. These 2 subtypes of lesions

differed from each other only in the deposition of complement and antibodies, which occurs in pattern II. Patterns III and IV also contained macrophages and T cells; however, these lesions differed from patterns I and II in that they contained evidence of oligodendrocyte apoptosis or perturbation. Features of pattern III lesions which set them apart include: lack of immune cells centering around inflamed blood vessels, ill-defined lesion contours, significant oligodendrocyte apoptosis, and preferential loss of myelin-associated glycoprotein (MAG) compared to other myelin proteins. Pattern III was detected mainly in tissue obtained with in 2 months of disease onset, leading to the conclusion that oligodendrocyte apoptosis may be an early stage of lesion formation. It is tempting to speculate that pattern I and II lesions might represent older and more developed lesions. However the lack of a follow up study of patients presenting with pattern III lesions left this possibility open-ended. Pattern IV lesions were relatively rare in this study, occurring in only 3 of the 200 patients with a variant of primary progressive MS. Pattern IV lesions looked similar to patterns I and II, except for extensive loss of oligodendrocytes, lack of remyelination shadow plaques, and presence of DNA fragmentation in oligodendrocytes, indicating perturbation of oligodendrocytes.

Lucchinetti's study is not the only report suggesting that newly formed MS lesions may be induced by oligodendrocyte death. In 2004, Barnett and Prineas described in intricate detail the lesion of a young patient who died within 17 hours of an acute attack, allowing the rare opportunity to study a very newly formed lesion [7]. Interestingly, this lesion was characterized by extensive oligodendrocyte apoptosis, the presence of activated microglia, relatively little loss of myelin sheaths, and absence of T cells and phagocytic macrophages. However, other older actively demyelinating lesions in this patient displayed the classical

pattern of T cell and phagocytosing macrophage involvement, suggesting that oligodendrocyte apoptosis in the absence of T cells may represent a very early stage of lesion formation in MS patients. These authors were able to identify 9 other similar lesions in 6 out of 11 patients who also died shortly after an acute attack, whereas no apoptotic lesions of this kind were found in additional 6 patients with established chronic MS. While intriguing, the reports of Lucchinetti and Barnett and Prineas remain controversial and the established dogma is that MS lesions are induced by immune attack against myelin antigens.

1.2   Sex Differences in MS

A prevalence of disease susceptibility in the female is common to several autoimmune diseases including multiple sclerosis, rheumatoid arthritis, Grave's disease, systemic lupus erythematosus, myasthenia gravis, Sjogren's syndrome and Hashimotos thyroiditis [8, 9]. In MS, the most often reported sex ratio is two females per one male [10-12]. Although subtle and not as widely accepted as a difference in susceptibility, there is also evidence that MS displays sex differences with respect to disease severity. A natural history study of greater than 1800 patients demonstrated that female gender was associated with a slower progression of disease [13]. In addition, MRI scans of 281 female and 132 male MS patients showed that men had a lower number of contrast-enhancing lesions, which represent areas of active inflammation, but a higher number of "black hole" lesions, which mark axonal loss [14]. This indicated that males with MS may be more likely to develop lesions that are less inflammatory, but result in greater axonal destruction. Possible mechanisms for

sex differences include influences by sex hormones on the immune system and/or the CNS, influence of sex chromosomes or sex-dependant effects of allelic variants, microchimerism (a recently introduced suspect in autoimmunity), or some combination of the above.

Immunological basis for sex differences.

On the whole, females exhibit more robust cell-mediated and humoral-mediated immune responses to antigenic challenges, such as vaccination and infection, compared to males [15]. Numbers of CD4$^+$ T cells are higher in women [16] and T regulatory (T$_{Reg}$) cell numbers fluctuate with the ovarian cycle [17]. This may be important for autoimmune diseases, as T$_{Reg}$ functional deficits have been implicated in MS and rheumatoid arthritis [18, 19]. Furthermore, estrogen was shown to increase the expression and responsiveness of CC-chemokine receptor 5 (CCR5) and CCR1 in CD4$^+$ T cells, which has important implications for T cell homing [20]. A gender difference in the T helper 1/ T helper 2 (Th1/Th2) balance is one hypothesis offered by researchers to explain differences in autoimmune disease susceptibility or severity between the sexes.

MS is normally considered to be a Th1-mediated autoimmune disease based on the expression of Th1 cytokines interfereon gamma (IFNγ) and tumor necrosis factor (TNF) by myelin antigen-specific T cells isolated during active disease [21-24]. In one study, freshly isolated PBMC from MS patients stimulated with various control and myelin antigens revealed an IFNγ (Th1) skewing in females and an interleukin 5 (IL-5) (Th2) skewing in males [25, 26]. Sex differences could also be mediated through innate immune cells such as microglia and macrophages that also secrete pro- and anti-inflammatory molecules, and may be involved in the pathology of MS [27-29]. The effects of estrogen on innate immune

6

responses are generally repressive [30]. In particular, *in vitro* administration of estrogen reduced the production of pro-inflammatory cytokines IL-1β, IL-6, and TNFα in macrophages [31] and nitric oxide (NO) and TNFα in microglia (reviewed in [29]).

Microchimerism.

Microchimerism is the stable presence of a small number of non-host cells in an individual. Microchimerism can develop as a consequence of maternal-fetal and fetal-maternal transmission of haematopoeitic cells through the placenta. Fetal DNA has been detected in women for as long as 27 years following the birth of their last child [32] and maternal cells have been detected in adult offspring up to 28 years of age [32, 33]. Other avenues of developing microchimerism include: blood transfusion, bone-marrow or organ transplant, and twin gestation. Within the last few years, there has been interest in the concept of linking microchimerism to autoimmunity [34]. While there is some evidence for increased microchimerism in a few autoimmune diseases, notably systemic sclerosis, the correlation remains vague for many others (reviewed in [35]). The incidence of microchimerism in MS patients has not been studied directly. However, given that microchimerism is common in women who have given birth, a logical assumption is that if microchimerism is a risk factor for the development of MS, then women who have given birth should have a higher incidence of MS. To the author's knowledge, only two studies have addressed this and have indicated that neither pregnancy [36], nor bearing children from more than one father [37] is a risk factor for developing MS. Therefore, there is no convincing evidence to date that fetal-maternal microchimerism plays a role in the female preponderance of MS susceptibility.

Chromosomal influence.

Recently, an elegant study by the Voskuhl group demonstrated the importance of sex chromosome makeup in animal models of MS and lupus [38]. In male mammals, the Y chromosome –linked gene *Sry* is responsible for testes development and subsequent determination of the male sex. This was shown by experiments in which expression of an *Sry* transgene in an XX mouse resulted in testes development [39] and deletion of *Sry* from the Y chromosome (Y⁻) led to ovary development [39]. A model system has been developed using these Y⁻ mice in which *Sry* was added back as a transgene on an autosome to produce XY⁻*Sry* males [40]. When bred to normal XX females, four genotypes were produced: XX and XY⁻ ovary-bearing females, and XY⁻*Sry* and XX *Sry* testes-bearing males [41]. The end result of these genetic manipulations was the ability to study the effect of the sex chromosome makeup on the background of both female and male hormones. Using this model in SJL mice, Smith-Bouvier and colleagues [38] assessed the influence of sex chromosome makeup in two distinct animal models of autoimmune disease, experimental autoimmune encephalomyelitis (EAE) and pristane-induced lupus, both of which are known to display increased susceptibility in SJL females [42, 43]. The results indicated that the XX chromosome makeup produces more severe disease compared to XY⁻ in mice that developed on both the female and male hormonal background, in both autoimmune models. However, this does not rule out an additional effect of sex hormones, because the authors performed these experiments on gonadectomized animals, to avoid any masking of the chromosome influence. Interestingly, similar experiments using the EAE model in C57BL/6 mice, which do not display gender differences in EAE [44-46], did not show any difference in XX or XY-

8

chromosomal makeup, revealing an interaction between sex chromosome makeup and genetic background [38].

Parental effects.

Evidence supporting a parent-of-origin effect, whereby the sex of the affected parent would influence the risk of developing MS in offspring, has been controversial. In 2004, Hupperts and colleagues studied concordant parent-child families with MS and concluded that there was no evidence for a parent of origin effect distorting sex ratios in affected offspring [47]. However they did report a potential effect on disability and disease course in offspring. Since then, some studies have shown an increased risk from an affected father [48], conversely an affected mother [49, 50], or no effect based on the sex of the affected parent [51].

Allelic variation.

Allelic variants in several genes have shown sex-dependent associations with MS susceptibility. Gene polymorphisms in IFNγ were associated with susceptibility to MS in males but not females [52-56]. Brain-derived neurotrophic factor (BDNF) is another gene for which allelic variation showed a sex-dependent influence on MS susceptibility [57]. In this study of a Polish population with a relatively small sample size, one polymorphism of BDNF was associated with increased risk for MS in both males and females, while another was associated with increased risk only in females. In addition, the *AT* haplotype on chromosome 16 chemokine cluster was indicated to be protective to MS susceptibility in males but not females [58]. A polymorphism in the IL-4 promoter (-589C/T) was associated

with MS in females but not males [59]. Analysis of a polymorphism in the matrix

metalloproteinase-9 (MMP-9) promoter (-1562C/T) indicated a protective effect of the T

allele for MS susceptibility only in females of a Serbian population[60]. However, a similar

analysis of a Swedish population indicated no influence of MMP-9 polymorphisms in MS

susceptibility.   The mechanism for how these polymorphisms may contribute to sex

differences in susceptibility are not known; however, one hypothesis which remains to be

tested is that the polymorphisms may alter binding of sex hormone transcription factors and

thus alter gene expression in a sex-dependent manor.

1.3   Sex Differences in Animal Models of Demyelination

Experimental autoimmune encephalomyelitis (EAE).

EAE, the dominant animal model of MS, is induced by immunization with myelin

proteins or T cells that have been primed to myelin proteins and adoptive transferred into

susceptible hosts [61]. The resulting pathology is similar to that seen in MS, with

demyelinated lesions in the spinal cord and brain highly infiltrated with inflammatory

macrophages and T cells. Disease severity of the rodent is measured by varying degrees of

limb or tail paralysis. Sex differences occur in EAE, and these vary by strain, suggesting a

genetic influence. Sex differences in the course of EAE disease progression were first

reported in Lewis rats, in which females but not males exhibited relapses [62]. Similarly,

female SJL mice have also demonstrated a relapsing disease course compared to a

monophasic course in males, as well as increased susceptibility and severity of EAE [42, 63,

64]. This sex difference has been attributed in a large part to differences in male and female

T cells during the induction phase of the disease as well as the influence of sex hormones on Th1/Th2 cytokines [65-67]. In addition, a comparison of seven different mouse strains immunized with various myelin peptides demonstrated that female SJL and ASW mice displayed greater disease severity, whereas disease was more severe for males of the B10.PL and PL/J strains [46]. Female SJL and NZW had increased disease incidence compared to male counterparts, but C57BL/6 and NOD mice did not exhibit any sex differences [46]. Thus, in EAE, the effect of sex appears to be specific to particular strains of mice and it is not reflected specifically on the type of immune T cells or antigenic peptide.

Theiler's murine encephalomyelitis virus model.

Intracerebral inoculation of rodents with Theiler's murine encephalomyelitis virus (TMEV) leads to an acute encephalomyelitic phase followed by a chronic demyelination phase [68]. Demyelinated lesions of the spinal cord exhibit infiltration primarily of T cells, some macrophages, and a few B cells and plasma cells (reviewed in [68]). Functional deficits such as hind limb paralysis, spasticity, ataxia, and incontinence mirror those described in MS [68]. In the Theiler's virus model, both male and female SJL male mice are highly susceptible to disease development, however, it has been reported that males exhibit more severe neurological deficits during the chronic phase of disease, possibly due to lower levels of anti-viral IgG antibodies and increased infectious virus titers in males compared to females during the acute phase of disease [69]. In the C57L/J strain, male mice are susceptible to disease formation, but females are completely resistant, which was shown to be correlated with increased anti-viral responses in female mice [70]. Thus, in TMEV, two strains of mice show sex differences in symptoms or resistance to disease.

Toxin models of demyelination.

Toxin-induced demyelinating lesions offer a reproducible model for studying oligodendrocyte loss and remyelination without the complication of autoimmune induction. In the ethidium bromide demyelination model injection of ethidium bromide into the caudal cerebellar peduncles creates a focal area of demyelination that undergoes spontaneous remyelination [71, 72]. Comparisons of remyelination in young and old male and female rats revealed an age-dependent sex difference, in that young male and female rats remyelinated similarly, but older females remyelinated more efficiently than older male counterparts [73]. This sex difference in remyelination was not due to circulating gonadal steroids, as castration did not affect remyelination in young or old rats of either sex. Despite this, a role for gonadal hormones has been suggested due to the fact that progesterone administration induced a slight increase in remyelination in older male rats [74]. For cuprizone intoxication, a model which will be discussed in detail later, no direct comparisons of sex differences have been reported.

1.4   The Role of Sex Hormones in MS and Animal Models of Demyelinating Disease.

Sex steroid hormones that circulate in the blood are produced in the adrenal glands as well as the ovaries of premenstrual women, and testes of men [75]. In addition, many cell types in various tissues of the body, including the brain, have the ability to metabolize sex hormone precursors, or produce them *de novo* from cholesterol [76]. Sex steroids are formed by a series of conversions by steroidogenic enzymes [77]. Synthesis begins with conversion

of cholesterol to pregnenalone in the mitochondria by the cytochrome P450ssc enzyme (scc for side-chain cleavage).   Pregnenalone is then metabolized to progesterone, which can be further metabolized to testosterone, and finally to 17β-estradiol by the enzyme aromatase.   In addition to 17β-estradiol (estradiol, E2), there are two other biologically active estrogens in mammals: estrone (E1), and estriol (E3) [75]. The most abundant and potent estrogen in premenopausal women is estradiol, with estrone production by nonovarian tissues becoming the predominant source of estrogen in postmenopausal women [75]. Estrone can be converted to estradiol in the ovaries or other target tissues [75]. Estriol is produced by the metabolism of estrone and estradiol and becomes important mainly during pregnancy where it is produced by the placenta and is at that time the most abundant estrogen in maternal circulation [75]. Though estrogens and progestins are considered "female" hormones, and testosterone a "male" hormone, all three are produced in both genders.

Sex hormones in MS.

A role for sex hormones in MS was suggested by the observation that disease may be altered by pregnancy. This observation was confirmed in a large prospective study which demonstrated a decrease in disease relapse rate during the third trimester of pregnancy (when sex hormone levels are high) [78]. Furthermore, the relapse rate was significantly increased in the first three months post-partum, when hormone levels drop, then returned to the pre-pregnancy rate [78]. Interestingly, a clinical trial of a small number of MS patients showed that taking a dose of oral estriol sufficient to mimic the levels seen during pregnancy decreased active lesions (as seen by MRI) compared to pre-treatment baselines in relapsing-remitting patients [79]. Estriol treatment also reduced the delayed-type hypersensitivity

response to tetanus in these patients and reduced expression of IFNγ from isolated peripheral blood mononuclear cells (PBMCs) [79]. Furthermore, PBMCs collected from these patients and stimulated *ex vivo* indicated that estriol treatment led to increased production of the anti-inflammatory cytokines IL-5 and IL-10 and decreased production of the pro-inflammatory cytokine TNFα [80]. The somewhat paradoxical observation that women are more susceptible to MS and yet it is ameliorated during pregnancy when female hormones are high, may be explained by a estrogen's biphasic effect on the immune system. Low concentrations of estrogen facilitate cell-mediated immunity, but high concentrations, such as during pregnancy, inhibit cell-mediated immunity [81].

Though the age of onset of MS in women tends to be during early adulthood (ages 18-30) the onset in men is slightly later (ages 25-40) which corresponds with the beginning of the decline in bioavailable testosterone [82-84]. This observation may indicate that testosterone has a protective effect in young males [79]. Recently, a small clinical trial in male MS patients demonstrated that one year of testosterone administration improved cognitive performance and slowed brain atrophy, but did not affect the number or volume of contrast-enhancing lesions [85]. A conclusion for an anti-inflammatory role of testosterone could not be made in this study, since the male patients enrolled had a relatively low level of baseline inflammation as measured by MRI. However, the results of this study do indicate a neuroprotective function for testosterone.

Effects of estrogen on EAE.

Additional evidence linking sex steroids to autoimmune demyelinating disease has been gleaned from the EAE model. For example, female Lewis rats treated with the synthetic

estrogen 17α-ethinylestradiol (EE) beginning 4 days before disease induction and continuing through the disease course reportedly experienced a partial suppression of EAE symptoms, as well as a reduction in spleen and thymus weights [86]. Castration of female B10.RII mice led to earlier onset of EAE, whereas administration of pregnancy levels of estradiol or estriol delayed onset [87]. Since these initial studies were published, several other studies have also demonstrated the beneficial effects of estrogens in EAE in several rodent strains, and leant support to the hypothesis that estrogen mediates a Th1 toTh2 immune shift. For instance, estriol-mediated reduction of EAE severity in female SJL mice was associated with increased production of serum IgG1 and splenocyte secreted IL-10 (Th2 associated phenomenon), but it did not change splenocyte production of IL-2, IL-5 or IL-10 [66]. Another study demonstrated that estriol ameliorated disease in male as well as female C57BL/6 mice, and led to suppression of several Th1 associated pro-inflammatory cytokines (TNFα, INFγ, IL-2, IL-6) from stimulated splenocytes of both genders as well an increase in the Th2-associated cytokine IL-5 from male splenocytes [45]. Furthermore, estradiol limited behavioral impairment and inflammation in female rats [88]. Interestingly, even low doses of estradiol and estriol reduced EAE severity in male and female SJL and female B10 mice, but only if administered before disease onset [89]. This study also demonstrated that estrogen treatment led to a reduction in IFNγ, IL-12 and TNFα production by stimulated lymph node cells or splenocytes, as well an increase in IL-10, although the results did not reach statistical significance. In summary, both estriol and estradiol appear to down-modulate peripheral immune responses and have a beneficial affect when administered before the onset of symptoms.

In addition, estradiol treatment led to a significant reduction in recruitment of macrophages and T cells into the CNS as well as a reduction in the percentage of these cells that produced TNFα, however, it did not have a significant influence on the production of TNFα by resident microglia [90]. The potential importance of a regulation of TNFα is demonstrated by the finding that EAE severity is significantly diminished in TNFα-deficient mice [91]. Interestingly, the ability of estradiol to reduce EAE severity was not altered in IFNγ, IL-10 or IL-4-deficient mice.   Thus, estradiol appears to primarily affect the number of immune cells in the CNS and their production of TNFα.

The use of estrogen receptor-deficient mice and selective agonists led to the finding that estradiol's ability to reduce inflammation and EAE symptoms is mediated by the alpha estrogen receptor (ERα) and not the beta estrogen receptor (ERβ) [92, 93]. Furthermore, using T cell-deficient mice or chimeras, it was determined that this effect of estradiol was not mediated through ERα in T cells, but instead must be another, as yet undetermined, cell type [94, 95]. The chemical production of estrogen receptor-specific agonists has further increased knowledge of the role of ERα and ERβ in demyelinating disease. Recently, use of the ERβ agonist diarylpropionitrile (DPN) confirmed that signaling through ERβ did not reduce EAE clinical scores or reduce inflammation during the time frame of disease generally studied, however, it did decrease symptoms later in disease [96]. In addition, both ERα and ERβ ligands exhibited neuroprotective effects by reducing neuronal loss in the gray matter, and axonal loss in the white matter during EAE [96].

Effects of progesterone on EAE

The data on progesterone in EAE is somewhat conflicting. Recently, two doses of continuously administered progesterone, beginning one week prior to active EAE induction (leading to a 10-fold or 20-fold increase in serum levels) were shown to have protective effects in female C57BL/6 mice, generating a slight delay in disease onset and reduction of clinical scores [97]. Histologically, progesterone treatment during EAE resulted in a decrease of infiltrating inflammatory cells and a preservation of myelin proteins. In contrast, administration of progesterone beginning 4 days prior to active EAE induction (resulting in a 10-fold increase in serum levels) in female Lewis rats led to an increased severity of sensorimotor deficits, cell death in the gray and white matter, and increased accumulation of inflammatory cells [88]. Progesterone administration at two different doses had no effect on adoptive transfer of T cell-induced EAE in female SJL mice when administered continuously beginning three weeks before disease induction [66]. However, serum levels of progesterone were not confirmed in these mice, making comparisons to other experiments difficult. Therefore, it is not clear whether progesterone has a beneficial affect and if it does, it appears to be minimal.

Effects of testosterone on EAE.

Several studies have shown benefits of testosterone on demyelinating disease. Male SJL mice exhibit less severe EAE compared to females [42, 46, 63, 64]. Interestingly, castration of male SJL mice was shown to increase EAE severity, suggesting that testosterone has a protective effect [65, 98]. However, castration did not alter disease in C57BL/6, a strain which does not display sex differences in EAE [98]. Administration of

testosterone one week prior to EAE induction, at a dose which resulted in a 10-fold increase in serum levels, resulted in reduced disease severity in males of both the SJL and C57BL/6 strains [98]. Similarly, administration of testosterone to female SJL mice two weeks prior to EAE induction resulted in reduced clinical symptoms and also increased production of the anti-inflammatory cytokine IL-10 by T cells [99]. Thus, it appears that the role of endogenous testosterone may be strain dependent, and that similar to estrogen, high doses of testosterone may provide a protective effect during EAE.

1.5   The Role of Sex Steroids in Brain Glial Cells.

Cells of the CNS may be influenced directly by circulating hormones in the blood stream, or they can metabolize these hormones to active steroid derivatives (reviewed in [76, 77, 100]). In addition, the proteins necessary to perform de novo synthesis of steroids directly from cholesterol have been demonstrated to be upregulated after brain injury [101-107]. Furthermore, steroid signaling can occur through multiple mechanisms. The classical mechanism of steroid action is through binding of the ligand binding domain on steroid hormone receptors, which act as transcription factors to modulate gene expression. Steroids have been shown to exert rapid "non-genomic" effects such as modification of intracellular levels of calcium [108-110] or regulation of the activity of kinases, influencing signaling pathways such as the phosphoditidylinositol 3-kinase/protein kinase B (PI3K/Akt) [111] and mitogen-activated protein kinase (MAPK) pathways [112, 113]. It is plausible that synthesis of steroids in the CNS may differ among gender and in different strains of mice. For instance, 3β-hydroxysteroid dehydrogenase-1 (3β-HSD-1), the enzyme which converts pregnenalone

18

to progesterone, was found to have reduced mRNA levels in female hypothalamus compared to male mice [114]. In addition, the regional distribution of aromatase (which converts testosterone to estradiol) mRNA was found to be similar in male and female rats, however, the number of aromatase expressing cells in each region was lower in the females [115].

Sex steroid receptor expression..

The current knowledge of expression of steroid hormone receptors in neurons or glial cells has been reviewed recently by Garcia-Ovejero and colleagues [116] and will be summarized below in addition to a few reports which have come out since that time. *In vitro* studies indicate that all brain cell types (neurons, astrocytes, oligodendrocytes and microglia) express both estrogen receptors ERα and ERβ (reviewed in [116]) and that astrocytes also express the progesterone receptor (PR) and androgen receptor (AR) *in vitro* [117]. *In vivo* co-localization in subpopulations of neurons has been identified for ERα, ERβ, AR [118, 119], and PR [120]. Astrocytes have been co-localized *in vivo* with ERα, ERβ, AR (reviewed in [116]); and PR [121]. ERβ is expressed in oligodendrocyte cell bodies and the myelin sheath *in vivo* [122, 123]. Furthermore, oligodendrocytes express AR in primates [124], but there is as yet no convincing *in vivo* co-localization of ERα or PR. *In vivo* evidence for steroid hormone receptors in microglia is more sparse, however, adult microglia isolated ex vivo have been shown to express ERα mRNA and *in vivo* by electron microscopy [125]. Moreover, ERβ and AR were detected in microglia after brain injury [126, 127]. Many of these studies revealed that the steroid hormone receptors are expressed differentially in various brain areas, and were regulated in response to a variety of stimuli. Therefore, it is

important to determine exactly which receptors are expressed in the cells of the region of interest and under the specific parameters of any experiment.

Sex steroid functions in myelination and inflammation

Sex steroids have been shown to provide trophic and protective functions in the CNS and PNS and have the potential to affect the processes of demyelination, remyelination and axonal damage in MS [77, 100, 128]. While much is known about the effects of sex steroids on peripheral myelin and, in particular, the role of progesterone and its derivatives on peripheral myelination [129], the effects of sex steroids on central myelin and oligodendrocytes have been less well studied. The following sections below review the effects of sex steroids on particular glial cells of the CNS, with specific emphasis on myelination and inflammation.

Sex steroid effects on oligodendrocytes.

In 1966, Curry and Heim reported that estrogen administration causes an increase in myelination of neonatal rat brain [130]. Comparisons of oligodendrocyte progenitors from male versus female neonatal rat brains revealed increased yields of oligodendrocytes in female cultures [131]. Furthermore, studies of cultured oligodendrocytes and rat cerebellar organotypic cultures demonstrate that myelin basic protein (MBP) expression was enhanced upon administration of estradiol or progesterone [117, 132] and that estradiol promoted proliferation of OPCs as well as membrane sheet formation, while progesterone treatment increased cellular branching [131].

In addition, estradiol was shown to protect primary oligodendrocyte cultures from cytotoxic death induced by a peroxynitrite free-radical donor, and this effect was mediated by ERs, though no further mechanism was elucidated [133]. However, a recent study demonstrated that estradiol prevented hyperoxia-induced death of primary oligodendrocytes by regulating several mediators of apoptosis [134]. This group found that estradiol prevented hyperoxia-induced proapoptotic Fas-upregulation and caspase-3 activation as well as antagonized the inactivation of key kinases of the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) cell survival promoting pathways. *In vivo,* estradiol attenuated the loss of MBP in rat pups exposed to oxygen [134]. A role for sex hormones in remyelination is indicated by the finding that progesterone administration increased remyelination after toxin-induced demyelination in older male rats; however, the mechanism for this was not elucidated [74].

Sex steroid effects on microglia and astrocytes.

Sex steroids appear to inhibit microglia activity. *In vitro* studies with the N9 microglia cell line indicated that estradiol, but not progesterone, attenuated superoxide release, phagocytic activity, and inducible nitric oxide synthase (iNOS) protein expression, and these effects were dependent on ER binding [135]. Furthermore, estradiol induced rapid phosphorylation of MAP kinase and a MAP kinase inhibitor blocked the anti-inflammatory effects of estradiol. However, another study indicated that not only did estriol and estradiol have anti-inflammatory effects, but progesterone also reduced NO and TNFα in N9 cells. [136]. In the BV-2 microglia cell line, which expresses ERβ and not ERα, estradiol treatment led to a decrease in LPS-induced NO production, but in contrast to the reports for

N9 cells, it increased TNFα mRNA [137]. It is not known why estradiol produced the unexpected increase in TNFα mRNA, and could be a peculiarity of the BV-2 cell line.

Studies with primary microglia cultures activated by LPS indicated that estradiol reduces production of the inflammatory mediators iNOS, prostaglandin-E$_2$ and matrix metalloproteinase 9 (MMP9) [138] and that estriol and progesterone reduced nitrite production [136]. In an *in vivo* model of brain inflammation (induced by intracerebroventricular injection of LPS), systemic estradiol administration inhibited morphological activation of microglia and infiltration of macrophages into the brain [139]. Using estrogen receptor-deficient mice, it was demonstrated that ERα, but not ERβ, mediated this anti-inflammatory affect and was associated with a decrease in mRNA expression of MMP-9 [139]. Thus, in primary microglia, ERα may be an important mediator of anti-inflammatory effects induced by estradiol.

The role of sex steroid modulation of inflammation in astrocytes is less well studied. Astrocyte cultures derived from neonatal mouse midbrain or cortex displayed regional differences in response to sex steroid modulation of TNFα. Estrogen reduced TNFα in astrocytes from the midbrain but not cortex-derived astrocytes in response to LPS [140]. More recently, astrocytes given ERβ agonist reduced IL-1β, TNFα and MMP-9 but ERα agonist only attenuated IL-1β [141]. Similar analyses were conducted on microglia and it was concluded that compounds that bind ERβ are more effective at attenuating pro-inflammatory cytokines in both astrocytes and microglia.

1.6   The Cuprizone Model of Demyelination

Mechanism of cuprizone action.

Cuprizone (biscyclohexanone oxalydihydrazone) is a copper chelator originally produced for use in clinical chemistry. It is presumed, although not proven, that cuprizone's effects *in vivo* are due to copper binding and preventing the availability of copper for biochemical processes. In support of this, cuprizone intoxication resulted in strong inhibition of enzymatic activity of the copper-dependent enzymes monoamine oxidase and cytochrome oxidase in the brain [142], and cytochrome oxidase in liver [143]. Enlarged mitochondria were detected in oligodendrocytes and hepatocytes of cuprizone-treated animals, indicating a perturbation to mitochondrial function [144-146]. Recently, it was shown that 3 and 6 weeks of cuprizone treatment diminished the activities of complex I-III and II-III of the respiratory chain from mitochondria isolated from brain tissue [147]. Therefore, it is presumed that the toxic effects of cuprizone to oligodendrocytes are due to perturbation of mitochondrial function (reviewed in [148]). Why the oligodendrocytes are more sensitive to cuprizone toxicity than other cell types is not known, but could be due to their high metabolic demand in order to maintain the extensive myelin sheath.

Historical perspective.

Studies of cuprizone in animals were first performed in 1955 to study the systemic effects of copper chelation [149]. Dietary administration of 0.5% cuprizone for 7 weeks to male weanling mice was shown to produce severe brain edema characterized by vacuole formation, hydrocephalus, and demyelination [150]. Many of the subsequent studies focused

on the status spongiosis and astrocytosis which developed, though perturbations to myelin, oligodendrocytes, and axons were noted [145, 151]. Detailed ultrastructural studies of cuprizone-induced demyelination and remyelination in the superior cerebellar peduncle were first performed by Blakemore in the early 1970s [152-154]. In these studies, weanling male ICI mice were fed 0.5% cuprizone for up to 5 to 8 weeks to study demyelination and 6 or 7 weeks followed by 2 or 4 weeks of recovery to study remyelination. Degenerating oligodendrocytes were reported at weeks 2 through 4, with almost no oligodendrocytes present by 5 weeks of cuprizone treatment. The presence of phagocytosing migroglia and astrocytes were reported to be significantly increased at 3 and 4 weeks, while at 5 weeks, large areas of demyelinated axons were present, especially in the superior cerebellar peduncle which was 90% demyelinated.

Remyelination is also an important feature of this model. At the same time as demyelination, the appearance of remyelinating oligodendrocytes was also noted at 5 weeks [154]. Two weeks of recovery from cuprizone after 7 weeks of intoxication resulted in 20% remyelination, whereas 4 weeks of recovery lead to 80-90% remyelination [154]. In another study, weanling male Swiss mice fed 0.6% cuprizone were reported to undergo consistent demyelination of superior cerebellar peduncle, with oligodendrocyte degeneration, accumulation of microglia, astrocytes and immature oligodendrocytes, and remyelination beginning within one week after removal of cuprizone from the diet [146]. These changes occurring with cuprizone intoxication did not result in breakdown of the blood-brain barrier [155, 156].

Strain differences.

From the early studies of cuprizone intoxication, it was noted that mice were more susceptible than rats or guinea pigs [157], and that even within the same strain, mice maintained at separate facilities displayed marked differences in tolerance to cuprizone [158]. FGF2 KO on a 129 Sv-Ev:Black Swiss background required 0.3% cuprizone to induce demyelination compared to C57BL/6 mice that demyelinate with just 0.2% cuprizone [159]. BALB/cJ mice treated with 0.2% cuprizone along with C57BL/6 mice revealed a similar pattern of demyelination in the corpus callosum, but less demyelination and more microglia activation in the cortex as compared to C57BL/6 [160]. Lastly, 129SvJ mice required only 0.1% of cuprizone to induce demyelination at week 5 (Matsushima, personal communication). The results from these studies highlight the importance of titrating the cuprizone dose for each new strain that gives optimal demyelination while limiting systemic toxicity.

Cuprizone-induced demyelination/remyelination in C57BL/6 mice.

The advent of transgenic and knockout mice provides a significant advancement for the study of demyelination and remyelination. It is imperative to standardize the genetic background of mice so that comparisons among genetic mutants can be assessed for relative importance and function. Since a majority of mutant mice are bred to the C57BL/6 background, characterizing the effects of cuprizone on this strain became highly desirable. In the past decade, cuprizone intoxication and demyelination/remyelination of the corpus callosum white matter has been characterized by Matsushima and colleagues for the C57BL/6 mouse strain [148, 161]. Using a 0.2% cuprizone dose (which mitigates the

substantial weight loss, mortality and liver toxicity reported for the higher doses used in earlier studies [161]), these mice experienced demyelinating lesions of the corpus callosum, oligodendrocyte loss, active gliosis and accumulation of oligodendrocyte precursors [161, 162]. During the first week of cuprizone treatment, the gene expression of several myelin proteins, including myelin-associated glycoprotein (MAG), myelin basic protein MBP), and ceramide galactosyltransferase (CGT), was drastically reduced [163]. The second and third weeks of treatment were characterized by the onset of oligodendrocyte cell death and the appearance of astrocytes and microglia/macrophages [161, 162]. By 5 weeks of treatment, nearly all of the mature oligodendrocytes were depleted from the midline corpus callosum, and there was a 90% reduction of myelinated axons [164]. Oligodendrocyte precursors were detected in the lesion during the first few weeks of demyelination, and increased to maximal levels at 4 and 5 weeks, after which these populations declined, presumably due to their differentiation to mature oligodendrocytes [162, 165].

Partial remyelination was achieved at 6 weeks, during continued intoxication, but was not sustained unless the toxin was removed from the diet [166]. Remyelination was near complete after 4 to 6 weeks off cuprizone treatment [163, 166]. Many cytokines and growth factors were produced in the demyelinated lesion during this process and several, specifically interleukin 1β (IL-1β), insulin-like growth factor 1 (IGF-1), and TNFα, have been shown to be important in the repair process [164, 165, 167].

One advantage of the cuprizone model as characterized by Matsushima and colleagues is that it produces a stereotypical pathological pattern, allowing for quantified comparisons of temporal data sets. In addition, it allows for the study of primary oligodendrocyte death and recovery, in an environment free of T cells involvement [165,

168] and other immunological complications.   However, although this model has been used

extensively to study the cell types and cytokines involved in the process of demyelination

and remyelination in male C57BL/6, much less is known about the cuprizone response of

female C57BL/6 mice. In fact, to the author's knowledge, no comprehensive gender

comparisons have been made in any mouse strain treated with the cuprizone intoxication

model. The little that is known regarding cuprizone intoxication in female mice is discussed

below.

Gender differences in the cuprizone model.

Historically, early cuprizone studies were carried out in male mice, although there

were some reports published using female mice also. Ludwin and colleagues exposed female

Swiss mice to cuprizone but reported that they appeared not to be susceptible to cuprizone-

induced demyelination [146]. Elsworth and Howell cited using equal ratios of male and

female albino mice for their studies, but did not note whether any gender differences were

observed [158]. Female BSVS weanlings were used for cuprizone studies, but only

spongiosis was analyzed, not demyelination [169].

Perhaps because of the lack of scientific evidence using female mice, the vast

majority of cuprizone-induced studies since these earlier studies have also been performed in

male mice. It was noted in early studies of C57BL/6 mice that females did undergo

cuprizone-induced demyelination [148], however, data from female mice were not published.

Since this report, several investigators in the last few years have used female mice for

cuprizone-induced demyelination studies [170-175] without full knowledge of the temporal

pattern of demyelination or the cellular profiles. Most recently, two studies examining

cuprizone-intoxication on knockout mice backcrossed to the C57BL/6 background reported treating and combining data from male and female mice [176]. In both these studies it was noted in the text that no gender differences were observed.

Given the importance of sex differences and sex hormones in MS, it is critical that we gain a better understanding of the role of gender and sex hormones in the demyelination and remyelination processes. A toxin model of primary oligodendrocyte death and demyelination may provide important insights to complement the work being done in autoimmune models of MS. The first part of this dissertation work focuses on sex differences in the cuprizone model. With the use of two different mouse strains, SJL and C57BL/6, we demonstrate that sex differences are dictated by the genetic background of the strains. Furthermore, we find that the pattern of demyelination differs between the strains in location and in the rate at which demyelination occurs. While it is possible that several hormones or derivatives may have an effect on demyelination or remyelination in the cuprizone model, the fourth chapter of this dissertation is focused on the influence of estradiol in modulating oligodendrocytes and demyelination. It was demonstrated that estradiol administration attenuated cuprizone-induced oligodendrocyte loss and demyelination.

CHAPTER 2

SJL Mice Exposed to Cuprizone Intoxication Reveal Strain and Gender Pattern Differences in Demyelination.

2.1 ABSTRACT

The role of mouse strain and the influence of gender on demyelination was explored for the first time in SJL mice using the cuprizone intoxication model. We document here that SJL mice display a unique pattern of demyelination that did not follow the profile that is well characterized in C57BL/6 mice. SJL mice did not readily demyelinate at the midline within the corpus callosum but showed greater demyelination immediately lateral to midline. During continuous exposure to cuprizone, demyelination was not complete and appeared to plateau after week 7. Importantly, female mice were partially resistant to demyelination, while male mice were more severely demyelinated. Differences in the number of mature oligodendrocytes were consistent with the extent of demyelination; however, microglia, astrocyte and oligodendrocyte precursor cell populations did not differ between male and female mice. Thus, genetic factors and gender influence susceptibility to demyelinating disease in the cuprizone model, which may provide additional insights into the variability observed in human demyelinating diseases such as multiple sclerosis.

2.1 INTRODUCTION

Multiple sclerosis (MS) is a degenerative disease of the central nervous system (CNS) in which autoimmune damage to myelin is a primary pathological finding. Each year ten thousand individuals in the United States are diagnosed with MS, with women outnumbering men by at least two-fold. In addition to gender differences in susceptibility, MS may also

display subtle differences in disease severity between men and women. For instance, MRI scans of 281 female and 132 male MS patients showed that men had a lower number of contrast-enhancing lesions, which represent areas of active inflammation, but a higher number of "black hole" lesions, which mark axonal loss [14]. This suggests that men with MS may develop less inflammatory, but more destructive lesions. Possible explanations for these gender differences include effects of gonadal hormones, inherent differences between male and female immune systems, or genetic factors that may or may not involve sex chromosomes.

In the experimental autoimmune encephalomyelitis (EAE) animal model for demyelinating disease, several strains of rodents display gender differences in disease incidence and severity ([46], [63] [64] [42]). However, the nature of the gender difference is highly dependent upon specific strain characteristics and the method of disease induction. A comprehensive comparison of EAE clinical disease scores in several mouse strains revealed that female SJL and ASW have more severe EAE than males when induced by active immunization [46]. In addition, female NZW show a higher incidence of EAE than males, but male B10.PL and PL/J have more severe EAE than females. There was no appreciable sex difference in active EAE in C57BL/6 or NOD mice. This indicates that genetic variability has a strong influence on gender differences in EAE. The influence of gender has also been explored in Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease in mice. In this model, male SJL/J display more severe neurological deficits than female mice [69]. The fact that SJL mice show gender differences in both the EAE and TMEV models indicates that the SJL strain is a logical choice for gender studies in the cuprizone model.

Early studies characterizing CNS pathology resulting from cuprizone treatment in ICI, Swiss and Swiss-Webster mice used large doses of cuprizone, in the 0.5-0.6% range, to induce demyelination [146, 151, 152]. In these early cuprizone studies male mice were used, and it was noted that female Swiss mice were not susceptible to cuprizone-induced demyelination [146]. In contrast, Elsworth and Howell cited using equal ratios of male and female albino mice for their studies, but did not note whether any gender differences were observed [158]. These studies focused on characterization of demyelination, oligodendrocyte depletion and the numbers of microglia and astrocytes in the cerebellar peduncles using electron microscopy [146]. Demyelination appeared complete by the fifth week of treatment. In each strain, differences in cuprizone dosage, time course of demyelination and cellular changes were noted with a high degree of accuracy. In addition, staining for MBP and MAG by immunohistochemistry showed changes that correlated with demyelination and remyelination in the superior cerebellar peduncle [177]. However, several limitations, including the genetic background prevented full utility of the model.

In the C57BL/6 strain, initial studies indicated that a lower dose of 0.2% of cuprizone was optimal [161] to induce full demyelination of the midline corpus callosum at week 5. Coincident with mature oligodendrocyte apoptosis, demyelination was accompanied by accumulation of microglia and astrocytes within the developing lesion. Interestingly, this environment appears to promote infiltration of oligodendrocyte precursor cells (OPCs) presumably from the subventricular zone [148, 162]. When cuprizone is removed from the diet, OPCs differentiate into mature oligodendrocytes and remyelination occurs over the next 5-7 weeks. Thus, acute exposure to cuprizone results in a consistent temporal pattern of

demyelination and remyelination that is marked by specific cellular, biochemical and molecular changes.

In contrast, the continuous exposure of C57BL/6 mice to cuprizone results in a pattern of two cycles of demyelination and remyelination, followed by chronic demyelination from which animals do not recover [148, 166]. The first remyelination is robust but incomplete and the second remyelination is limited [148]. In this strain, the inability to remyelinate is associated with a depletion of OPCs and can be stimulated by injection of functional OPCs into the lesion [178]. Thus, the C57BL/6 cuprizone model has been useful to our understanding of the limitations to repair that occur in chronically demyelinated lesions in multiple sclerosis [179, 180].

Our initial studies of the role of gender in C57BL/6 mice in the cuprizone model (soon to be published), and the lack of sex differences in EAE in C57BL/6 mice indicate that this strain may not be a good choice for the study of gender factors in the cuprizone model. Here the focus is on SJL mice, with the goal of determining whether gender influences the pattern of demyelination and remyelination in cuprizone toxicity. The data indicate that SJL mice show a different temporal and spatial pattern of cuprizone-induced demyelination than C57BL/6 mice. In addition, unlike C57BL/6 mice, female SJL mice show less severe oligodendrocyte loss and demyelination than male SJL mice.

2.3   MATERIALS AND METHODS

Animals and cuprizone treatment:

Adult male and female SJL/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used for experiments at 8 weeks of age. An initial dose titration was performed with 0.1%, 0.2%, 0.3%, 0.4% and 0.5% cuprizone (oxalic bis(cyclohexylidenehydrazide)) (Sigma-Aldrich) mixed into ground or Purina mouse chow and fed ad libitum for 5 weeks. In order to determine the temporal pattern of cuprizone induced demyelination, both male and female mice were either untreated, or treated with 0.2% cuprizone (as was determined to be the optimal dose) for 3, 4, 5, 6, 7, 8, 9, or 10 weeks. The data for this time course is presented as a combination of two separate experiments, each of which included 4 male and 4 female mice at each time point, for a total of 8 animals of each gender at each time point. Untreated mice were fed ground Purina chow during the time that experimental mice were being treated with cuprizone. A preliminary study of remyelination during the second time course was performed with 4 male and 4 female mice, which were treated with 0.2% cuprizone for 7 weeks then switched to a normal diet for 3 weeks. Also, 4 additional mice of each gender were treated with 0.2% cuprizone for 3, 6, or 8 weeks in order to assess oligodendrocyte progenitor cells, which require frozen sections. In addition, 4 male C57BL/6J mice bred in a pathogen-free facility at University of North Carolina-Chapel Hill (UNC-CH), were treated with 0.2% cuprizone for 5 weeks along with the SJL mice as a control for the expected pattern of demyelination as previously described [148, 161]. A comparison of these mice with SJL is shown in Figure 2.1. All animal breeding and use was performed in compliance

with the NIH *Guide for Care and Use of Laboratory Animals* and approved by the UNC-CH

Institutional Animal Care and Use Committee.

Tissue Preparation: For mice treated to look at oligodendrocyte precursor cells, brains were

removed, submerged in freezing media and immediately frozen in a supercooled isopentane

bath. All other mice were deeply anesthetized and intracardially perfused with 0.15M

phosphate buffer followed by 4% paraformaldehyde (PFA) solution. Brains were removed,

post fixed overnight in PFA, and embedded in paraffin. 5μm coronal brain sections were cut

at the fornix region of the corpus callosum (approximately Bregma -0.5mm to -0.7mm) and

corresponding to Figure 37 of *The Mouse Brain In Sterotaxic Coordinates* [181].

Luxol Fast Blue – Periodic Acid Schiff's (LFB-PAS) stain: To examine demyelination and

remyelination, paraffin sections were stained with Luxol fast blue (Sigma, St. Louis, MI),

which stains myelin blue, and periodic acid-Schiff (Sigma, St. Louis, MI), which stains

microglia/macrophages and demyelinated axons pink. Sections were scored blinded based

on the amount of blue or pink fibers in the corpus callosum, on a scale from 3 (complete

myelination equal to an untreated mouse) to 0 (complete demyelination, as seen during peak

cuprizone demyelination). The regions analyzed were the midline corpus callosum, and a

region just lateral to midline (illustrated in Figure 2.1).

Immunohistochemistry: The detection of mature oligodendrocytes was performed with

antibody to the Pi isoform of glutathione *S*-transferase (GSTπ) (Biotrin, Newton, MA).

Paraffin sections were rehydrated and permeabilized with 0.1% Triton X-100/ 2% normal

goat serum in phosphate buffered saline (PBS) for 20 minutes at room temperature. Tissue was unmasked in 0.1% calcium chloride/ 0.1% trypsin in 0.05M Tris, pH 7.4 for 15 minutes at 37°C. Sections were rinsed in PBS and incubated with GSTpi antibody (1:1000) or isotype control overnight at 4°C. Following appropriate rinsing, sections were incubated for 1 hour at room temp with a goat anti-rabbit IgG AlexaFluor conjugated secondary antibody (1:400) (Molecular Probes, Eugene, OR), rinsed and cover slipped with Vectasheild plus DAPI (Vector Laboratories Inc, Burlingame, CA) to counter stain nuclei.

Microglia/macrophages were detected with biotinylated lectin *Ricinus communis* agglutin-1 (RCA-1) (Vector Laboratories Inc, Burlingame, CA). Paraffin sections were rehydrated and unmasked with 0.025% protease, type XIV (Sigma-Aldrich) for 2 minutes at 43°C. Following a brief rinse in PBS they were blocked with 0.1% Triton X-100/ 1% bovine serum albumin in PBS for 1 hour at room temperature. Sections were then incubated with RCA-1, 1:500 in blocking solution or blocking solution alone as a control, overnight at 4°C. Following appropriate rinsing, sections were incubated for 1 hour at room temp with a streptavidin AlexaFluor conjugated secondary reagent (1:400) (Molecular Probes, Eugene, OR), rinsed and cover slipped with Vectasheild plus DAPI (Vector Laboratories Inc, Burlingame, CA) to counter stain nuclei.

Astrocytes were detected with antibody to glial fibrillary acidic protein (GFAP) (Invitrogen). Paraffin sections were rehydrated and unmasked with 0.025% protease, type XIV (Sigma-Aldrich) for 2 minutes at 43°C. Following a brief rinse in PBS they were blocked with 0.1% Triton X-100/ 2% normal goat serum in PBS for 1 hour at room temperature. Sections were then incubated with GFAP antibody (1:200) or isotype control overnight at 4°C. Following rinsing, sections were incubated for 1 hour at room temp with a

36

goat anti-rat IgG AlexaFluor conjugated secondary antibody (1:400) (Molecular Probes, Eugene, OR), rinsed and cover slipped with Vectasheild plus DAPI (Vector Laboratories Inc, Burlingame, CA) to counter stain nuclei.

Oligodendrocyte precursor cells were detected with a rabbit antibody to NG2, a kind gift from Dr. W.B. Stallcup (BIMR, La Jolla, CA). Five micrometer frozen sections were fixed in 95% ethanol before being stored at -80 ^{o}C. Upon removal from the freezer, sections were post-fixed in cold acetone, rinsed in KPBS, and blocked with 0.1% Triton X-100/ 5% normal goat serum in KPBS for 1 hour at room temperature. Sections were then incubated with NG2 antibody (1:500 in blocking solution) or isotype control overnight at 4 ^{o}C. Following rinsing, sections were incubated for 1 hour at room temp with a goat anti-rabbit IgG AlexaFluor-conjugated secondary antibody (1:600) (Molecular Probes, Eugene, OR), rinsed and cover slipped with Vectashield plus DAPI (Vector Laboratories Inc, Burlingame, CA) to counter stain nuclei.

All comparative analyses were focused in the corpus callosum at the midline or just lateral to midline (Figure 2.1). Immunohistochemically positive cells were analyzed using a Nikon (Tokyo, Japan) Optiphot FXA microscope with epifluorescence optics and quantified per square mm. Positive-stained cells were counted only if a nucleus was observed.

Statistical analysis: Statistical comparisons between time points for each gender separately were carried out using a one-factor ANOVA and Tukey's test for multiple comparisons. Statistical analysis to determine whether there was a gender effect was made using a two-factor ANOVA and Bonferroni correction for comparisons between time points. The data are expressed as mean $\pm$ SEM.

2.4 RESULTS

Localization of Demyelination within the Corpus Callosum of SJL mice.

We first examined brains of SJL mice for demyelination at 5 weeks following exposure to cuprizone, the time when demyelination is complete in the C57BL/6 mouse [148, 161, 164]. Coronal sections were stained with LFB-PAS to determine the extent of demyelination. Robust demyelination did not occur at midline as observed in C57BL/6 mice, but instead, localized immediately lateral to midline (Figure 2.1). In addition, there appears to be two different layers of myelinated fibers, each showing distinct patterns of demyelination, with the lower lateral layer, adjacent to the fornix, showing less demyelination than the upper lateral layer (Figure 2.1B). Myelination scores reflect a combination of both of these layers. This pattern is more similar to that occurring in the caudal corpus callosum in the C57BL/6 mice at the level of the hippocampus, in which three myelin layers are sometimes observed (unpublished observations). Although the lateral areas appear to be more susceptible to demyelination than the midline in SJL mice, we have characterized the temporal pattern of demyelination in both regions.
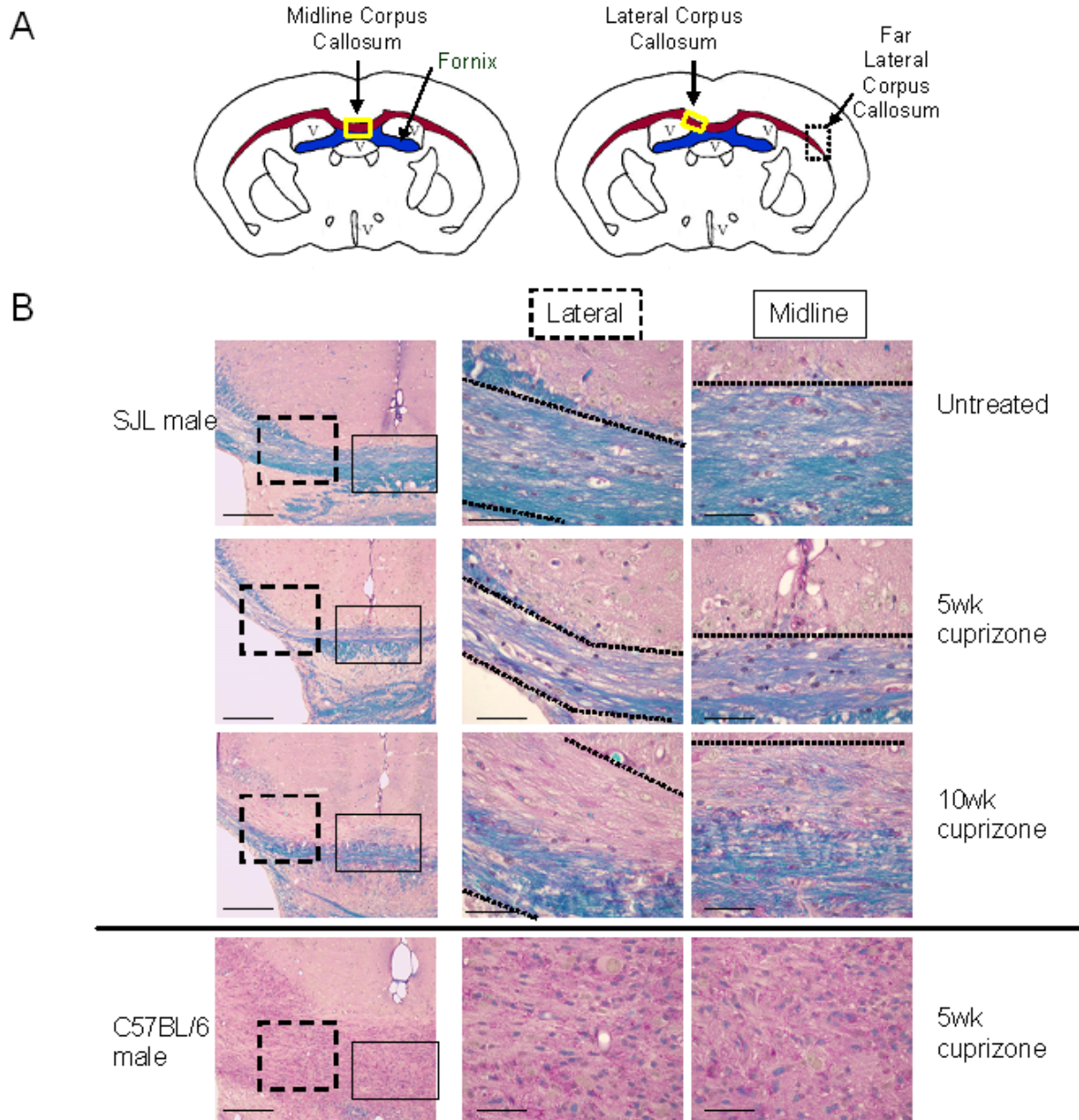
Figure 2.1. Illustration of areas scored for myelination in SJL and C57BL6 corpus callosum.
A. Midline scores were derived from coronal sections of the SJL brain above the fornix
(Bregma -0.5 to -0.7) as indicated by the solid box in the left figure. Lateral scores were
immediately adjacent to the midline areas of the corpus callosum that is depicted by the solid
box in the right figure. Far lateral areas indicated by the dotted box also showed
demyelination but were not scored. The letter "V" indicates a ventricle.
B. Comparison of demyelination in midline versus lateral corpus callosum of SJL and
C57BL/6 mice. LFB-PAS stain for myelin (blue fibers), microglia/macrophages (pink
cytoplasm) and demyelinated axons (pink fibers). Left column depicts the regions that were
scored for demyelination: midline and lateral, scale bars represent 200 μM length. In the

right two columns, the corpus callosum is outlined by dotted lines and shows representative images of lateral and midline regions from untreated, 5 week and 10 week cuprizone treated animals. Right two column scale bars represent 50 micrometer length. Note the presence of two layers of fiber tracts in SJL, especially evident in the 10 week treated brain. The bottom row shows midline and lateral areas of 5 week treated C57BL/6 mice that were treated along side the SJL mice and are typical of the robust demyelination and gliosis previously reported in this strain.

Titration of cuprizone in SJL mice

SJL mice were exposed to different concentrations of cuprizone (0.1, 0.2, 0.3, 0.4, 0.5%) in the diet to determine the amount of cuprizone required to induce demyelination without significant toxicity. Again, mice were sacrificed at week 5, corresponding to the time of full demyelination in C57BL/6 mice. Mice on a diet of 0.3% or greater exhibited overt toxic effects of cuprizone indicated by diminished weight, lethargy and lack of grooming. Over half of the animals from these groups succumbed to the higher doses, similar to C57BL/6 mice. 0.1% cuprizone induced very little demyelination, not significantly different from untreated. Therefore, we determined that 0.2% provided the optimal cuprizone dosage to administer to SJL mice.

Temporal pattern of demyelination at midline and lateral areas of the corpus callosum.

Male and female SJL mice were treated continuously with 0.2% cuprizone for 3 to 10 weeks and analyzed at weekly intervals to determine the time frame of maximal demyelination and possible remyelinating episodes. Sections of the corpus callosum were stained for myelin with LFB-PAS and scored in a blinded manner both at midline and lateral regions. Demyelination was also observed far lateral in the corpus callosum, on the opposite

side of the ventricle (Figure 2.1A), However, the fiber density and variability made scoring less reliable in these regions, so they were not included in the illustrated assessments.   As shown in Figure 2, demyelination in male and female mice continuously exposed to cuprizone occurred gradually, from week 3 to week 10. In the midline region (Figure 2.2A) cuprizone-treated male SJL mice showed significantly greater demyelination at weeks 8-10 compared with untreated mice (p<0.05; one factor ANOVA with Tukey's post-test).   In female SJL mice in the midline region, demyelination reached a statistically significant difference from untreated mice only at weeks 9 and 10 (p<0.001). Comparison of male and female mice using two-factor ANOVA showed a significant gender effect on demyelination over the entire observed time period (p=0.0010). The midline corpus callosum showed only partial demyelination and remyelination was not observed during the course of cuprizone administration.

The same coronal sections of brains from SJL mice continuously exposed to cuprizone were scored for demyelination immediately lateral to the midline area (See Figure 2.1). As shown in Figure 2.2B, the temporal pattern of demyelination in the lateral areas is dramatically different than at midline. Mild demyelination was detected in male SJL mice beginning at week 3, reaching statistical significance compared with untreated mice at weeks 5 and 6 (p<0.05). More robust demyelination was observed in males at week 7 (p<0.001), reaching a plateau through week 10. The partial demyelination present at week 10 persists as long as week 13 (data not shown). Thus, unlike male C57BL/6 mice, which show three cycles of demyelination while on continuous exposure to cuprizone over 13 weeks [166], SJL mice appear to demyelinate gradually once during these time points; however, the demyelination is incomplete.

Equally important in female SJL mice, demyelination showed a slow, delayed course of demyelination (Figure 2.2B), which is moderate from weeks 3 through 6 and does not reach a statistically significant difference from untreated until week 7 (p< 0.001). This is in contrast to males which reached statistically significant demyelination by week 5. From weeks 5 to 10, female SJL mice exhibit less demyelination than male counterparts, and this gender effect over the entire observed time period is significant (two factor ANOVA p=0.0305). Thus, female SJL mice appear to be less susceptible to demyelination than male mice. In both male and female SJL there is no apparent remyelination during this time period.

A preliminary study of remyelination was performed by removing cuprizone from the diet at the 7 week time point, and allowing the mice to recover for 3 weeks. Interestingly, when compared to the 7 week time point, significant remyelination had not occurred in either the midline or lateral regions (Figure 2.2A and 2.2B). Furthermore, there was no significant gender difference in myelin score at this time point.
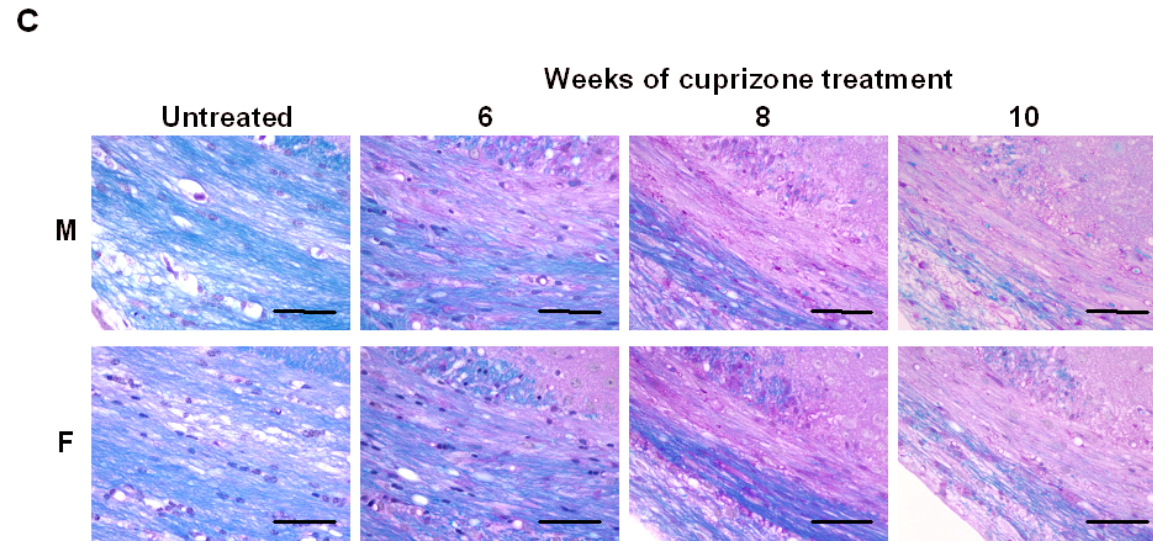
A

B

C
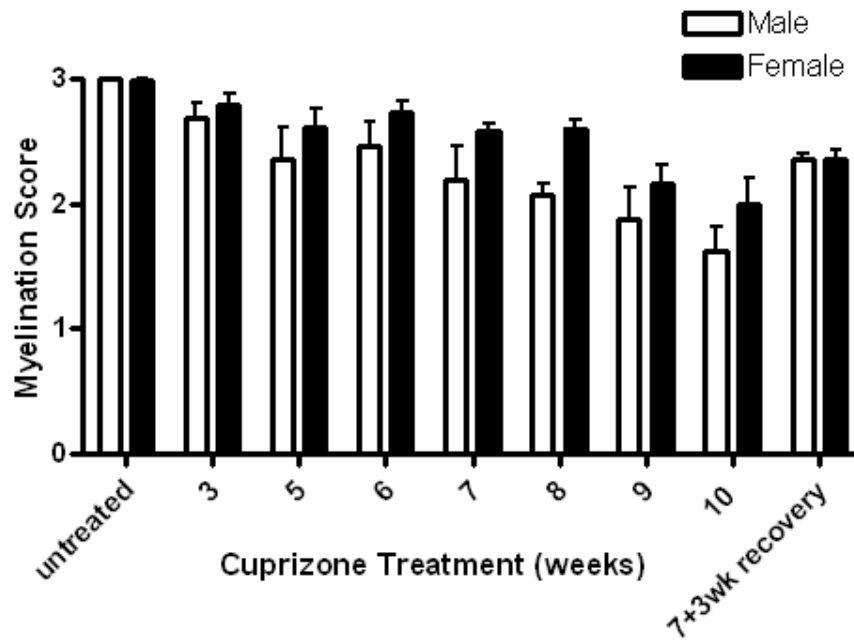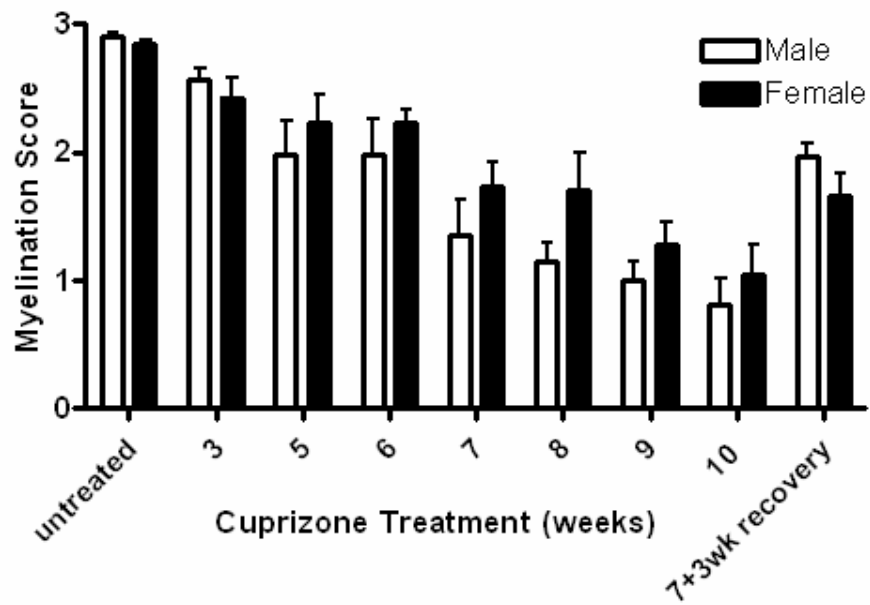
Figure 2.2. Cuprizone-induced demyelination of the SJL corpus callosum.
Myelination scores of the corpus callosum from male and female SJL mice continuously
exposed to cuprizone (8 mice per gender per group, except the recovery time point which is
composed of 4 mice per gender per group).
A. Midline corpus callosum: two-factor ANOVA indicates a significant gender effect
(p=0.0010). B. Lateral corpus callosum: two-factor ANOVA indicates a significant gender
effect p=0.0305.   C. Representative images of LFB-PAS stain in male (M) and female (F)
lateral corpus callosum at selected time points. Scale bar equals 50 μM

Quantification of mature oligodendrocytes.

In C57BL/6 mice continuously exposed to cuprizone, mature oligodendrocytes are

depleted by week 5 and recover temporarily at week 6 before diminishing a second time

[178]. Here, we quantified the mature oligodendrocyte population in the midline and lateral

areas. Untreated male and female mice begin with similar numbers of oligodendrocytes in

the corpus callosum (Figure 2.3). As shown in Figure 2.3A, the number of mature

oligodendrocytes at midline is initially diminished at week 3 but did not reach a statistically

significant difference from untreated mice until week 5. Thereafter the number of mature

oligodendrocytes appears to plateau through week 10. Except for week 7, female mice show a trend for greater numbers of mature oligodendrocytes than male mice from week 3-10, particularly at week 8. The overall gender effect by two-factor ANOVA is statistically significant (p=0.0158). Thus, similar to demyelination in Figure 2.2, there are significantly more mature oligodendrocytes in female mice than male mice.

In the lateral regions of the corpus callosum, a gradual depletion of mature oligodendrocytes is observed in both male and female mice, beginning at week 3, compared with untreated mice (p<0.01; Figure 2.3B). Female mice have more oligodendrocytes than male counterparts at all time points, and this gender effect is highly significant (p<.0001). Interestingly at weeks 8 and 9, there is a trend for recovery of mature oligodendrocytes in both males and females. By week 10, the number of mature oligodendrocytes again diminishes. However, similar to demyelination, full depletion of mature oligodendrocytes is not observed during this time period.

When cuprizone administration was discontinued at week 7 and mice were allowed to recover for 3 weeks, a modest increase in oligodendrocytes was observed at both midline and lateral regions; however, statistical significance was observed only in the lateral region (Figure 2.3B, p<0.05 for both male and females). However, there is no gender difference in the number of oligodendrocytes during this recovery time point (7+3 week). This increase in oligodendrocytes indicates that SJL mice are capable of recovery from cuprizone-induced oligodendrocyte loss and correlates with the remyelination observed in Figure 2.2A and 2.2B.

A

B

46
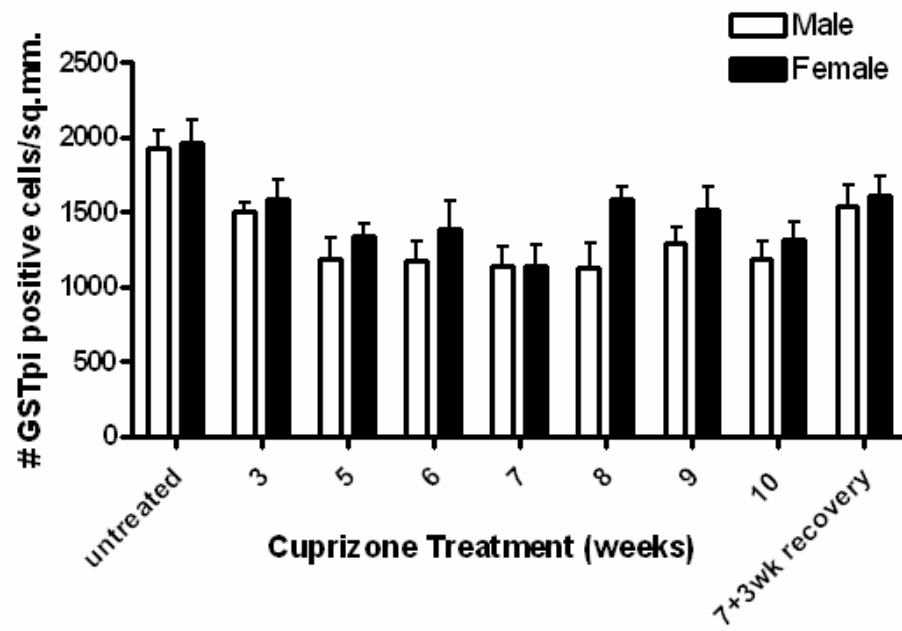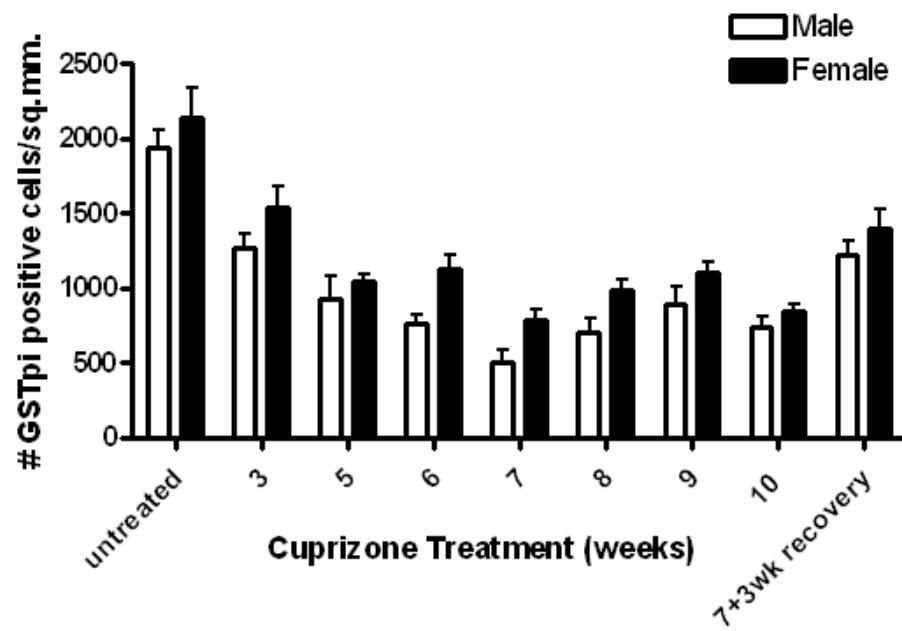
Figure 2.3. Mature oligodendrocytes in the corpus callosum of SJL mice.
Male and female mice were treated with cuprizone continuously over the time course
indicated (8 mice per gender per group, except the recovery time point which is composed of
4 mice per gender per group).
A. Numbers of GSTpi[+] oligodendrocytes were quantified from the midline of the corpus
callosum. Two-factor ANOVA indicates a significant gender effect $p=0.0158$.
B. Numbers of GSTpi[+] oligodendrocytes were quantified from lateral areas of the corpus
callosum. Two-factor ANOVA indicates a significant gender effect $p<0.0001$.
C. Representative images of mature oligodendrocytes identified by immunoreactivity to
GSTpi in male (M) and female (F) lateral corpus callosum at selected time points. Scale bar
equals 50 μM.

Oligodendrocyte precursor cells accumulate in the demyelinated lesion.

The accumulation of oligodendrocyte precursor cells (OPCs) in demyelinated lesions has been noted since the earliest cuprizone studies in both ICI and Swiss mice [146, 152]. In these studies OPCs were identified by ultrastructural characteristics and were detected at 5 weeks of cuprizone treatment when the lesions in superior cerebellar peduncles were nearly fully demyelinated. The availability of antibodies to the NG2 protein, which is commonly used as one marker for OPCs, has allowed for easier quantification of these cells. In C57BL/6 mice, NG2-positive OPCs are present in limited numbers in untreated mice, accumulate during cuprizone-induced demyelination and reach maximal numbers at 4 and 5 weeks of intoxication [162]. During recovery, OPC numbers decline slightly, presumably due to their maturation into oligodendrocytes that are responsible for the remyelination of the lesion [162, 165]. However, chronic administration of cuprizone (8-12 weeks), leads to progressive depletion of OPCs from the lesion [178] Here, we used the NG2 marker to identify the pattern of OPC accumulation in SJL male and female mice at select times during demyelination. A quantification of NG2-positive OPCs indicates that a few of these cells are present in the untreated corpus callosum, in similar numbers between male and female SJL (Figure 2.4). During cuprizone intoxication, OPC numbers increase gradually, becoming statistically significant at the 8 week time point. Unlike the response of mature oligodendrocytes, the numbers of OPCs are very similar in the midline and lateral regions of the corpus callosum. In addition, OPCs seem to differ from mature oligodendrocytes in that there is no statistically significant gender difference in their numbers (Figure 2.4).

C

Weeks of cuprizone treatment

Untreated   3   6   8

M

F

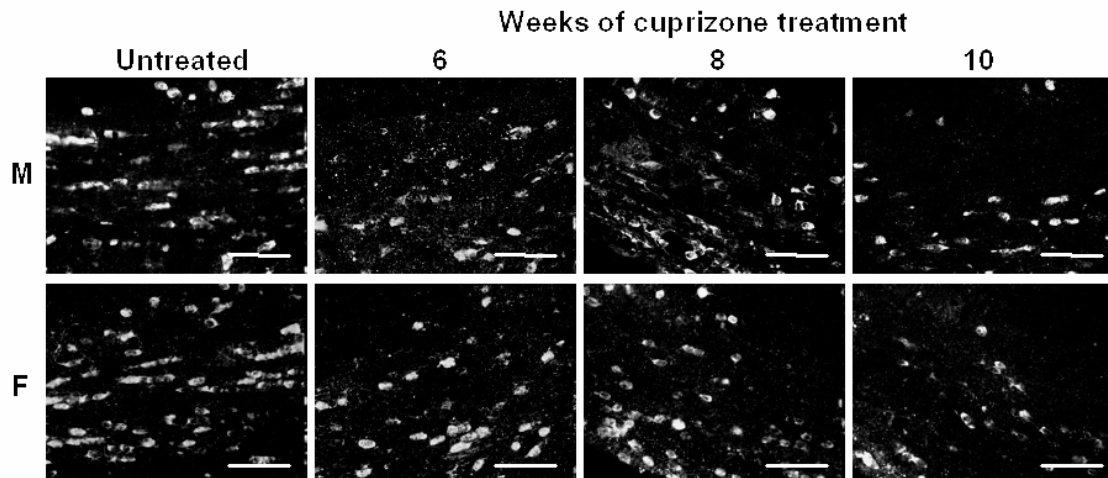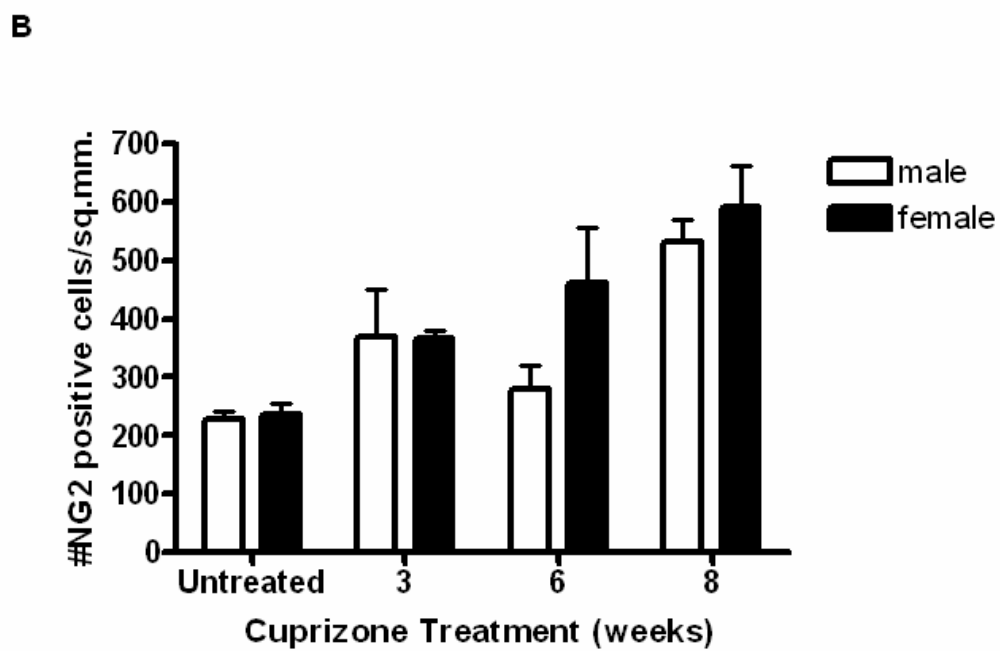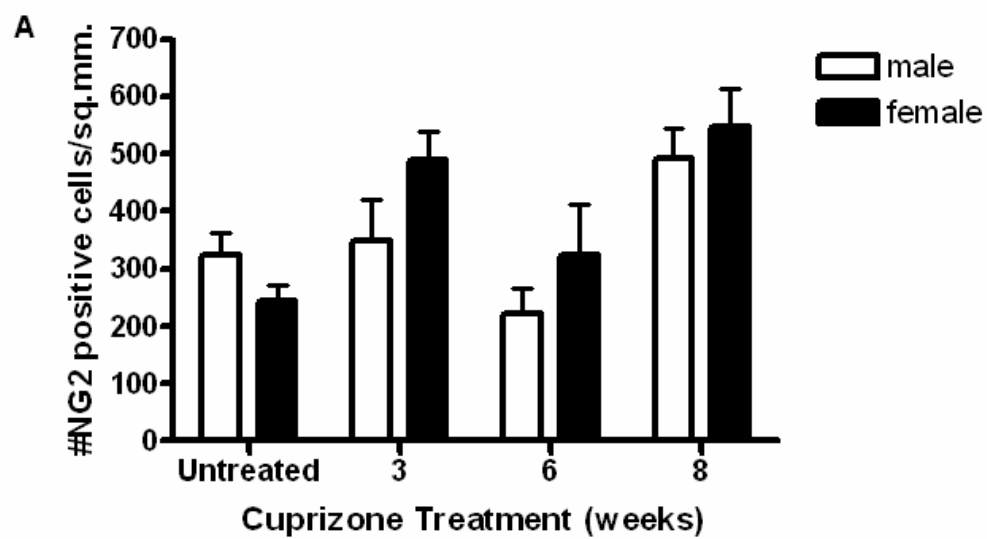Figure 2.4. Oligodendrocyte precursor cells (OPCs) in the corpus callosum of SJL mice.
Male and female mice were treated with cuprizone continuously over the time course
indicated (4 mice per gender per group).
A. Numbers of NG2$^+$ OPCs were quantified from the midline of the corpus callosum. Two-
factor ANOVA indicates there is no significant gender effect.
B. Numbers of NG2$^+$ OPCs were quantified from the lateral corpus callosum. Two-factor
ANOVA indicates there is no significant gender effect.
C. Representative images of OPCs identified by immunoreactivity to NG2 in male (M) and
female (F) lateral corpus callosum at selected time points. Scale bar equals 50 μM

Quantification of microglia

Microglia appear during demyelination and typically disappear with remyelination [165, 178, 182]. Here we assessed the microglial response at midline and lateral regions to determine if their numbers correlated with the extent of demyelination. As show in Figure 2.5A, microglia accumulation at midline in both male and female mice is detected at weeks 3 and 5 and becomes statistically significant compared to untreated during weeks 6 through 10 ($p<0.05$). In female mice there appears to be a plateau from week 6 through week 10, whereas male mice exhibit an upward trend in microglial numbers through week 9. At a few time points microglia accumulation appears to be less robust in female mice; however, there is not a statistically significant gender effect.

In the lateral regions, female and male mice show microglia accumulating between week 3 through week 6 and then the number of microglia appear to level off (Figure 2.5B). There are no significant differences among male and female mice in terms of microglial responses to demyelination. Interestingly, the number of microglia in the lateral regions is greater in number compared to the midline, probably due to the greater demyelination observed in Figure 2.2B.

A

B

C

Weeks of cuprizone treatment

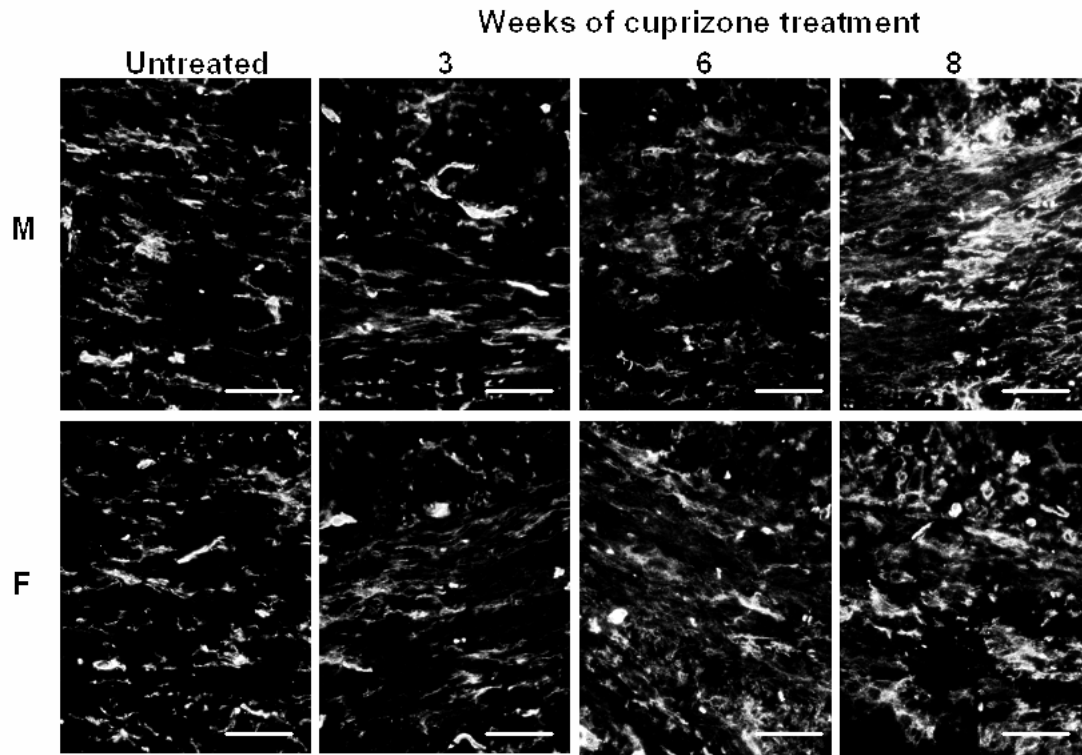| Untreated | 6 | 8 | 10 |
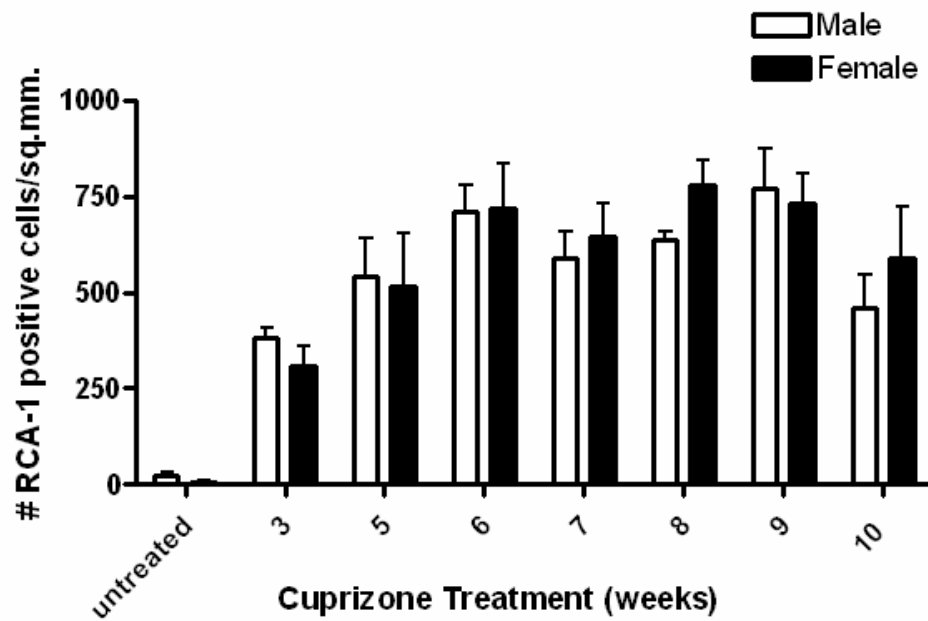
Figure 2.5. Microglia in the corpus callosum of SJL mice.
Male and female mice were treated with cuprizone continuously over the time course
indicated (8 mice per gender per group).
A. Numbers of RCA-1$^+$ microglia were quantified from the midline of the corpus callosum.
Two-factor ANOVA indicates there is no significant gender effect.
B. Numbers of RCA-1$^+$ microglia/macrophages were quantified from the lateral corpus
callosum. Two-factor ANOVA indicates there is no significant gender effect.
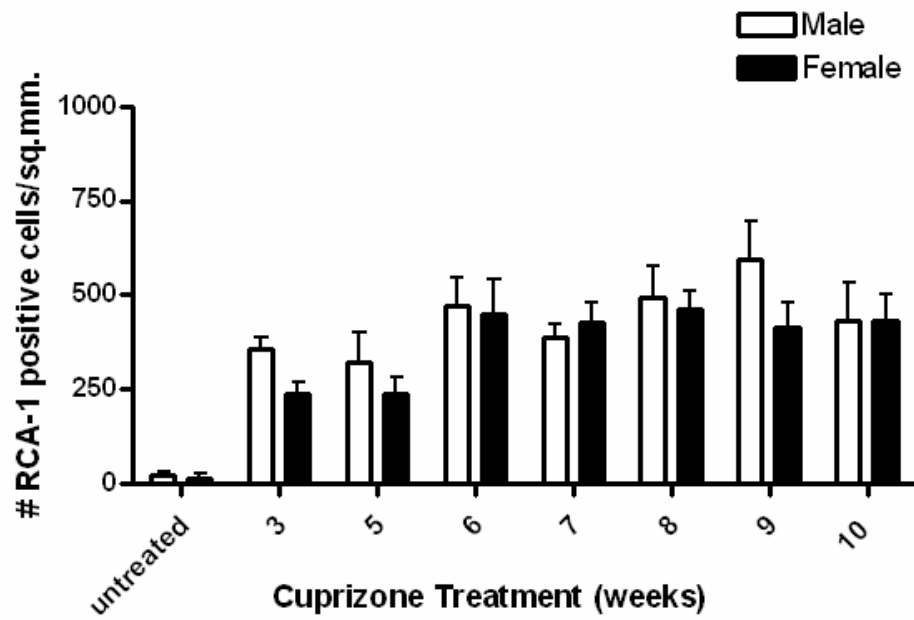C. Representative images of microglia/macrophages identified by reactivity to the lectin
RCA-1 in male (M) and female (F) lateral corpus callosum at selected time points. Scale bar
equals 50 μM

Quantification of astrocytes

Similar to microglia (though fewer in number), astrocytes infiltrate the demyelinated lesion, but unlike microglia, typically persist through remyelination [182]. Here we assessed whether differences observed for demyelination and mature oligodendrocyte scores could be partly explained by the astrocyte populations. At midline, an endogenous population is present as expected and there is no difference between male and female mice. After exposure to cuprizone, a higher number of astrocytes begin to populate the midline of the corpus callosum and by week 5, they appear to have reached maximal numbers in both male and female mice (Figure 2.6A). There is no difference in the astrocyte response between male and female mice at midline.

In the lateral areas, the appearance of astrocytes is very similar to the midline scores. Interestingly, unlike the greater number of microglia found in the lateral areas, astrocyte numbers (~500 cells/mm^2) in the lateral regions are similar to the numbers at midline. Although female mice appear to have a slight trend for higher numbers of astrocytes at several time points, the overall differences are not statistically significant.
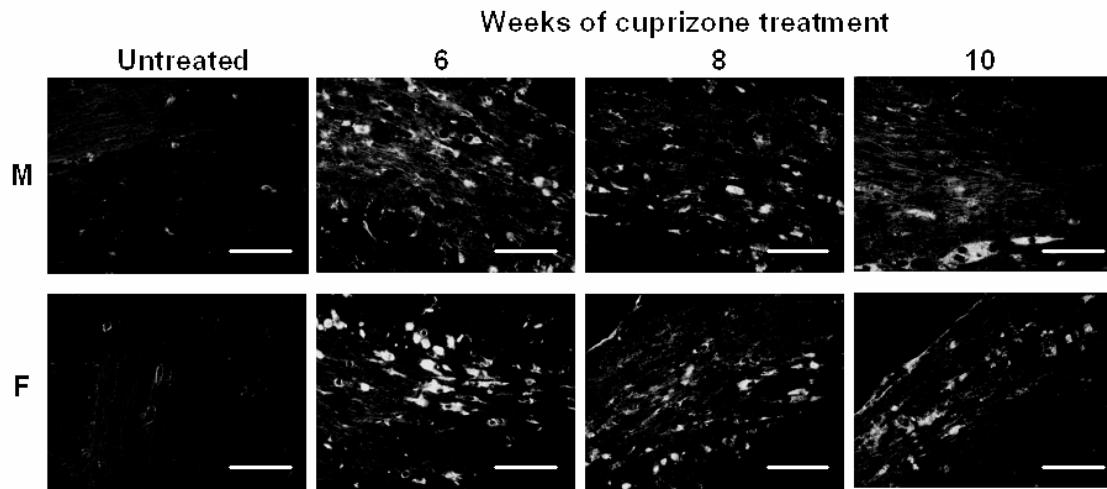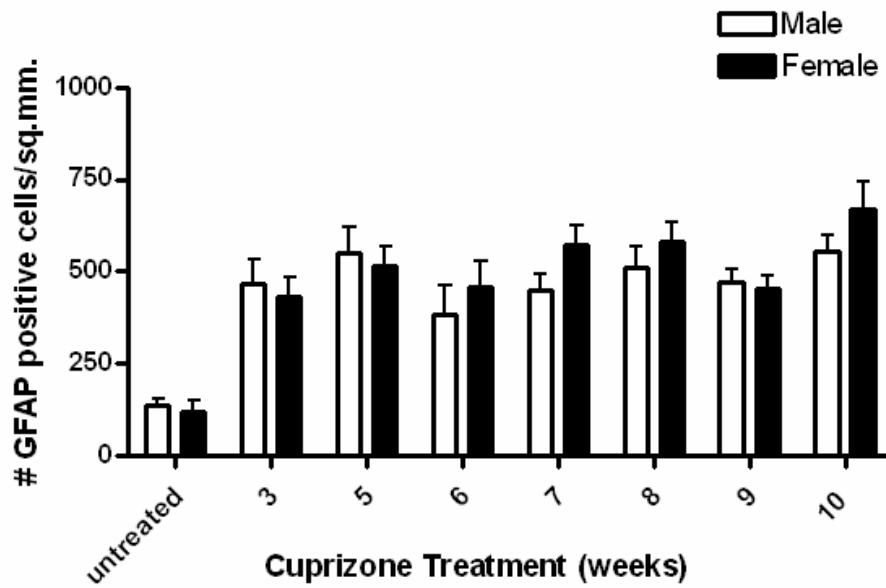
C
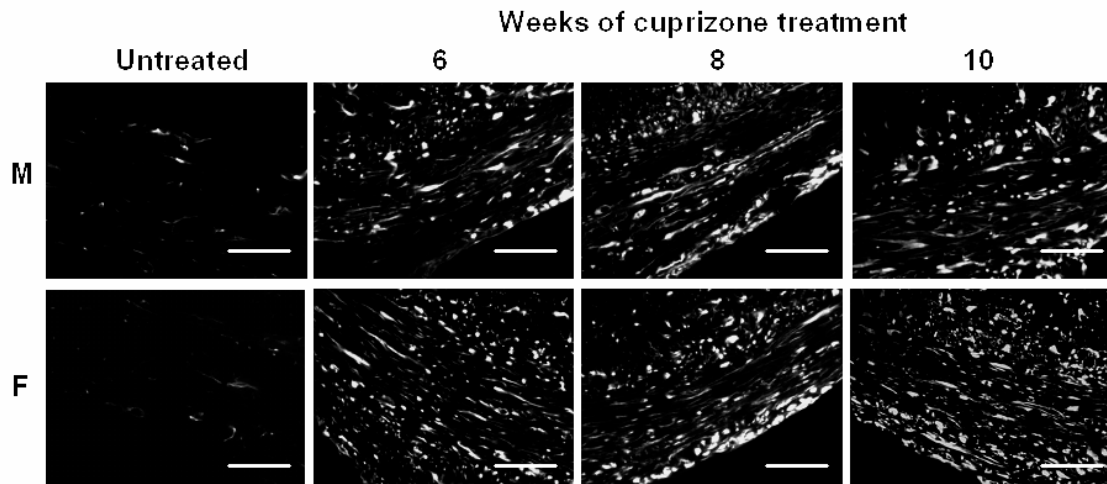
Figure 2.6. Astrocytes in the corpus callosum of SJL mice.
Male and female mice were treated with cuprizone continuously over the time course
indicated (8 mice per gender per group).
A. Numbers of GFAP$^+$ astrocytes were quantified from the midline of the corpus callosum.
Two-factor ANOVA indicates there is no significant gender effect.
B. Numbers of GFAP$^+$ astrocytes were quantified from the lateral corpus callosum. Two-
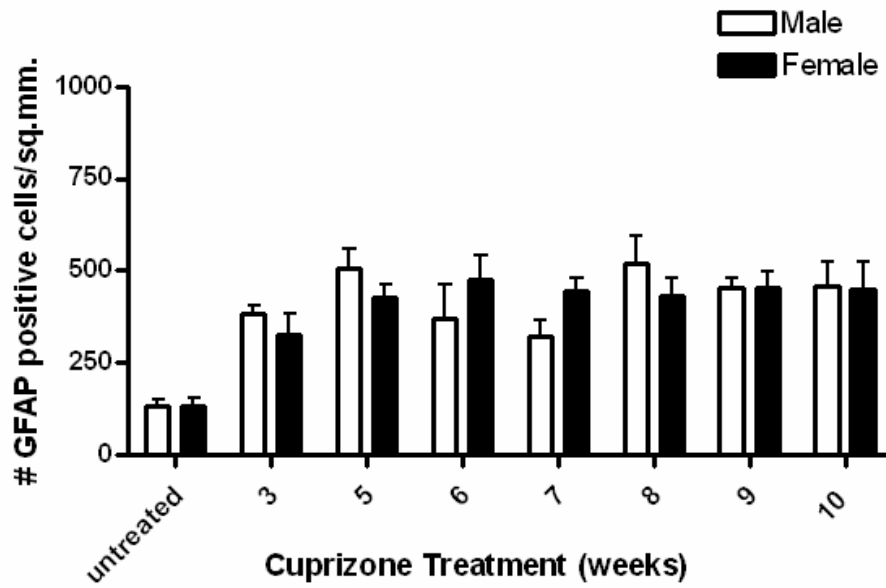factor ANOVA indicates there is no significant gender effect
C. Representative images of astrocytes identified by immunoreactivity to GFAP in male (M)
and female (F) lateral corpus callosum at selected time points. Scale bar equals 50 μM

2.5   DISCUSSION

The SJL strain of mice revealed several differences when compared to C57BL/6 mice

in the cuprizone model of demyelination. First, the location of appreciable demyelination

within the corpus callosum is immediately lateral to midline, rather than midline, as observed

in the C57BL/6 mouse. Second, demyelination is only partial in SJL mice through 10 weeks

of continuous treatment whereas full demyelination is observed at week 5 in C57BL/6.

Third, demyelination was less severe in female SJL mice than male mice. Fourth, mature

oligodendrocytes in female SJL mice were more resistant to depletion than male counterparts. There were no apparent differences between the sexes in the number of oligodendrocyte precursors, microglia or astrocytes in the demyelinated regions. Furthermore, unlike C57BL/6 mice which remyelinate quickly upon discontinuation of cuprizone, SJL mice which were treated with cuprizone for 7 weeks and then allowed to recover for 3 weeks showed only slight remyelination. However, SJL mice are similar to C57BL/6 mice in that a 0.2% diet of cuprizone promotes demyelination without the severe toxic effects observed at 0.3% diet of cuprizone or greater.

Interestingly, several strains of mice show differences in sensitivity to cuprizone intoxication. Studies of ICI and Swiss Webster mice used 0.5% to 0.6% cuprizone in their diet to induce demyelination [146, 183, 184]. Though not tolerated by all of these mice, many of them mice lived for months on the cuprizone diet. In contrast, C57BL/6 mice and SJL mice are more susceptible to cuprizone, showing demyelination at the lower 0.2% dose. Similar to C57BL/6 mice [161], SJL mice die or are lethargic at 0.3%, and higher doses of 0.4, 0.5 and 0.6% resulted in death within the first couple of weeks. It is not clear why there is strain variability in sensitivity to cuprizone. Recently, strain differences have been reported for cortical demyelination induced by cuprizone. In this study BALB/cJ mice were treated with 0.2% cuprizone and compared to C57BL/6 [160]. The BALB/cJ mice were reported to have a similar pattern of demyelination in the corpus callosum, but less demyelination and more microglia activation in the cortex as compared to C57BL/6.

The difference in response due to genetic background becomes important also when crosses of different mice are used to analyze specific genes. Unless there is an attempt to backcross to an established genotype such as C57BL/6, the background genes of the

knockout or transgenic mice are not homogeneous and could influence outcomes. This may be also true for 129 mice which have similar patterns of demyelination in the corpus callosum as C57BL/6, but demyelinate at a lower dose of 0.1% of cuprizone (data not shown). Thus, mice on the 129 background may show more severe effects if tested at the 0.2% cuprizone dose and mice of a mixed background may give a more complicated pattern. The data in this manuscript emphasize the importance of considering the influence of genetic background in the study of demyelination, regardless of the model, (EAE, viral or toxin-induced demyelination).

Genetic background clearly influences the morphologic and temporal pattern of demyelination. We found SJL mice demyelinate partially at midline (Figure 2.2A), while C57BL/6 mice show robust demyelination, particularly at week 5 [161, 164, 165]. SJL mice show greater demyelination in the lateral region of the corpus callosum immediately adjacent to the midline; however, two distinct layers appeared to demyelinate differently. The lower one third is more resistant to demyelination than the upper two thirds (Figure 2.1B), hence, myelin scores in the SJL mice are a bit more complex requiring a cumulative subjective combination. Scores in Figure 2.2B are reflective of the entire lateral corpus callosum region indicated in Figure 2.1. The utility of the LFB-PAS histological stain is illustrated here as myelin fibers that are stained blue versus unmyelinated fibers that stain pink can be estimated quickly for a large number of samples. It would be cumbersome to assess these sections by electron micrography since all axons within the tract would have to be counted because representative areas would be difficult to choose. Previous studies have shown that LFB-PAS scores are reflective to trends in demyelination when compared to percent myelinated fibers that are counted in electron micrographs [166, 185]. However, one limitation of the LFB-

PAS stain is that one cannot detect whether any remyelination is occurring during the demyelination period. In order to determine whether there is any remyelination occurring during the cuprizone treatment, electron microscopy could be employed to monitor thinly myelinated axons which are characteristic of remyelination [186]. However, in C57BL/6 mice, we do not detect any apparent remyelination in the midst of demyelination and remyelinated axons are not detected until after complete demyelination which occurs at week 5.

The reason for regional differences in demyelination of the corpus callosum observed in SJL mice is not known. There are many examples of regional differences in dysmyelination due to genetic manipulation of mice between areas such as the optic nerve, spinal cord, cerebellum, and corpus callosum. For example, laminin deficient *dystrophia muscularis (dy/dy)* mice exhibit hypomyelination in the corpus callosum and optic nerve, but not the spinal cord [187] whereas dominant negative β1 integrin ΔC mice are hypomyelinated in spinal cord and optic nerve, but not the corpus callosum [188]. Heterogeneity of oligodendrocytes is one possible explanation for such regional differences, however this seems unlikely in the present case, given that we are seeing differences within the corpus callosum rather than between different white matter regions. One possible explanation for the appearance of two different layers of demyelination (Figure 2.1B) in the lateral region of SJL corpus callosum during demyelination is that these may represent two different groups of axonal fibers coming from different types of neurons and are providing different levels of trophic support or conversely different levels of detrimental signaling. Similar layers that show differential demyelination in C57BL/6 mice occur in a more caudal regions of the corpus callosum next to the hippocampus (data not shown) and the reasons for

these differences is not clear. Another alternative explanation is that the cuprizone has better access to oligodendrocytes in the upper lateral layers or these oligodendrocytes are hypersensitive to insult.

Our temporal analysis of demyelination in the lateral corpus callosum indicates 7 weeks of cuprizone treatment is optimal for detecting marked demyelination in SJL mice. From 7 weeks on, there is only modest continued demyelination. In addition, 7 weeks is when the greatest loss of oligodendrocytes was observed in both genders. One of the more striking differences in SJL mice is the lack of total demyelination during a 10 week period of continuous exposure to cuprizone. This is very different than C57BL/6 mice which fully demyelinate by week 5. Even out to week 13 of continuous exposure to cuprizone, demyelination scores were approximately 0.75 for males and 1.0 for females at the lateral region, similar to week 10 scores in Figure 2.2B. Although higher doses may induce full demyelination, we could not examine SJL mice at doses of 0.3% or greater as they are susceptible to systemic toxicity. In addition, C57BL/6 mice experience a spontaneous partial remyelination after 6 weeks and then continue to demyelinate further when exposed continuously to cuprizone [148, 166]. Despite a trend for an increase in the oligodendrocyte population at weeks 8 and 9, we did not detect any remyelination during continuous cuprizone exposure in SJL mice.

An additional interesting difference of SJL mice from C57BL/6 mice, is the lack of robust remyelination after 3 weeks of recovery from the cuprizone diet. C57BL/6 mice show greater than 50% remyelination within 2 weeks of discontinuation of cuprizone [163, 165], which is in stark contrast to the reduced remyelination report here for SJL. However, it should be emphasized that the remyelination analysis performed here is limited and a more

thorough examination to determine whether remyelination progresses further would require later time points. The quantification of mature oligodendrocytes indicates that there is some recovery of these cells after 3 weeks of removal from cuprizone, and it is possible that this would lead to greater remyelination at later time points. A less robust remyelination in SJL could be explained by the less robust infiltration of microglia and astrocytes which are known to produce factors such as IGF-1 and TNFα that are important for promoting remyelination [164, 165, 189].

Another important finding is the difference between male and female SJL mice during demyelination. At many time points, male mice were more severely demyelinated than female mice in both midline and lateral regions, and the overall gender effect was statistically significant. In addition, when we analyzed the oligodendrocyte populations at midline and lateral regions, we found a statistically significant preservation of oligodendrocytes in female mice compared to males (Figure 2.3). Thus, female SJL mice are more resistant to oligodendrocyte loss and demyelination, and this trend was observed at most of the time points between weeks 5 through 10. A difference in the size of corpus callosum or numbers of oligodendrocytes in untreated mice is unlikely to explain the resistance to toxicity reported here. As shown in Figure 2.3, untreated male and female SJL mice have similar numbers of oligodendrocytes in the corpus callosum. Although, the size of the corpus callosum was not measured here, data published by Bishop and Walsten [190] indicate that unlike in humans or rats, mice do not display a gender difference in corpus callosum size. The lack of difference in numbers of OPCs (Figure 2.4) and remyelination (Figure 2.2) between male and female SJL suggest that there is no gender difference in

proliferation, migration, or maturation of oligodendrocytes. Additional future experiments to further characterize remyelination in SJL mice may clarify this point.

It is possible that female gender affords a protection from toxicity in general, which is supported by our observations during the cuprizone dose titration that male mice died more quickly when exposed to higher doses of cuprizone (data not shown). Analysis of liver sections indicated that at the 0.2% cuprizone dose, there is little indication of toxicity in either gender as indicated by vacuolation or necrosis, but male mice do exhibit an increase in binucleated hepatocytes compared to untreated mice, whereas females do not (data not shown). Whether this indicates that female mice may handle cuprizone differently than males, or whether mechanisms for protecting oligodendrocytes in female brains are superior to those in male mice remains to be determined. A final note, Swiss female mice have been reported to be resistant to cuprizone-induced demyelination; however, the mechanism for such differences compared to male Swiss mice is not known [146]. Studies of dietary copper deficiency in rats indicate that females exhibit less severe symptoms of weight loss, anemia, and lethality [191-193]. The mechanism of this gender difference is not known and the potential role of endogenous sex hormones is controversial. One study found an exacerbation of some copper deficient symptoms, such as weight loss, in ovariectomized females [194] but no effect on severity by male or female sex hormones was detected in another study [193]. It is possible that the protective effect from dietary copper deficiency or cuprizone-chelated copper deficiency may be similar in these female subjects; however, the inherent mechanism remains elusive.

Nevertheless these findings of gender differences are in contrast to those in the EAE model, in which female SJL are both more susceptible to disease induction, and exhibit more

severe neurological deficits [42, 46, 63]. The fact that female SJL mice show increased severity in EAE, but decreased severity in cuprizone intoxication may reflect a key difference between EAE and cuprizone models: EAE has an autoimmune etiology, while cuprizone delivers a toxic injury to oligodendrocytes. The increased severity in EAE may be due to T cell immune mechanisms which are not induced by cuprizone intoxication [165],[168]. Thus, in spite of a low threshold for autoimmunity in SJL females, female SJL oligodendrocytes appear to exhibit a high threshold, or resistance, to toxic injury. The utility of both models for the study of MS is supported by heterogeneity in MS lesions, reported by Lucchinetti and colleagues [6], in which type III and IV lesions suggest primary oligodendrocyte dystrophy, while type I and II lesions are consistent with T- or B-cell associated autoimmune mechanisms. Similar to our findings in the cuprizone model, female SJL mice infected with TMEV show less severe neurological deficits and a moderate (though not statistically significant) resistance to demyelination compared to males [69].

Future studies to determine the mechanism of protection of female oligodendrocytes will be an important contribution to the growing body of work addressing the role of gender in neuropathological conditions. There is much evidence for a role of sex hormones in demyelination and oligodendrocyte function. In MS there is a clear protection from relapse during the third trimester of pregnancy when several hormones, especially sex steroids, reach a peak [78]. In EAE, administration of estrogens lead to functional benefits [66, 86, 89, 195]. In addition, sex hormones have been shown to have direct effects on oligodendrocyte proliferation and maturation [131, 196] as well as survival [133, 134]. Cuprizone intoxication of SJL mice will be a useful model to study the role of sex hormones in primary oligodendrocyte disruption.

We also monitored the microglia and astrocyte populations in the male and female mice. In the midline corpus callosum microglia accumulation began by week 3 and reached statistically significant numbers compared with untreated mice by week 6, then remained unchanged through week 10, in both male and female mice. In the lateral regions, the microglial response was more robust, with nearly 30% more microglia/mm^2 than at midline, and there was a slight decrease at week 10. Overall there was not a difference between male and female mice. Lastly, when we measured the astrocytic response both at midline and in the lateral regions, we observed an increase in astrocytes until week 5, when they stabilized in number. Unlike microglia, astrocytes numbers in the lateral regions remained similar to numbers at midline and they did not increase appreciably. Thus, unlike microglia and astrocytes in C57BL/6 mice that respond vigorously to demyelination [148, 161, 165], these cell types in SJL mice have a guarded response at midline and in the lateral region, which correlates with subdued demyelination. There was no difference in microglia or astrocyte numbers between male and female SJL mice. This study did not address specific functions of microglia or astrocytes, which may also be influenced by sex hormones [29, 136, 140, 197-199] . Future studies to address differences in cytokine and growth factor production that may provide protection for oligodendrocytes are warranted and would shed important light on the role of these cells in demyelinating disease.

In conclusion, male and female SJL mice did not fully demyelinate during a period of 10 weeks of continuous cuprizone exposure, when C57BL/6 mice would have undergone two rounds of full demyelination, indicating a clear genetic influence on susceptibility to demyelination. Importantly, SJL female mice are more resistant than males to loss of oligodendrocytes and demyelination, though there were no gender differences in microglial

or astrocytic accumulation. Further study of gender and hormonal influences in SJL mice using the cuprizone model will be an important complement to EAE studies given the heterogeneity of disease pathology in human MS.

CHAPTER 3

C57BL/6J Mice Exhibit No Sex Difference in Cuprizone-induced Demyelination or Remyelination

3.1 ABSTRACT

Demyelinating diseases such as multiple sclerosis may be influenced by gender dimorphisms. Cuprizone-induced demyelination and remyelination have been extensively characterized in male mice, but a direct comparison with female mice has not been conducted. We have undertaken a comprehensive characterization of the morphological and cellular processes that occur in female C57BL/6J mice during cuprizone-induced demyelination and subsequent remyelination, and compared this to age-matched male counterparts. We find that the pattern of demyelination and remyelination is similar between genders, and that there is little to no difference in the loss or repopulation of mature oligodendrocytes, or accumulation of reactive glia. Interestingly, we found that cuprizone treatment disrupts estrous cyclicity in female mice, possibly interfering with potential hormone influences on demyelination and remyelination. Therefore, cuprizone- induced demyelination in C57BL/6J mice may have limitations as a model for the study of sex differences.

3.2 INTRODUCTION

Destruction of the myelin sheath and subsequent neurological impairments are hallmark characteristics of the degenerative disease multiple sclerosis (MS). MS is thought to be an autoimmune disease, due to the presence of myelin reactive T cells present in demyelinated lesions. Women are 2-3 times more likely to develop MS, a trend that is shared with many autoimmune diseases. Gender may also have subtle effects on disease

severity, illustrated by a natural history study of greater than 1800 patients in which female

gender showed an association with slower progression of disease [13]. Also, MRI scans of

281 female and 132 male MS patients revealed that men had a higher number of "black hole"

lesions, which mark axonal loss [14]. A role for high levels of sex hormones is demonstrated

by the finding that females have fewer relapses during pregnancy, when several sex

hormones are elevated [78]. Given these gender dimorphisms in MS, it is critical to

understand the mechanisms of sex differences in demyelinating disease.

The most frequently used animal model for MS is experimental autoimmune

encephalomyelitis (EAE). Sex differences occur in this model, and these vary by animal

strain, suggesting a genetic influence. Female SJL mice, commonly used in EAE studies,

show a greater incidence and severity of disease, and are more likely to exhibit relapses than

males [42, 46, 63-65]. However, other strains exhibit increased severity in males (B10.PL

and PL/J) or no sex differences (NOD/lt and C57BL/6) [46]. Another autoimmune model of

MS, intracerebral inoculation with Theiler's murine encephalomyelitis virus (TMEV) also

displays sex differences that vary by strain. Male C57L/J mice are more susceptible than

females to TMEV-induced demyelinating disease [200], and male SJL mice exhibit more

severe neurological deficits than females [69].

Models that involve chemically induced death of oligodendrocytes and demyelination

are useful for the study of primary oligodendrocyte perturbation, which is hypothesized to

occur as a component of the pathology in type 3 and 4 MS lesions [6]. These models are also

very useful in the study of remyelination and recovery from oligodendrocyte loss. Using the

ethidium bromide toxin model, it was shown that young male and female rats display a

similar rate of remyelination, but in older rodents where remyelination occurs more slowly [201, 202], females remyelinated more efficiently than male counterparts [73].

Another toxin model that produces a well-defined and highly reproducible pattern of demyelination and remyelination is the cuprizone model. Cuprizone is a copper chelator that has been used to induce demyelination in several strains of mice. More recently, this model has been well characterized in male C57BL/6 mice. Exposure to a low dose of cuprizone induces death of oligodendrocytes and loss of myelin in the corpus callosum fiber tract [161, 162] as well as some degeneration and axonal loss, especially in older mice [174]. In the demyelinated lesion, oligodendrocyte loss is accompanied by active gliosis and accumulation of oligodendrocyte precursors [162]. Upon discontinuation of cuprizone treatment, full remyelination is achieved (reviewed in [148]). Although most earlier studies used male mice, partly because it was thought that female mice do not demyelinate [146], demyelination in female mice has been noted [148]. As more investigators are beginning to use female mice in cuprizone studies [170-175], it is important to have a similar knowledge base of the processes occurring in female mice during cuprizone treatment. Furthermore, to our knowledge, no direct comparisons between male and female C57BL/6 mice have been made in the cuprizone model. In this communication, we present the results of experiments designed to provide baseline knowledge of cuprizone-induced demyelination and remyelination, comparing morphological and cellular changes in male and female C57BL/6 mice. Although the data indicate no significant sex differences in several measures of demyelination and remyelination, we observed that cuprizone intoxication disrupts estrous cyclicity in female mice. Thus, cuprizone may interfere with potential endogenous hormone influences on demyelination and remyelination in this strain, perhaps interfering with innate

69

sex differences.

3.3   MATERIALS AND METHODS

Animals and cuprizone treatment: Adult male and female C57BL/6 mice were purchased

from Jackson Laboratories (Bar Harbor, ME). At 8 weeks of age mice were fed ad libitum

0.2% cuprizone (oxalic bis(cyclohexylidenehydrazide)) (Sigma-Aldrich) mixed into ground

or pelleted Purina mouse chow for 3 weeks to induce partial demyelination or 5 weeks to

induce maximal demyelination. In keeping with previous studies [162, 165, 166],

remyelination was assessed by returning mice to a diet of normal Purina pellet chow for one

week, after 6 full weeks of cuprizone treatment. Untreated control mice were fed ground or

pelleted Purina chow during the time that experimental mice were being treated with

cuprizone.

Tissue Preparation: For light microscopy, mice were deeply anesthetized and intracardially

perfused with 0.15M phosphate buffer followed by 4% paraformaldehyde (PFA) solution.

After overnight fixation in PFA, brains were removed, dehydrated and embedded in paraffin.

Tissues were processed and embedded at the UNC Lineberger Animal Histopathology Core

Facility. 5μm coronal brain sections were cut at the fornix region of the corpus callosum,

(approximately Bregma -0.5mm to -0.7mm) and corresponding to Figure 37 of *The Mouse*

Brain In Sterotaxic Coordinates [181]. For electron microscopy, mice were deeply

anesthetized and intracardially perfused with 0.15M phosphate buffer followed by a 4%

glutaraldehyde/ 2% PFA solution. Brains were removed, post fixed for two weeks and

embedded in resin. A coronal block of brain was cut at the fornix region of the corpus callosum, as described above. A sagittal cut was then made at the midline, and tissue was embedded in resin such that the cross sections of the axonal fibers could be visualized.

Luxol Fast Blue – Periodic Acid Schiff's (LFB-PAS) stain: To examine demyelination and remyelination, paraffin sections were stained with Luxol fast blue (Sigma, St. Louis, MI), which stains myelin blue, and periodic acid-Schiff (Sigma, St. Louis, MI), which stains microglia/macrophages and demyelinated axons pink. Sections were scored blinded based on the ratio of blue or pink fibers in the corpus callosum, on a scale from 3 (complete myelination equal to an untreated mouse) to 0 (complete demyelination, as seen during peak cuprizone demyelination). The region of corpus callosum analyzed is midline, directly above the fornix in a coronal section at approximately Bregma -0.5mm to -0.7mm . See manuscript by Mason and colleagues [162] for a cartoon of the region analyzed.

Immunohistochemistry: For immunohistochemistry, 5um paraffin sections were deparaffinized and rehydrated. Phosphate buffered saline (PBS) was used in all rinsing steps. Mature oligodendrocytes were detected with a polyclonal antibody to the Pi isoform of glutathione *S*-transferase (GSTpi) (Biotrin, Newton, MA). A biotin conjugated lectin, *Ricinus communis* agglutin-1 (RCA-1) (Vector Laboratories Inc, Burlingame, CA), was used to detect microglia/macrophages. Astrocytes were detected with antibody to glial fibrillary acidic protein (GFAP) (Invitrogen).

For GSTpi staining, sections were first permeabilized/blocked with 0.1% Triton X-100/ 2% normal goat serum in PBS for 20 minutes at room temperature. Antigen retrieval was performed with 0.1% trypsin/ 0.1% calcium chloride in 0.05M Tris, pH 7.4 for 10

minutes at 37ºC. Following rinsing, sections were incubated with GSTpi antibody (1:1000 in block) or isotype control overnight at 4ºC. For RCA-1 and GFAP staining, sections underwent antigen retrieval with 0.025% protease, type XIV (Sigma-Aldrich) for 2 minutes at 43 ºC. After rinsing, sections were incubated in blocking solution (0.1% Triton X-100 and 1% bovine serum albumin for RCA-1 or 2% normal goat serum for GFAP in PBS) for 1 hour at room temperature. To detect microglia, sections were then incubated overnight at 4ºC with RCA-1, 1:500 in blocking solution, or blocking solution alone as a control. For GFAP staining, sections were incubated with GFAP antibody (1:200 in blocking solution) or isotype control overnight at 4ºC.

After incubation in the primary detection reagents described above, sections were incubated with the appropriate AlexaFluor conjugated secondary antibody (1:400) (Molecular Probes, Eugene, OR), for 1 hour at room temp. Sections were then rinsed and mounted with Vectasheild medium containing the nuclear counter stain 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc, Burlingame, CA). Fluorescent staining was visualized using a Nikon (Tokyo, Japan) Optiphot FXA microscope with epifluorescence optics. All comparative analyses were focused in the median corpus callosum in a region of 0.21mm wide and 0.15mm to 0.30mm high (the corpus callosum area becomes larger with infiltrating cells during demyelination). Positive-stained cells were counted only if a nucleus was observed and were quantified per square mm.

Detection of apoptotic oligodendrocytes: Apoptosis of oligodendrocytes in the corpus callosum was determined by labeling DNA strand breaks by Terminal deoxynucleotidyl transferase (TUNEL reaction) co-localized with GSTpi immunostaining as a marker for

72

mature oligodendrocytes. GSTpi immunohistochemistry was performed as described above, followed by detection of apoptotic nuclei using the In Situ Cell Death Detection Kit, Fluorescein (Cat# 11 684 795 910) (Roche Diagnostics, Mannheim, Germany) with a one hour incubation at 37°C in TUNEL reaction mixture prepared as described in manufacturer's protocol except for a 1:3 dilution of Enzyme solution in TUNEL Dilution Buffer (cat# 11 966 006 001) (Roche Diagnostics, Mannheim, Germany).

Electron microscopy: Ultrathin sections were cut, stained with lead citrate and uranyl acetate and electron micrographs obtained using Zeiss EM910 Transmission Electron Microscope with a digital camera at the Microscopy Services Laboratory in the UNC Department of Pathology and Laboratory Medicine. Greater than 500 axons (0.3µm or greater diameter) from each animal were counted as myelinated or unmyelinated and the percentage of myelinated axons out of total axons is given as percent myelination.

Statistical analysis: Statistical comparisons were made using two-way ANOVA with Bonferroni post-test or, where indicated, by Student's *t* test. The data are expressed as mean $\pm$ SEM.

Monitoring of estrous cyclicity by vaginal cytology: Samples were collected daily by vaginal lavage with PBS, analyzed by phase-contrast microscopy and scored for the stage of the estrous cycle, based on number of each cell type, as described by Allen [203], as follows: proestrus = mostly smooth epithelial cells, estrus = cornified epithelial cells, diestrus = mostly leukocytes, metestrus = all three cell types.

3.4   RESULTS

Female C57BL/6J mice demyelinate and remyelinate to a similar extent as male counterparts.

Five weeks of cuprizone treatment is known to produce maximum demyelination in the midline corpus callosum of male C57BL/6 mice [148] and remyelination begins by week 6 of cuprizone treatment [166, 174, 204]. Remyelination continues if cuprizone treatment is withdrawn after week 6 [165, 166]. Our first objective was to determine whether this pattern is similar in female mice. Eight week old mice of each sex were fed either normal chow (untreated), or 0.2% cuprizone to induce demyelination, assessed at 3 or 5 weeks, or treated for 6 weeks followed by one week of normal chow to assess remyelination (represented in the figures as "6+1wk"). At each time point, mice were sacrificed and brains collected for light microscopy or electron microscopy analysis.

For an initial assessment of myelination, paraffin-embedded brain sections were stained for myelination with the Luxol Fast Blue-Periodic Acid Schiff's (LFB-PAS) stain. Myelinated fibers are stained blue (Figure 3.1A, Untreated), and unmyelinated fibers appear pink (Figure 3.1A, 5 wk cuprizone). The sections were scored blinded, in the midline region of the corpus callosum using a scale from 0 to 3 in which 3 represents normal myelination, and 0 represents complete demyelination. We found that females exhibit less demyelination at the 3 week time point than males, though this difference was not statistically significant (Figure 3.1A and Figure 3.1B). By 5 weeks, both males and females reached nearly full demyelination, with no difference between the sexes. At the 6+1 week remyelination time point, in which the mice have been off the cuprizone diet for 1 week, partial remyelination

74

occurred similarly with no statistical difference between males and females (Figure 3.1A and Figure 3.1B).

To obtain a more accurate and quantitative measure of myelination, we analyzed cross-sections of corpus callosum axons by electron microscopy. For each animal, greater than 500 axons were counted as myelinated or unmyelinated and the percentage of myelinated axons was computed and averaged for each group. The results shown in Figure 3.2A and Figure 3.2B confirm the light microscopy data in Figure 3.1. Prior to treatment, male and female mice have similar numbers of myelinated axons. Upon exposure to cuprizone, the number of myelinated fibers at week 3 and maximal demyelination observed at week 5 were similar. Furthermore, remyelination progressed similarly between male and female mice. Thus, no significant differences in demyelination or remyelination were observed between male and female mice at the time points tested.
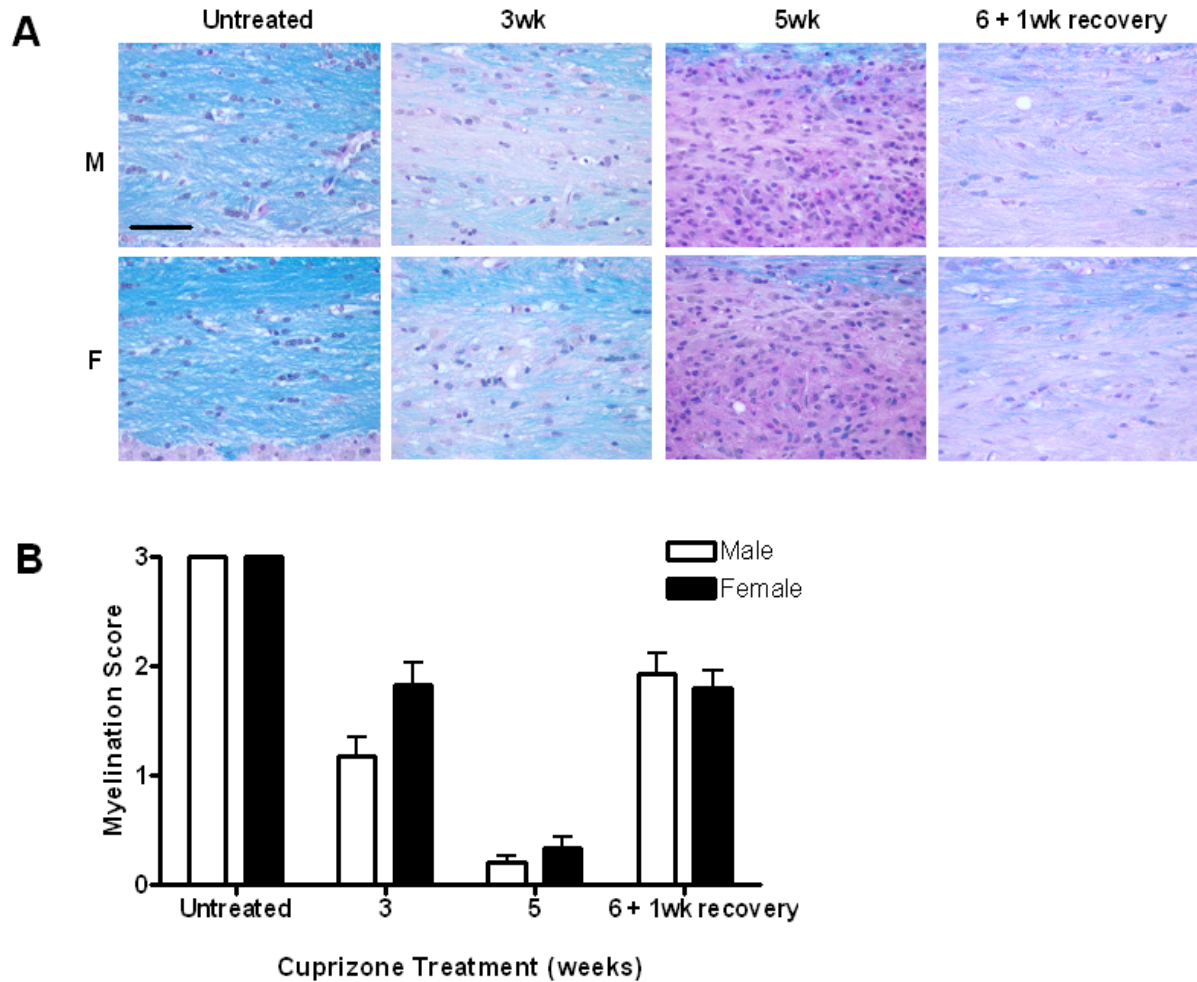
Figure 3.1. Cuprizone-induced demyelination and remyelination in male and female
C57BL/6 mice.
A. Representative images of LFB-PAS stained midline corpus callosum sections.
Magnification is 40x and scale bar represents 50 micrometers.
B. Myelination scores obtained by blinded scoring of midline corpus callosum sections
stained with LFB-PAS. A score of 3 reflects normal myelination in an untreated mouse,
whereas a score of 0 reflects the absence of myelin, similar to 5wk (as depicted in part A).
Mean and SEM bars are plotted for 10-16 animals per sex at each time point.
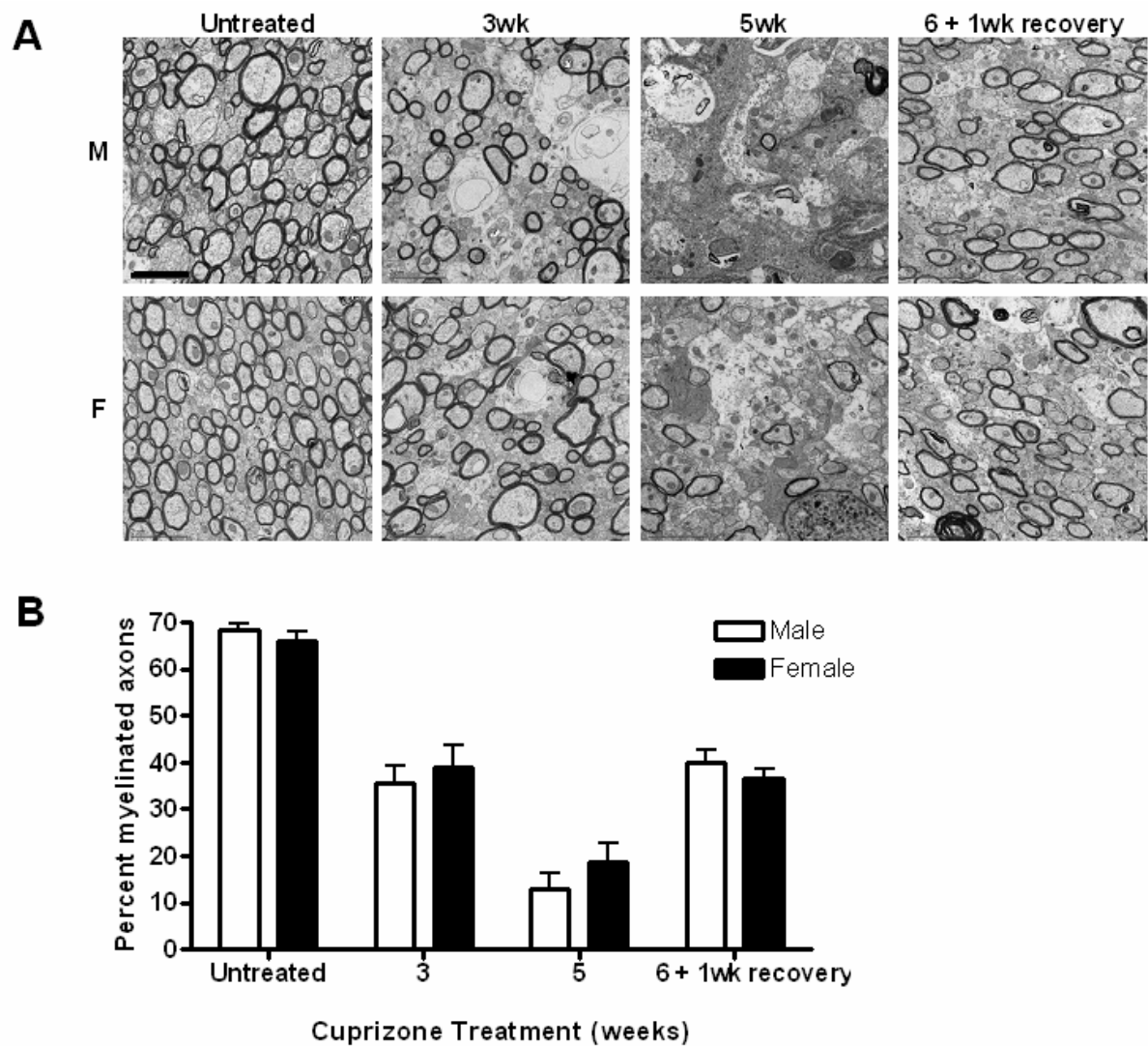
Figure 3.2. Myelinated and demyelinated axons in male and female C57BL/6 mice.
A. Representative electron micrographs of corpus callosum axons during cuprizone treatment. Magnification is 10,000x and scale bar represents 2 micrometers.
B. Percent myelinated axons in corpus callosum electron micrographs obtained by counting greater than 500 axons per animal. Mean and SEM bars for percent of total axons that are myelinated are plotted for 8-12 animals per sex at each time point.

Female C57BL/6 exhibit no differences in mature oligodendrocytes loss during demyelination or oligodendrocyte repopulation during remyelination compared to male counterparts.

Depletion of mature oligodendrocytes is a feature of cuprizone-induced demyelination in male C57BL/6 mice [162]. Mature oligodendrocytes were detected by immunohistochemistry with an antibody to glutathion-*S*-transferase pi isoform (GSTpi) and those associated with a nucleus were quantified. The number of GSTpi-positive cells in the midline corpus callosum was similar in untreated male and female mice (Figure 3.3A and 3.3B). After 3 weeks of cuprizone treatment, male and female mice exhibit a similar loss of oligodendrocytes in the corpus callosum, as determined by the total number of oligodendrocytes (Figure 3.3A and 3.3B) as well as the number of apoptotic oligodendrocytes (Figure 3.3C). At 5 weeks, the time of maximal demyelination, very few mature oligodendrocytes are found in the midline corpus callosum of either sex. During remyelination, female and male mice exhibit a similar extent of repopulation of mature oligodendrocytes in the corpus callosum (Figure 3.3A and 3.3B).
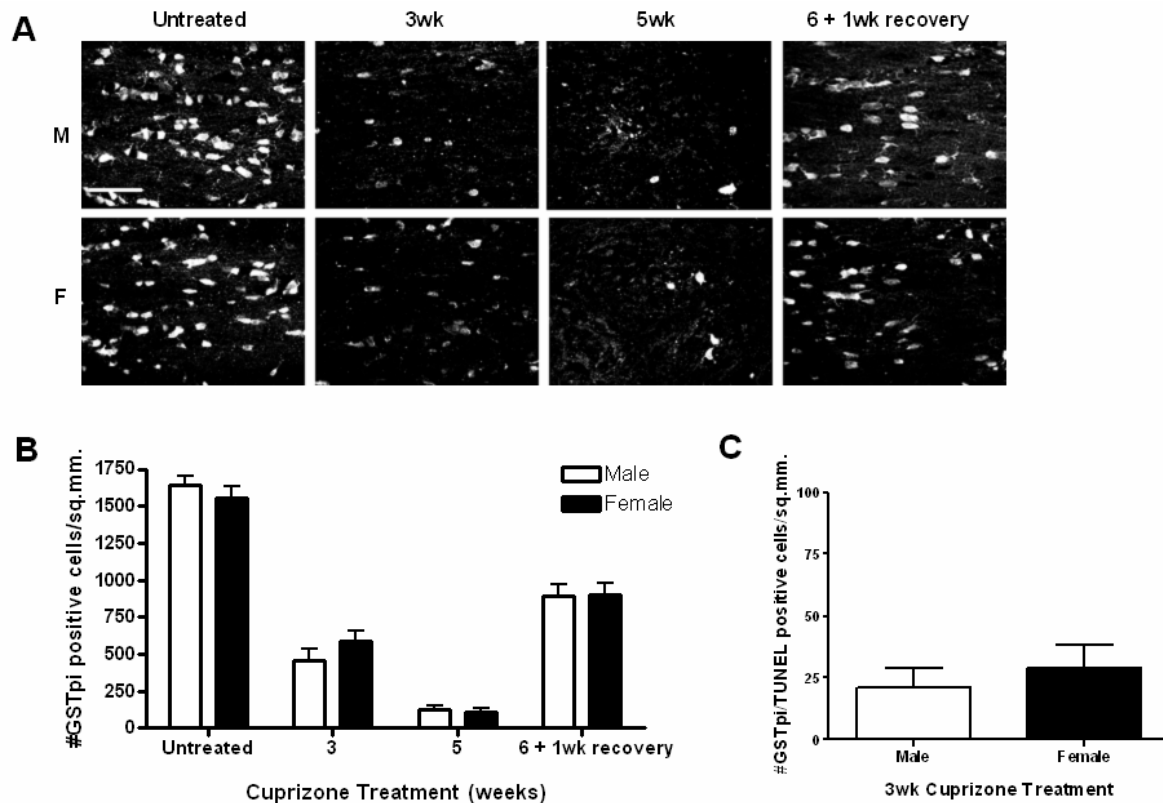
Figure 3.3. Cuprizone-induced loss of oligodendrocytes and subsequent repopulation during recovery in corpus callosum of male and female C57BL/6 mice.

A. Representative images of GSTpi[+] mature oligodendrocytes in the corpus callosum of male and female mice during cuprizone treatment and recovery. Magnification is 40x and scale bar represents 50 micrometers.

B. Quantification of GSTpi[+] mature oligodendrocytes in the corpus callosum of male and female mice during cuprizone treatment and recovery. Mean and SEM bars plotted for 10-16 mice per sex at each time point.

C. Quantification of apoptotic oligodendrocytes in the corpus callosum. Mean GSTpi[+]/TUNEL^{+} cells per square millimeter and SEM bars plotted for 12 animals per sex after 3 weeks of cuprizone administration.

Female C57BL/6 exhibit no difference in the accumulation of microglia in the corpus callosum during demyelination or remyelination compared to male counterparts.

Microglia accumulate in the corpus callosum presumably to clear debris during demyelination and provide trophic factors that support remyelination. Histochemical staining using the lectin RCA-1 (a marker for microglia/ macrophage) was performed on paraffin-embedded brain sections. The number of RCA-1-positive cells in the midline corpus callosum were counted and averaged for each group. There are very few microglia in the corpus callosum of normal male and female mice and the number of microglia are similar between the sexes (Figure 3.4A and 3.4B). During demyelination, microglia accumulate at week 3 and reach maximal numbers at week 5, when demyelination is also maximal. However, no significant difference was observed between male and female mice in the accumulation of microglia/macrophages in the corpus callosum during demyelination. During remyelination the presence of microglia/macrophages diminished to a similar extent in male and female mice (Figure 3.4).

Figure 3.4. Accumulation of microglia in the corpus callosum of male and female C57BL/6 mice.

A. Representative images of RCA-1 histochemical staining of microglia/macrophages in the corpus callosum of mice during cuprizone treatment and recovery. Magnification is 40x and scale bar represents 50 micrometers.

B. Quantification of RCA-1^{+} microglia/macrophages in the corpus callosum. Mean RCA-1^{+} cells per square millimeter and SEM bars plotted for 10-16 animals per sex at each time point.

Female C57BL/6 exhibit no difference in the accumulation of astrocytes in the corpus callosum during demyelination or remyelination compared to male counterparts

Astrocytes are present at low numbers in normal adult mice and accumulate in the corpus callosum similar to microglia during demyelination and remyelination. Immunohistochemical staining using an antibody to GFAP, a marker for astrocytes, was also performed here and the number of GFAP-positive cells in the midline corpus callosum was counted and averaged for each group. There are astrocytes in the corpus callosum of untreated mice; however, male and female mice have similar numbers (Figure 3.5A and 3.5B). During demyelination, astrocyte numbers increase at week 3 and reach a peak by week 5. There were no significant sex differences in the accumulation of astrocytes in the corpus callosum during demyelination, although there was a trend for a slightly higher number of astrocytes in female mice. Similarly, female mice had slightly higher numbers of astrocytes during remyelination (Figure 3.5). However, these differences did not reach statistical significance.
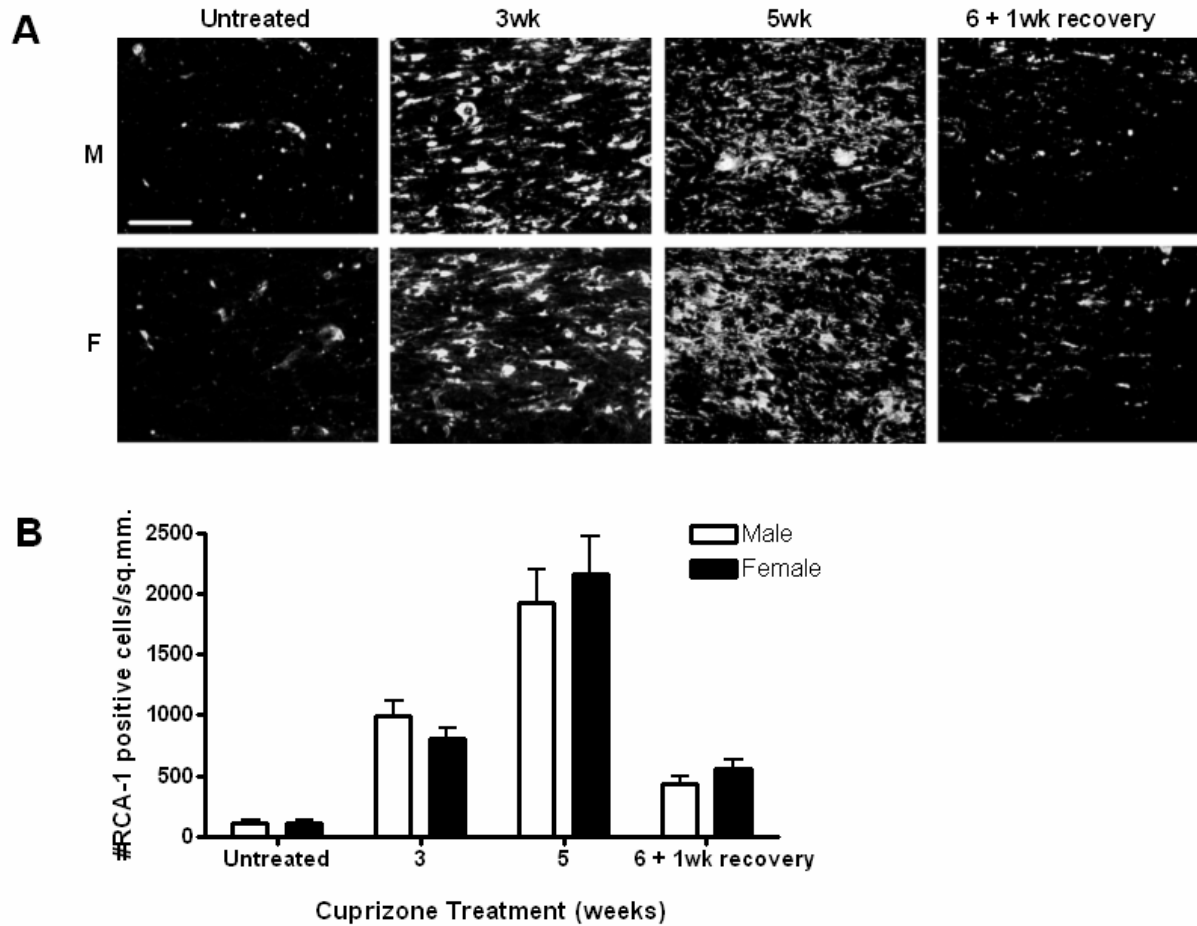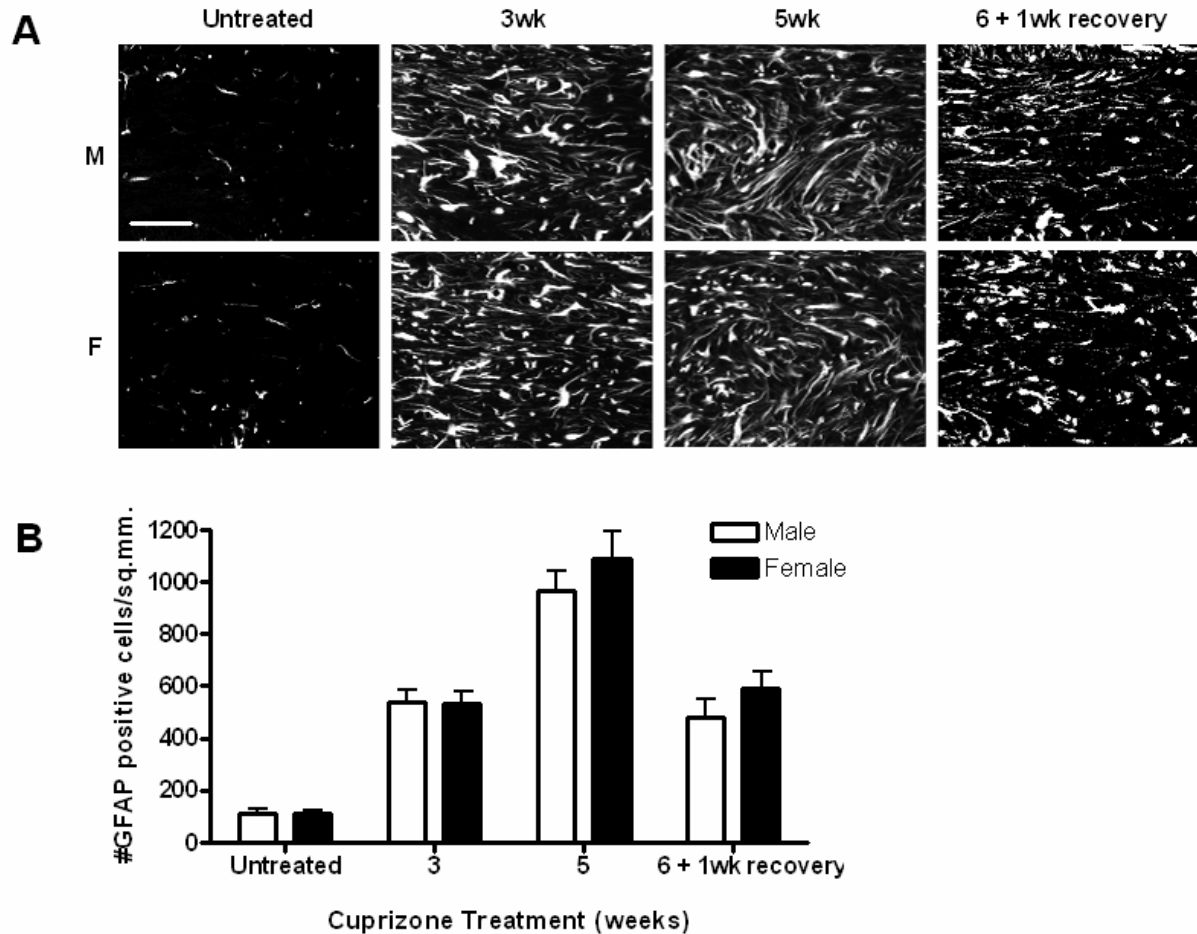
Figure 3.5. Accumulation of astrocytes in the corpus callosum of male and female C57BL/6 mice.
A. Representative images of GFAP immunohistochemical staining of astrocytes in the corpus callosum of mice during cuprizone treatment and recovery. Magnification is 40x and scale bar represents 50 micrometers.
B. Quantification of GFAP$^+$ astrocytes in the corpus callosum. Mean GFAP$^+$ cells per square millimeter and SEM bars plotted for 10-16 animals per sex at each time point.

Cuprizone treatment disrupts estrous cyclicity in female mice.

A previous study in our laboratory determined that a 0.2% dose of cuprizone treatment in male C57BL/6J mice causes minimal weight loss and no histologically detectable liver toxicity [161]. However, to our knowledge, gonadal tissues and function

have not been assessed for cuprizone toxicity in any strain or sex of rodent. In female mice, this can be readily approached using an *in vivo* assay of hormone status; i.e., by monitoring estrous cyclicity by vaginal cytology [108]. This technique is well established in rodents and is performed by daily collection of vaginal cells by gentle saline lavage of the vaginal cavity. Phase contrast analysis of the number and type of cells present in the sample indicates which phase of the four to five day estrous cycle the mouse is undergoing (Figure 3.6A, top). We began by monitoring estrous cycling in female mice 2 weeks prior to cuprizone treatment. Because the standard procedure for cuprizone treatment is to use 8-10 week old mice, we began our vaginal cytology analysis in 6-7 week old mice. Mice of this age are just beginning to cycle regularly and there is some variability in the length of cycle. Only female mice that displayed a 4-6 day estrous cycle in the two weeks prior to cuprizone treatment were included in the study.

In order to determine whether cuprizone treatment adversely affects estrous cycling, vaginal cell samples were collected every day at the same time during the 6 week cuprizone treatment and compared to untreated mice that were monitored concurrently. The data are plotted in Figure 3.6B, as the number of estrus phases (the phase that corresponds to ovulation) detected every 7 days, so that we could assess the cycling pattern relative to number of weeks on cuprizone treatment. In a normal cycling mouse, an estrus phase should occur every 4-5 days, so this would result in a score of approximately 1.4 estrus phases per 7 days. Prior to cuprizone treatment, female mice displayed a relatively regular estrous cycle, with estrus occurring every 4-6 days. This pattern continued in the untreated mice. In contrast, in cuprizone-treated mice, a dramatic change in estrous cyclicity occurred, indicated by representative images of vaginal cell samples in Figure 3.6A and the drop in number of

estrus phases per week as shown in Figure 6B. By the second week of cuprizone treatment,

no estrus phases were detected for the duration of the 6 week treatment, with the exception of

2 out of 13 mice.

Upon removal of cuprizone from the diet, female mice began displaying an estrus

phase within the first week or two, and cycles became regular by the 3rd or 4th week off the

cuprizone diet (Figure 3.6B). This ability to recover estrous cycling indicates that the

reproductive tissue was not irreversibly damaged by cuprizone toxicity.
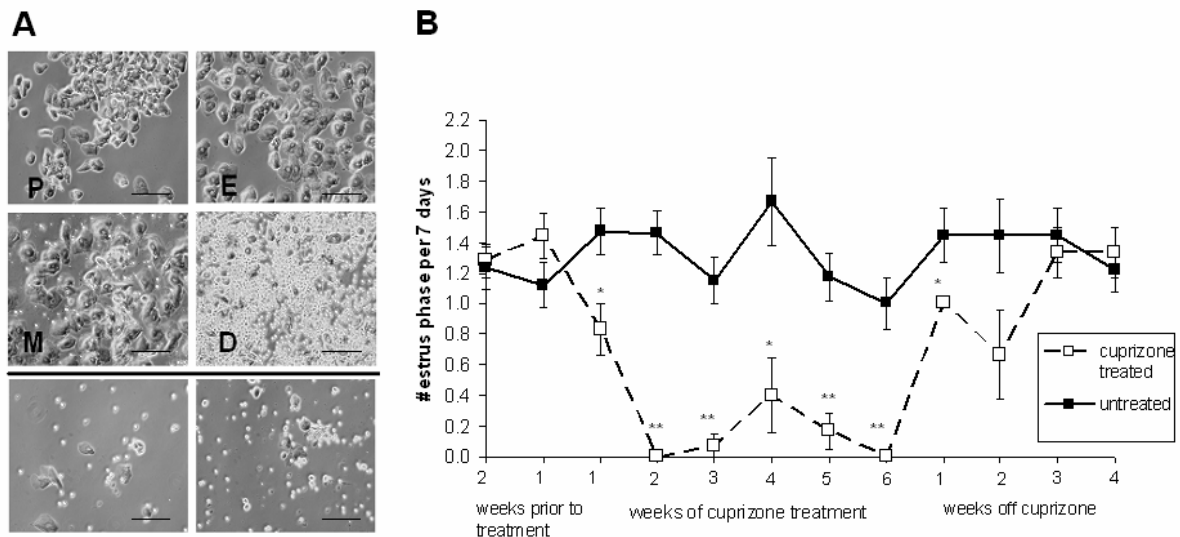
Figure 3.6. Cuprizone treatment reduces the number of estrus phases compared to the same mice before treatment or untreated mice monitored concurrently.
Estrous cycling was monitored by scoring of vaginal cytology.
A. Top: representative samples from untreated female mice (P = proestrus, E = estrus, M = metestrus, D = diestrus). Bottom: representative samples collected from cuprizone-treated female mice during weeks 2-6. Magnification is 40x and scale bar represents 50 micrometers.
B. Number of estrus phases detected per 7 days in mice monitored for 2 weeks before cuprizone, during 6 weeks of cuprizone treatment, and during recovery from cuprizone, or untreated mice. * p < 0.05, ** p < 0.005 as compared to untreated by Student's t-test.

85

Reproductive tissues of female mice are affected by cuprizone.

To corroborate the data obtained by analysis of vaginal cytology, female reproductive organs were weighed. It is known that uterine weight fluctuates with the estrous cycle and is reflective of estradiol levels, such that it is highest during proestrus, begins falling during estrus, is lowest during metestrus and rises again during diestrus [205, 206]. Furthermore, uterine weight is often used as a bioassay of estrogenic activity [207, 208]. The wet weights of uteri + ovaries were obtained from cuprizone-treated or untreated mice sacrificed at the 5 week time point when demyelination is maximal and estrous cycling was disrupted (Figure 3.6B). At sacrifice, uterine weights in untreated mice correlated with the phase of the estrous cycle (data not shown). All of the cuprizone-treated mice had reduced uterine + ovary weights (Figure 3.7B), with average weights showing a 2-3 fold reduction compared with untreated females (Figure 3.7A and 3.7B). Four weeks after cuprizone removal from the diet, uterine + ovary wet weights have returned to levels equivalent to untreated mice, mirroring the return of estrous cyclicity shown in Figure 3.6B. These observations of disrupted estrous cyclicity and reduced uterine weight suggest that circulating hormone levels may not be normal in cuprizone treated mice. Furthermore, testes weights were obtained for male mice treated with cuprizone for 5 weeks, and were compared to untreated and remyelinating mice (Figure 3.7C). There was no significant change in testes weights in the cuprizone treated male mice.
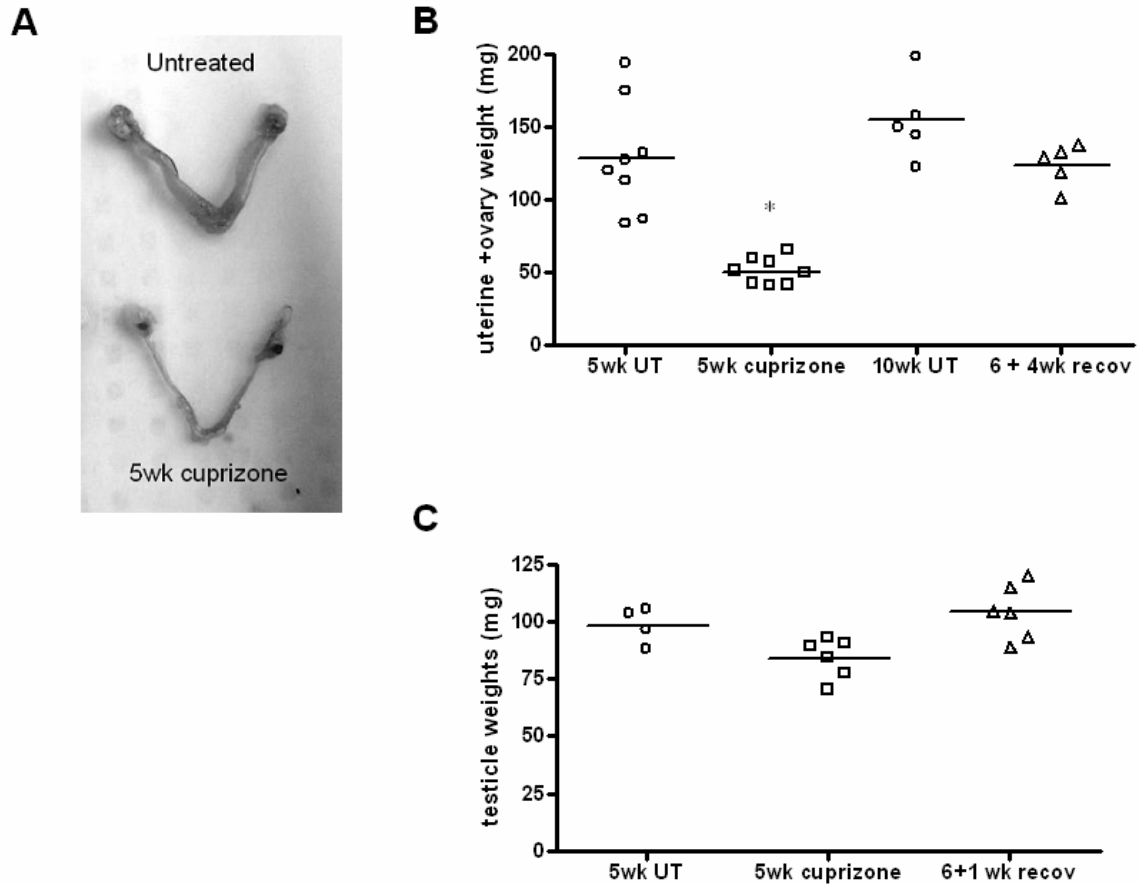
Figure 3.7. Cuprizone treatment results in diminished weights of female but not male sex organs.

A. Representative images of uteri + ovaries from untreated female mice (upper) and 5 week cuprizone treated female mice (lower).

B. Wet weights of uteri + ovaries obtained from untreated (5wk UT, circles) and cuprizone-treated female mice at 5 weeks (5wk cuprizone, squares) as well as 10 week untreated (10wk UT, circles) or mice that were treated with cuprizone for 6 weeks followed by 4 weeks of normal diet (6 + 4wk recov, triangles). Sex organ weights are significantly diminished by 5 weeks of cuprizone treatment (* $p < 0.001$ compared to 5wk UT by student's t test) but return to untreated weights 4 weeks after removal of cuprizone from the diet.

C. Wet weights of testes from 5 week untreated (5wk UT, circles), 5 week cuprizone treated (squares), and remyelinating (6 + 1 recov, triangles) mice which were treated with cuprizone for 6 weeks followed by 1 week of normal diet. There are no statistical differences in testes weights between groups.

3.5   DISCUSSION

Cuprizone intoxication in C57BL/6 male mice has been increasingly used as a model to study oligodendrocyte loss and demyelination, as well as remyelination, in the CNS. We noted previously that C57BL/6 female mice were susceptible to demyelination, in contrast to earlier reports for female Swiss mice [146, 148]. Although investigators have begun using female C57BL/6 mice to study cuprizone-induced CNS disease and have shown them to be susceptible to demyelination, to our knowledge, no direct comparisons between males and females of this strain have been made. We report here that, similar to male C57BL/6 mice, females develop extensive demyelination by week 5 of cuprizone treatment. Although there was a trend for less severe demyelination at week 3 in female mice, detected by LFB-PAS staining, the difference was not statistically significant. In addition, analyses at the electron microscope level indicated no differences in demyelination or remyelination between female and male mice. Similarly, oligodendrocyte depletion and recovery was the same between females and males. Microglial and astrocyte responses also appeared equal. Interestingly, cuprizone treatment disrupted estrous cyclicity, which may indicate a perturbation in the sex hormone status of cuprizone treated female mice. No change in male reproductive tissue was observed.

Sex differences have been characterized in other rodent models of demyelinating disease, particularly EAE. Most studies of sexual dimorphism in EAE are carried out the SJL mouse strain, in which females show an increase in susceptibility and severity of disease [42, 46, 63, 209]. However, in other strains, males exhibit increased susceptibility (B10.PL and PL/J) or no sex differences (NOD/lt and C57BL/6) [46]. Likewise, the TMEV model

displays sex differences that vary by strain. An early study in the C57BL/J mouse strain demonstrated that female mice are completely resistant to TMEV-induced demyelinating disease [200]. This was later shown to be due to a more robust anti-viral response to the initial infection by female mice [70]. In the SJL mouse strain, both males and females are susceptible, but male mice demonstrate more severe neurological deficits [69]. Given the variation by strain in sex differences in these models, it is possible that another mouse strain may show sex differences in the cuprizone model. Indeed, we have recently investigated sex differences in the cuprizone model in SJL mice and found that females exhibit less severe oligodendrocyte loss and demyelination [210].

Sex may also play a role in remyelination, as demonstrated by Li and colleagues [73]. In this study ethidium bromide was used to induce death of oligodendrocytes in young and old male and female adult rats. Remyelination is less efficient in older animals. While no sex difference in remyelination was observed in young adult rats, remyelination was better in older females compared with males. Removal of the gonads prior to demyelination did not affect remyelination in young or old male or female rats, suggesting that gonadal sources of sex hormones did not influence remyelination. However, another study using this model demonstrated a modest increase in remyelination in aged male rats treated with progesterone. Most cuprizone studies, including the present one, are performed in young adult rodents. Thus, it would be of interest to determine if sex differences in cuprizone-induced disease occur in older mice.

An interesting finding in this study is that cuprizone intoxication disrupts estrous cyclicity, accompanied by diminished weights in the sex organs in female mice. To our knowledge, this is the first indication that the estrous cycle is affected during cuprizone

treatment, and indicates that circulating sex hormone levels are not normal in treated female mice. Whether cuprizone directly affects gonadal tissue, or whether disruption of the estrous cycle is a side effect of the moderate loss in body weight that occurs, is not known. It is also possible that cuprizone alters steroid hormone biosynthesis or metabolism. Based on wet weight measurements of testes in male mice, it does not appear that cuprizone has a detrimental effect on male reproductive tissue. In the future, it would be of interest to measure the levels of circulating steroid hormones to more completely assess hormone status, and if specific changes are detected, to determine if they vary with mouse strain. These data suggest that future studies of sex differences in demyelination and remyelination using this model may require hormone replacement strategies and/or analysis of mechanisms of hormone metabolism. Another factor to consider is the possibility that cuprizone toxicity has a different effect on sex steroid synthesis in the brain, compared with the periphery. It is well known that CNS cells are capable of *de novo* synthesis and metabolism of sex steroids, and that the enzymes responsible for their synthesis are upregulated after brain injury (reviewed in [116]). Since female C57BL/6 mice appear to demyelinate and remyelinate similarly to male mice, it seems unlikely that endogenous sex hormones play a major role in the pathogenesis of cuprizone intoxication in this mouse strain.

Consistent with a lack of a sex difference in demyelination, we found that the microglial and astroglial responses were similar in female and male mice. The temporal pattern of oligodendrocyte depletion and recovery, and the accumulation of microglia and of astrocytes in female mice suggest a lack of inherent differences in these cells when compared to male mice. In conclusion, female C57BL/6 mice exhibit no significant sex differences in

cuprizone-induced demyelination or remyelination and this may be explained by the finding

that cuprizone treatment disrupts estrous cycling.

CHAPTER 4

17β-estradiol Protects Male Mice from Cuprizone-induced Demyelination and Oligodendrocyte Loss

4.1 ABSTRACT

In addition to regulating reproductive functions in the brain and periphery, estrogen also has trophic and neuroprotective functions in the central nervous system (CNS). Estrogen administration has been demonstrated to provide protection in several animal models of CNS disorders, including stroke, brain injury, epilepsy, Parkinson's disease, Alzheimer's disease, age-related cognitive decline and Multiple Sclerosis. Here, we use a model of toxin-induced oligodendrocyte death which results in demyelination, reactive gliosis, recruitment of oligodendrocyte precursor cells and subsequent remyelination to study the potential benefit of 17β-estradiol (E2) administration to male mice. The results indicate that E2 partially ameliorates loss of oligodendrocytes and demyelination of the corpus callosum, and this protection is accompanied by a delay in microglia accumulation as well as reduced mRNA expression of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) and the growth factor insulin-like growth factor -1 (IGF-1). E2 did not significantly alter the accumulation of astrocytes or oligodendrocyte precursor cells, or remyelination. This work sets the stage for more detailed analysis of the mechanism by which E2 protects oligodendrocytes from death and demyelination.

4.2 INTRODUCTION

Interest in the use of sex hormones for therapy to treat Multiple Sclerosis (MS) stems from the observation that disease is partially ameliorated during pregnancy. A large

prospective European PRIMS study of 227 female MS patients showed a significant decrease in disease relapses during pregnancy, especially during the third trimester, compared to the relapse rate in these same women before they became pregnant [13]. In addition, the relapse rate significantly increased in the 3 months post-partum, before returning to pre-pregnancy levels. This finding suggests a protective role for pregnancy-related factors, one of which could be sex steroids. In fact, the estrogen derivative estriol, which is produced by the placenta and is present in high levels in maternal serum during pregnancy, was recently examined for potential benefit to MS patients in a clinical trial [79]. The pregnancy level of oral estriol that was delivered to a small number of female MS patients was able to reduce the number and volume of lesions as seen by magnetic resonance imaging (MRI).

The data from animal models of demyelinating disease and *in vitro* studies provides further evidence for a potential benefit of estrogens. Two forms of estrogen, estriol and 17β-estradiol (E2), have been shown to reduce clinical symptoms of experimental autoimmune encephalomyelitis (EAE), an accepted model of MS [66, 88, 89]. A direct role for estrogen in oligodendrocytes, the cells responsible for central nervous system (CNS) myelination, has been demonstrated. Addition of E2 to rodent primary oligodendrocyte cultures led to increased proliferation of oligodendrocyte precursors and enhanced membrane sheet formation, [117, 131, 132]. E2 also prevents toxin and oxygen-mediated death of oligodendrocytes in culture and oxygen-mediated loss of MBP in neonatal rat white matter [133, 134]. Furthermore, sex steroids may have a role in mediating demyelinating disease through anti-inflammatory mechanisms. E2 has anti-inflammatory effects on microglia cultures, by reducing expression of inducible nitric oxide synthase and several other inflammatory mediators in response to LPS and to proinflammatory cytokines [135, 136,

94

138, 211]. Estrogen also protects against neuronal death from brain injury, neurodegeneration associated with Alzheimer's disease and Parkinson's disease, age-related cognitive decline, and epilepsy [212-216]. Furthermore, E2 and estrogen receptor ligands administered during EAE prevented axonal loss in the white matter and neuronal pathology in gray matter, highlighting the potential benefit of estrogen in preserving neuronal integrity in demyelinating disease [96, 217].

In an effort to determine the role of estrogen in primary oligodendrocyte death and CNS demyelination, we have administered E2 to male C57BL/6 mice during-cuprizone induced demyelination and remyelination. We used male mice to avoid estrogen fluctuation during cycling in female mice and because demyelination is well characterized in C57BL/6 males [161]. Cuprizone causes death of oligodendrocytes, demyelination and infiltration of reactive glia to the lesion, as well as accumulation of oligodendrocyte precursor cells (OPCs) [148, 161, 162]. Remyelination occurs spontaneously by six weeks of treatment, and proceeds rapidly if cuprizone intoxication is discontinued [163, 165, 166]. We report that E2 was effective in producing a partial amelioration of oligodendrocyte loss and demyelination, but did not alter remyelination. Furthermore, E2 treatment produced a delay in microglia accumulation and a reduction in levels of IGF-1 and TNFα mRNA.

4.3   MATERIALS AND METHODS

Animals and cuprizone treatment: Adult male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used for experiments at 8 weeks of age. Mice were

housed in DLAM facilities under sterile pathogen-free conditions. To induce demyelination, cuprizone (oxalic bis(cyclohexylidenehydrazide)) (Sigma-Aldrich) at a concentration of 0.125% was mixed into ground Purina mouse chow and fed *ad libitum* for 3 to 5 weeks. Remyelination was assessed by returning the mice to a diet of normal chow for one week following 6 weeks of cuprizone administration. Control mice (labeled "untreated") were fed pelleted Purina chow and were sacrificed at the same time as the 5 week cuprizone group. All animal use was performed in compliance with the NIH *Guide for Care and Use of Laboratory Animals* and approved by the UNC-CH Institutional Animal Care and Use Committee.

17β-estradiol administration: Continuous release 25 mg 17β-estradiol (E2) or placebo pellets (3x4 mm; Innovative Research of America, Sarasota, FL) were implanted subcutaneously by sterile surgical procedure on the backs of mice between the shoulder blades. These pellets are designed to release continuously over a 60 day period, which would calculate to 0.42 mg/day. Cuprizone treatment began 5 days after implantation.

Serum 17β-estradiol measurement: Serum was obtained from whole blood samples by centrifugation after removal of blood clot and stored at -80°C until use. E2 was measured by radioimmunoassay (RIA) using the double antibody estradiol kit (cat# KE2D5, Seimens, Los Angeles, CA) and following the manufacturer protocol.

Tissue Preparation: For histology, mice were deeply anesthetized using isoflurane and intracardially perfused with 0.15M phosphate buffer followed by 4% paraformaldehyde

(PFA) solution. Brains were removed, post-fixed overnight in PFA, and embedded in paraffin or post-fixed for 4 hours in PFA, followed by 1-2 days in 30% sucrose, and then embedded in Tissue-Tek® O.C.T.™ freezing media (Sakura Finetek, Torrance, CA) and frozen on a bed of dry ice. Five μm paraffin or frozen coronal brain sections were cut at the fornix region of the corpus callosum (approximately Bregma -0.5mm to -0.7mm) and corresponding to Figure 37 of *The Mouse Brain In Sterotaxic Coordinates* [181].

For the isolation of mRNA, corpus callosum tissue was obtained by making coronal cuts at approximately Bregma -0.25mm and -1.25mm. Then sagittal cuts through the cingulum, on the inside of each lateral ventricle were performed followed by a cut above and below the corpus callosum to remove the majority of cortex and fornix. This block of tissue was immediately submerged in RNA*later* solution (cat#AM7020, Applied Biosystems, Foster City, CA) and after overnight 4^oC incubation, stored at -80° C until use. The corpus callosum of five brains was combined for each sample.

Luxol Fast Blue – Periodic Acid Schiff's (LFB-PAS) stain: To examine demyelination and remyelination, paraffin or frozen sections were stained with Luxol fast blue (Sigma, St. Louis, MI), which stains myelin blue, and periodic acid-Schiff (Sigma, St. Louis, MI), which stains microglia/macrophages and demyelinated axons. Sections were scored blinded based on the amount of blue or pink fibers in the corpus callosum, on a scale from 3 (complete myelination equal to an untreated mouse) to 0 (complete demyelination, as seen during peak cuprizone demyelination at week 5). The region analyzed was the midline corpus callosum, as diagramed previously in Figure 1 of Mason et al. [162].

Immunohistochemistry: The detection of mature oligodendrocytes was performed with antibody to the Pi isoform of glutathione *S*-transferase (GSTπ) (Biotrin, Newton, MA). Paraffin or frozen sections were permeabilized with 0.1% Triton X-100/ 2% normal goat serum in phosphate-buffered saline (PBS) for 20 minutes at room temperature. Antigen retrieval to better expose the antibody-binding epitope was performed with 0.1% calcium chloride/ 0.1% trypsin in 0.05M Tris, pH 7.4 for 15 minutes at 37°C. Sections were rinsed in PBS and incubated with anti-GSTpi antibody (1:1000) or isotype control overnight at 4° C.

Oligodendrocyte precursor cells were detected with a rabbit antibody to NG2, generously supplied by Dr. W.B. Stallcup (Genomic Institute of Novartis Res. Foundation, CA). Five micrometer frozen sections were fixed in 95% ethanol before being stored at -80 ^{o}C. Upon removal from the freezer, sections were post-fixed in cold acetone, rinsed in potassium-phosphate-buffered saline (KPBS), and blocked with 0.1% Triton X-100/ 5% normal goat serum in KPBS for 1 hour at room temperature. Sections were then incubated with anti-NG2 antibody (1:500 in blocking solution) or isotype control overnight at 4° C.

Microglia/macrophages were detected with biotinylated lectin *Ricinus communis* agglutin-1 (RCA-1) (Vector Laboratories Inc, Burlingame, CA). Paraffin or frozen sections were unmasked with 0.025% protease, type XIV (Sigma-Aldrich) for 2 minutes at 43° C. Following a brief rinse in PBS, they were blocked with 0.1% Triton X-100/ 1% bovine serum albumin in PBS for 1 hour at room temperature. Sections were then incubated with RCA-1, 1:500 in blocking solution or blocking solution alone as a control, overnight at 4° C.

Astrocytes were detected with antibody to glial fibrillary acidic protein (GFAP) (Invitrogen). Paraffin sections were rehydrated and unmasked with 0.025% protease, type XIV (Sigma-Aldrich) for 2 minutes at 43° C. Following a brief rinse in PBS they were

98

blocked with 0.1% Triton X-100/ 2% normal goat serum in PBS for 1 hour at room

temperature. Sections were then incubated with anti-GFAP antibody (1:200) or isotype

control overnight at 4° C.

Following incubation in the primary detection agent, all immunohistochemistry was

completed by first rinsing sections three times in PBS, then incubating for 1 hour at room

temp with the appropriate secondary antibody (1:400) conjugated to AlexaFluor (Molecular

Probes, Eugene, OR). After rinsing, sections were cover-slipped with Vectasheild plus DAPI

(Vector Laboratories Inc, Burlingame, CA) to counter stain nuclei.

All comparative analyses were focused in the corpus callosum at the midline.   An

Olympus (Melville, NY) BX40 microscope, Olympus DP70 digital camera and

ImageProPlus software (Media Cybernetics, Silver Spring, MD) were used to obtain images.

The images of the fluorescent antibody staining were overlayed with DAPI images and

immunohistochemically positive cells which colocalized with a nucleus were quantified per

square mm.

Semi-quantitative real-time PCR: RNA was obtained from corpus callosum tissue (see

above), which was pooled for 5 animals per treatment group, by manual homogenization in

Trizol (Invitrogen, Carlsbad, CA) and cleaned up with RNeasy Mini kit (cat# 74104, Qiagen,

Valencia, CA). TaqMan 5' nuclease real-time PCR assays were performed using an ABI

Prism 7500 sequence-detection system (PE Applied Biosystems, Foster City, CA) in the

UNC Neuroscience Center Functional Genomics Core Facilty. For IGF-1 analysis the

Taqman® Gene Expression Assay system was used (ID# Mm00439559_m1), which includes

proprietary sequences (Applied Biosystems, Foster City, CA). For TNFα analysis custom

oligonucleotide primer and TMRA^{TM} probe sequences were as follows (Applied Biosystems, Foster City, CA):

TNFα primer-forward: CATCTTCTCAAAATTCGAGTGACAA;

TNFα primer-reverse: CTCCAGCTGCTCCTCCACTT;

TNFα Probe: CCTGTAGCCCACGTCGTAGCAAACCAC

Statistical analysis: Statistical analysis was performed using two-tailed Student's *t* test. Differences were considered significant if $p \leq 0.05$.

4.4   RESULTS

Serum levels of E2 in mice implanted with continuous release hormone pellets.

In order to determine the effect of E2 on demyelination and remyelination, male C57BL/6 mice were implanted subcutaneously with continuous release placebo or pellets containing 25 mg E2 designed to release a consistent amount of hormone over 60 days (0.42 mg/day). Blood was collected from a representative sample of mice 4 days following implantation and cuprizone treatment began the following day, except for control mice which continued on a normal diet. Serum was collected again prior to sacrifice after 3, 5 or 7 weeks of the demyelination experimentation. Serum levels of E2 were measured by RIA.

Four days after implantation, serum E2 rose rapidly and reached an average of about 3000 pg/mL in the groups of mice with E2 implants (Figure 4.1). In contrast, mice without implants did not show detectable levels of E2. After 3 or 5 weeks of cuprizone treatment, the

average serum E2 was 4000 to 5000 pg/mL and similar levels were reached in the mice not

receiving cuprizone. By the 7 week time point, average serum E2 had diminished slightly to

3500 pg/mL. These levels of serum E2 correspond to the lower range reported for pregnant

mice (5000-10,000 pg/mL) [218]. Serum E2 in the placebo implanted mice was nearly

always below the detection limit of the RIA assay, but when detectable, a range from 5-

10pg/mL was found, which is consistent with normal levels in males.
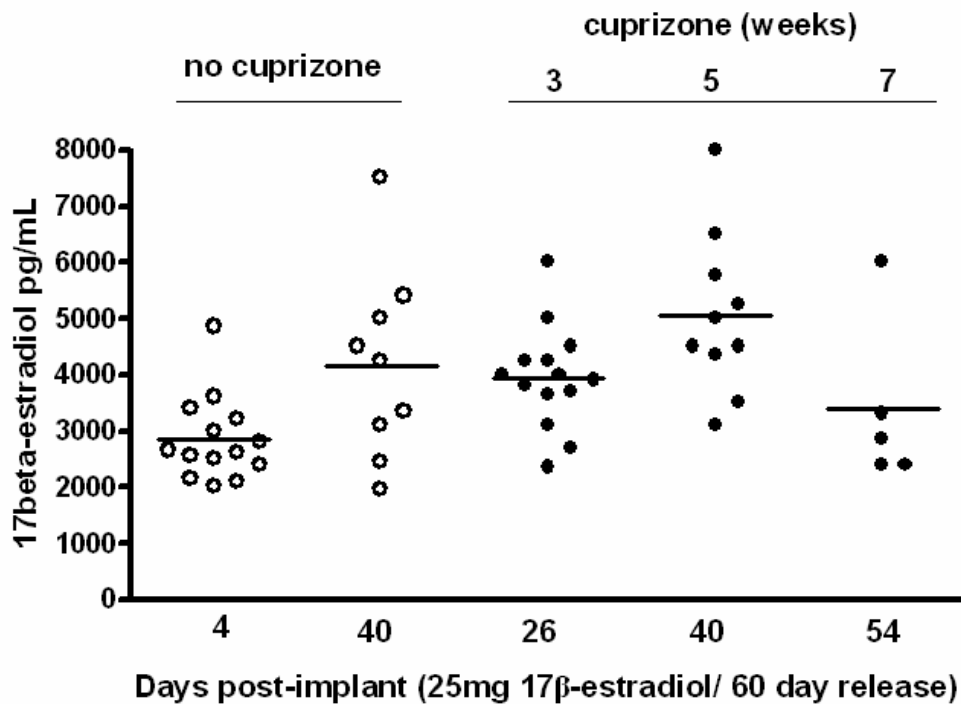
Figure 4.1. Serum E2 of male mice implanted with continuous release E2 pellets.
Serum was collected 4 days after subcutaneous implantation of 25 mg/60 day continuous
release E2 pellets, as well as at the time of sacrifice from mice that were not exposed to
cuprizone, or exposed to cuprizone for 3 weeks, 5 weeks, or 6 weeks plus 1 week of
discontinuation of cuprizone. Measurement of E2 in the serum was conducted by RIA.
Results indicate that serum levels of E2 approached the range reported for pregnant mice,
and remained relatively constant during the entire time course.

E2 partially ameliorates cuprizone-induced demyelination.

The affect of E2 on demyelination was assessed by exposing mice to cuprizone five days after implantation of placebo or E2 pellets. Mice were analyzed for demyelination after 3 or 5 weeks of cuprizone treatment when male C57BL/6 are known to be partially or fully demyelinated, respectively [161]. After 3 weeks of cuprizone administration, E2 treated mice are significantly less demyelinated than placebo controls (Figure 4.2A and 4.2B). This effect continues at the 5 week time point in which the E2-treated mice showed significantly less demyelination.

A visual analysis of the individual myelin scores during demyelination, indicate that E2 was effective in attenuating demyelination in a sub-population of the mice to which it was administered. These data points are indicated with gray circles in Figure 4.2B. At the five week time point, there is a clear difference between mice that are almost fully demyelinated (black circles) and those which had myelin scores greater than any of the placebo treated mice (gray circles). Although variance in myelin scores of placebo treated mice at the 3 week time point is greater, there is still a population of E2 treated mice which all have myelin scores greater than any of the placebo treated mice.

The reason for the variability in the protective effect of E2 is unclear, but could be a result of the confounding factor of the way cuprizone is administered, i.e. mixed into chow that is fed ad libitum. Thus, if some mice are ingesting more cuprizone than others, the increased exposure to the toxin may overcome the protective effect of E2. The effectiveness of E2 to attenuate demyelination did not correlate with the level of serum E2 measured at the time of sacrifice (data not shown). In the subsequent analysis of cellular populations of these

mice laid out in this manuscript, the mice that showed a protection from demyelination (gray circles) will continue to be colored gray so that their cellular profiles may be followed.

Interestingly, after 1 week of recovery from cuprizone, the placebo-treated mice that showed greater demyelination at week 5 began to remyelinate substantially. However, the E2-treated mice display an average myelin score very similar to their 5 week time point (Figure 4.2B). This suggested that remyelination may be partly delayed since demyelination was not as severe in the E2-treated mice.
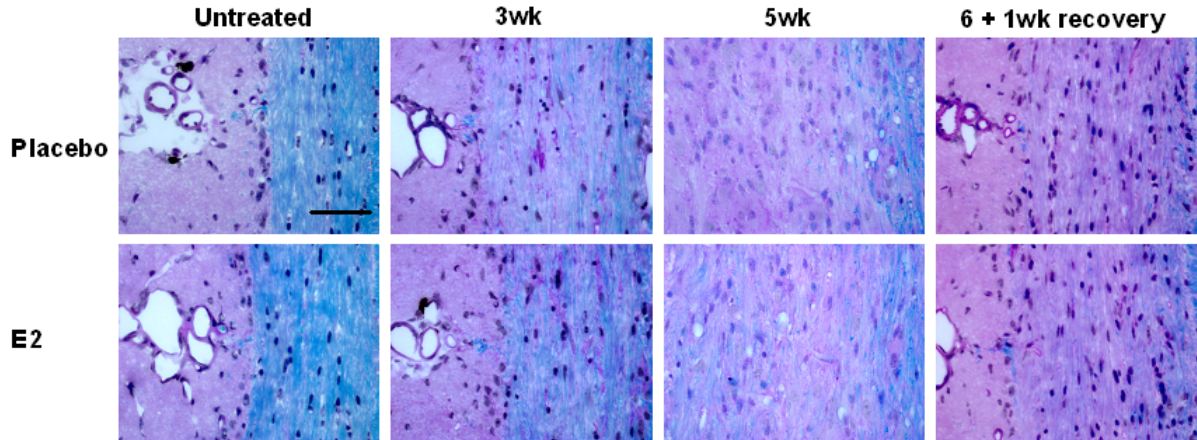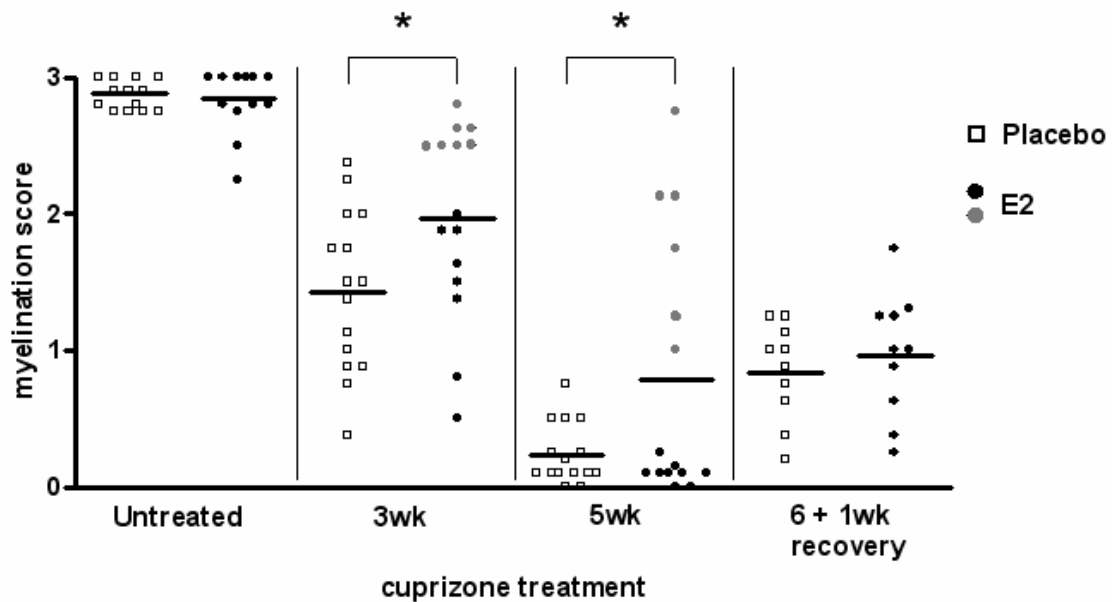
Figure 4.2. Demyelination and remyelination in placebo and E2-treated mice.
A. Representative images of LFB-PAS-stained midline corpus callosum sections.
Magnification is 40x and scale bar represents 50 micrometers.
B. Myelination scores obtained by blind-scoring of midline corpus callosum sections stained
with LFB-PAS. A score of 3 reflects normal myelination in an untreated mouse, whereas a
score of 0 reflects the absence of myelin. Individual data points and mean bars are plotted for
10-15 animals per group at each time point. * p < 0.05. At the 3 and 5 week time points, E2-
treated mice that exhibited the greatest protection from demyelination are indicated with gray
circles so that they may be followed in the subsequent figures of this manuscript.

E2 preserves oligodendrocytes.

In addition to myelination, the number of oligodendrocytes was examined in corpus callosum sections by immunohistochemistry to the marker GSTpi, which allows visualization of mature oligodendrocyte cell bodies (Figure 4.3A). Equal numbers of oligodendrocytes were detected in mice that did not receive cuprizone (Figure 4.3B). Similar to what was seen in the myelin histological stain (Figure 4.2B), more oligodendrocytes are present in E2-treated mice at 3 and 5 weeks of cuprizone treatment, though statistical significance was reached only at the 5 week time point ($p < 0.05$; Figure 4.3A and 4.3B). This suggests that this hormone results in partial protection of oligodendrocytes from cuprizone toxicity.

The E2-treated group of mice at week 5 in Figure 4.2B could be segregated into non-responders and those mice that responded to E2 treatment. A visual analysis of the E2-treated mice which showed a protection from demyelination (gray dots in Figure 4.2B) indicates that these mice also tended to have greater preservation of oligodendrocytes (Figure 4.3B), particularly at the 5 week time point. This would strengthen our correlation that responses to E2 treatment provide a noticeable protection for mature oligodendrocyte preservation.

The mature oligodendrocytes repopulating the demyelinated lesions were also quantified. After 1 week of recovery from cuprizone, oligodendrocyte numbers are restored to levels near that of the untreated mice. Although there are no statistically significant differences between the groups, there appears to be slightly fewer mature oligodendrocytes in the E2-treated mice (Figure 4.3B).
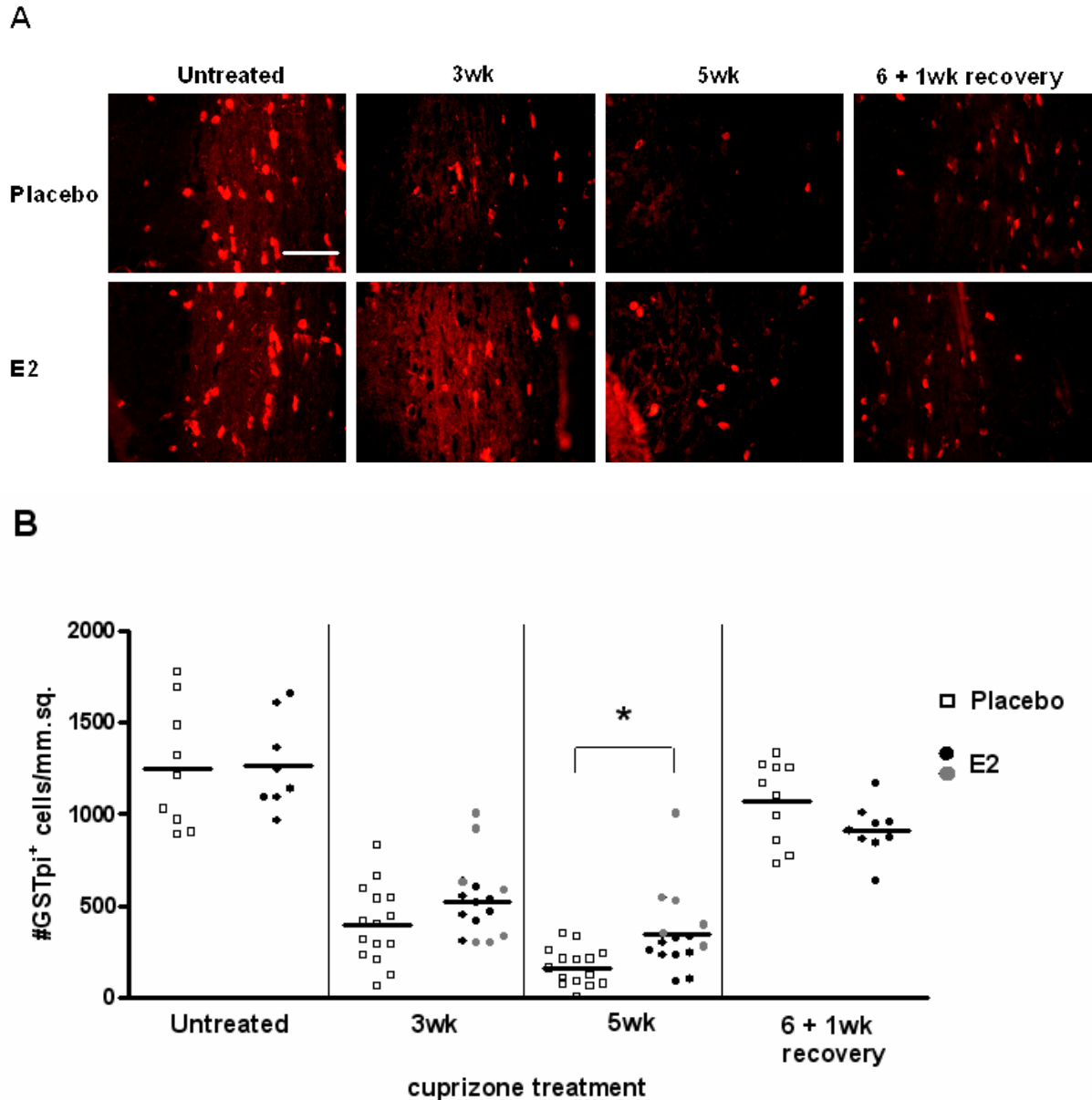
Figure 4.3. Cuprizone-induced loss of oligodendrocytes and subsequent repopulation during recovery in placebo and E2-treated mice.
A. Representative images of GSTpi⁺ mature oligodendrocytes in the corpus callosum of placebo and E2-treated mice during cuprizone administration and recovery. Magnification is 40x and scale bar represents 50 micrometers.
B. Quantification of GSTpi⁺ mature oligodendrocytes. Individual data points and mean bars are plotted for 10-15 animals per group at each time point. * $p < 0.05$. At the 3 and 5 week time points, E2-treated mice that exhibited the greatest protection from demyelination (Figure 4.2B) are indicated with gray circles.

E2 does not affect the number of oligodendrocyte precursor cells in demyelinating/remyelinating lesions of treated mice.

In vitro studies indicate that E2 can promote proliferation of oligodendrocytes [131]. Therefore, we hypothesized that numbers of oligodendrocyte precursor cells (OPCs) would be greater in demyelinated lesions of E2-treated mice. OPCs were detected by immunohistochemistry to the marker NG2. Contrary to our expectations, there was no statistically significant difference in numbers of OPCs in the lesions of E2-treated mice compared to placebo, at any of the time points (Figure 4.4). However, a following of the mice that showed protection from demyelination (gray circles) indicates that when E2 was most effective at reducing demyelination, there were fewer OPCs accumulating in the corpus callosum. As OPCs transition to mature oligodendrocytes at 1 week of recovery, they are reduced in numbers as expected and are diminished simlarly in placebo and E2-treated mice. Thus, overall, OPCs are not affected by E2 treatment and participate in remyelination normally.

A

B

Figure 4.4. Accumulation of OPCs during demyelination and remyelination in placebo and E2-treated mice.

A. Representative images of NG2$^+$ OPCs in the corpus callosum of placebo and E2-treated mice during cuprizone administration and recovery. Magnification is 40x and scale bar represents 50 micrometers.
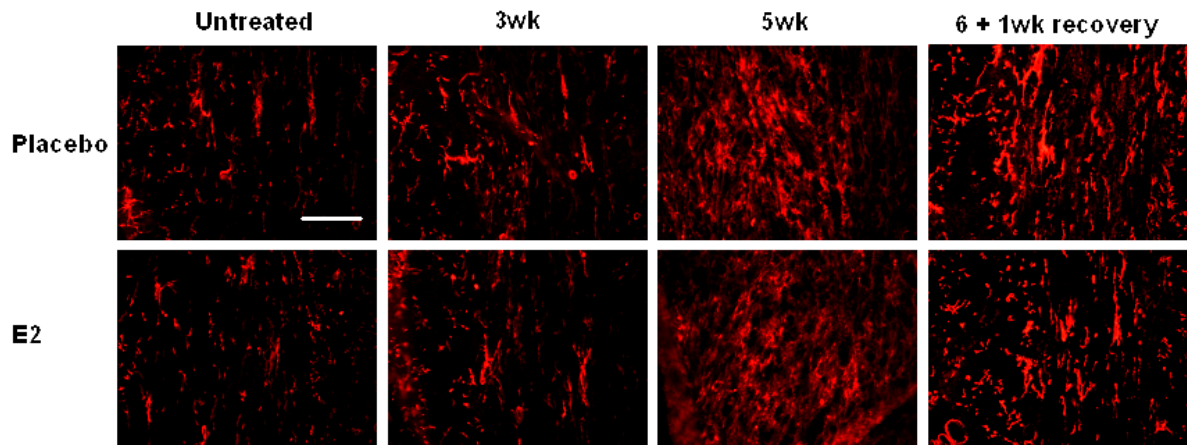
B. Quantification of NG2$^+$ OPCs. Individual data points and mean bars are plotted for 8-10 animals per group at each time point. There are no statistically significant differences between placebo and E2-treated mice at any time point. At the 3 and 5 week time points, E2-treated mice that exhibited the greatest protection from demyelination (Figure 4.2B) are indicated with gray circles.

Accumulation of microglia/macrophages is delayed in E2-treated mice.

Microglia, and to a lesser extent macrophage, activation and accumulation in demyelinated lesions is a hallmark feature in cuprizone-induced demyelination [161, 219]. These cells phagocytize myelin debris as well as produce a variety of cytokines and other molecules which may both exacerbate cuprizone-induced demyelination, and promote the repair process [147, 164, 165, 174, 189, 220-222]. Here, histochemical staining was performed with the lectin RCA-1, a marker for microglia/macrophages, in the corpus callosum during the time course of demyelination and remyelination. Very few microglia were present in mice that have not been exposed to cuprizone, and this was not altered by E2 treatment (Figure 4.5). After three weeks of cuprizone treatment, there were significantly fewer microglia in the demyelinated lesion of E2-treated versus placebo-treated mice. This pattern correlates with the decreased demyelination of E2-treated mice (Figure 4.2). However, by 5 weeks of cuprizone intoxication, there were similar numbers of microglia present in placebo and E2-treated mice, despite the decreased demyelination in E2-treated mice at this time point. An analysis of the mice that showed protection from demyelination (gray circles) indicated no clear pattern at the 3 week time point, but a distinct skewing towards fewer microglia in the less demyelinated lesions at 5 weeks. During remyelination, the microglia population was diminished to a similar extent in both placebo and E2-treated mice (Figure 4.5).
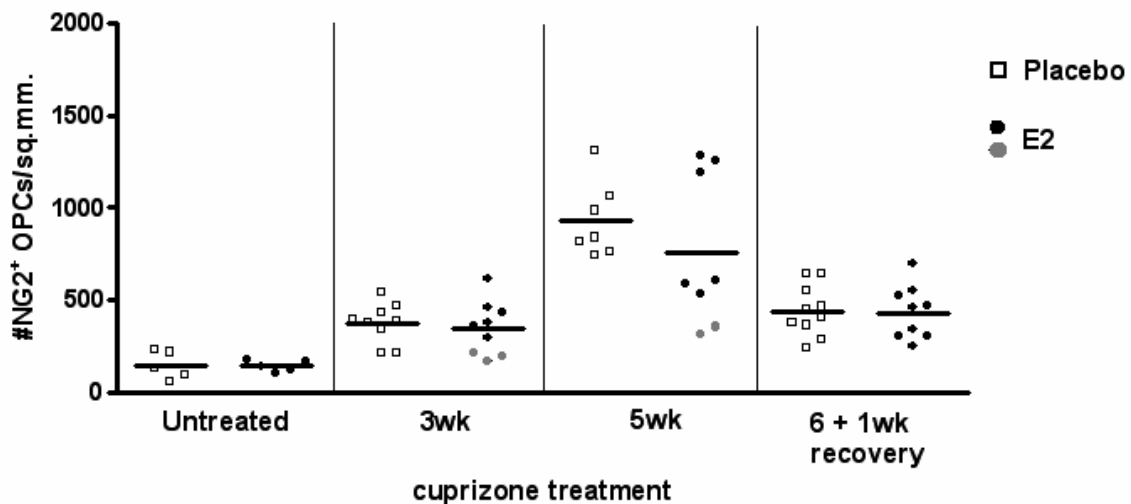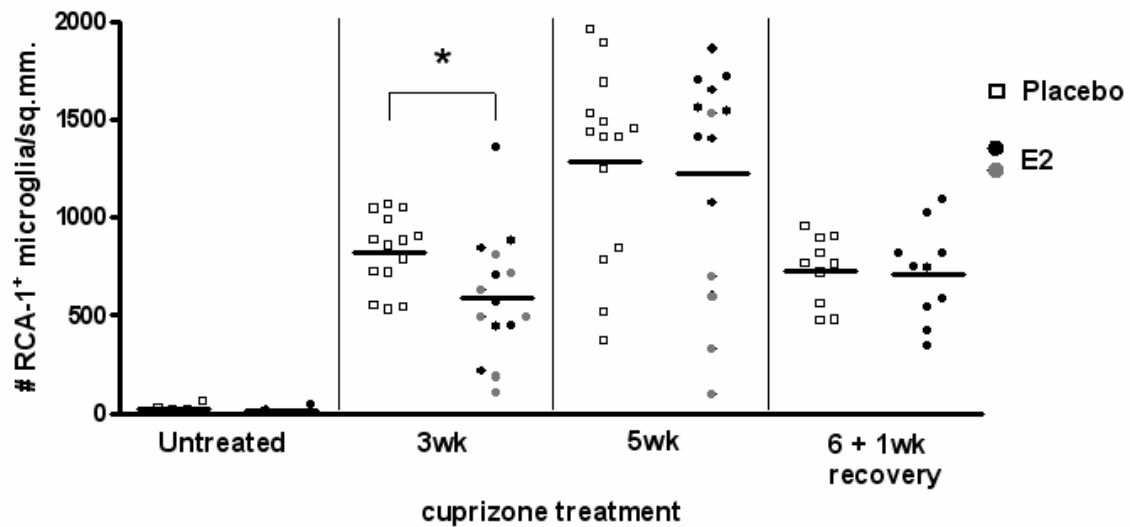
Figure 4.5. Accumulation of microglia during demyelination and remyelination in placebo and E2-treated mice.
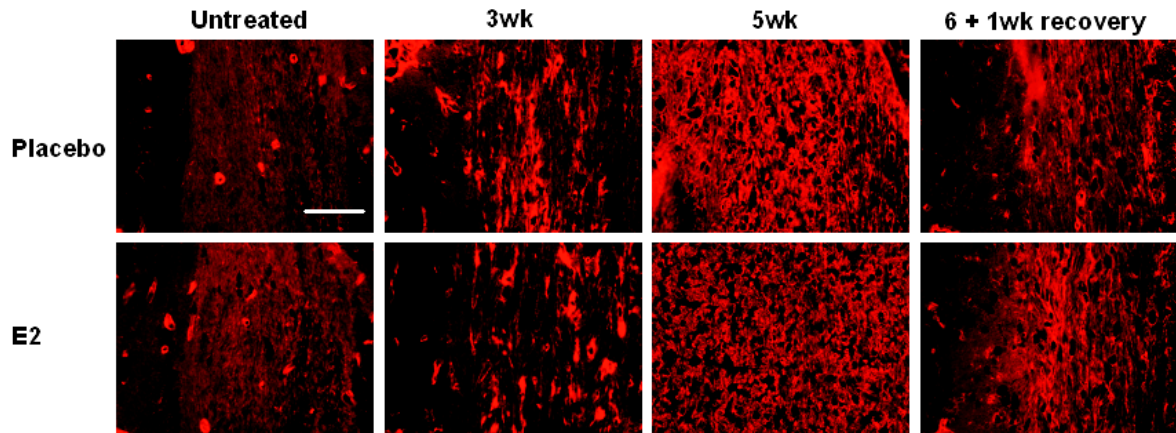
A. Representative images of RCA-1^{+} microglia/macrophages in the corpus callosum of placebo and E2-treated mice during cuprizone administration and recovery. Magnification is 40x and scale bar represents 50 micrometers.

B. Quantification of RCA-1^{+} microglia/macrophages. Individual data points and mean bars are plotted for 10-15 animals per group at each time point. * p < 0.05. At the 3 and 5 week time points, E2-treated mice that exhibited the greatest protection from demyelination (Figure 4.2B) are indicated with gray circles.

E2 does not affect numbers of astrocytes during demyelination or remyelination.

Astrocytes respond to demyelination and also secrete cytokines and growth factors that participate in cuprizone-induced demyelination and remyelination [162, 170, 182, 189, 221, 223]. Similar to microglia, immunostaining with the astrocyte marker GFAP was performed to examine whether E2 affects the numbers of these cells during demyelination and remyelination. In untreated mice, there are few astrocytes present in the corpus callosum. During demyelination, the astrocyte population increased similarly in placebo and E2-treated mice (Figure 4.6). Very similar to the observation of microglia accumulation, there appears to be no obvious correlation with protection from demyelination (gray circles) and astrocyte number at 3 weeks; however, at the 5 week time point, these mice showed a decreased astrocyte accumulation correlating with the less demyelinated lesions. E2 had no effect on astrocyte numbers in the corpus callosum during remyelination (Figure 4.6B).
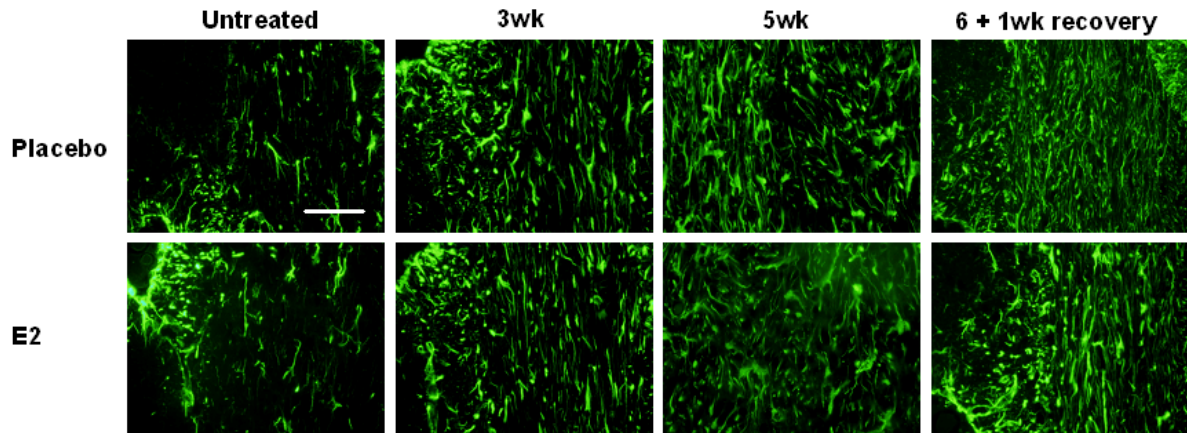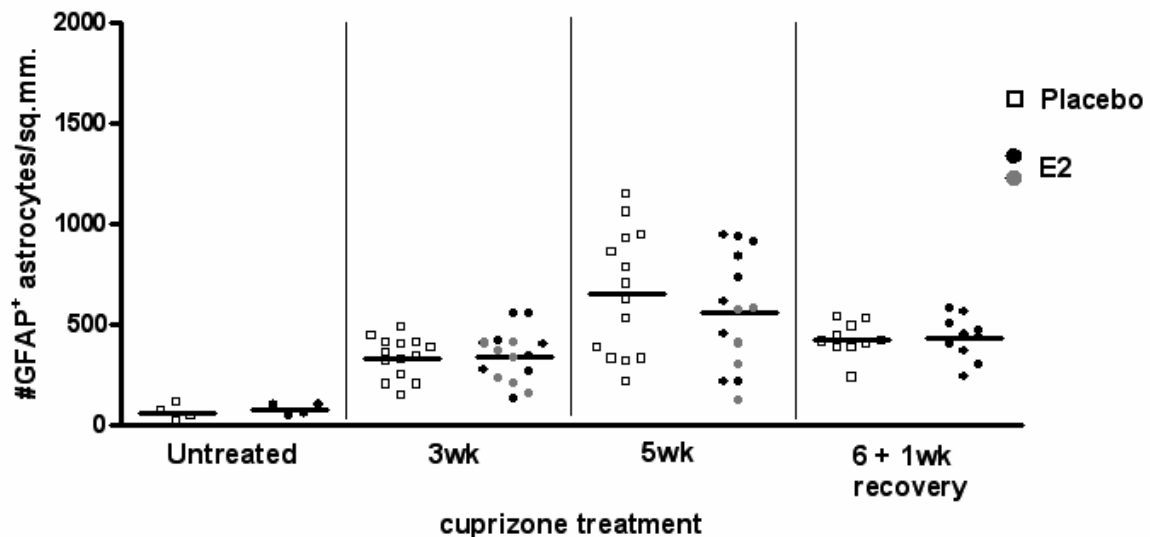
Figure 4.6. Accumulation of astrocytes during demyelination and remyelination in placebo and E2-treated mice.

A. Representative images of GFAP[+] astrocytes in the corpus callosum of placebo and E2-treated mice during cuprizone administration and recovery. Magnification is 40x and scale bar represents 50 micrometers.

B. Quantification of GFAP[+] astrocytes. Individual data points and mean bars are plotted for 10-15 animals per group at each time point. There are no statistically significant differences between placebo and E2-treated mice at any time point. At the 3 and 5 week time points, E2-treated mice that exhibited the greatest protection from demyelination (Figure 4.2B) are indicated with gray circles.

TNFα and IGF-1m RNA expression is diminished in E2-treated mice during demyelination.

In an effort to determine potential mechanisms of E2 protection against cuprizone-induced demyelination and oligodendrocyte loss, mRNA expression of two candidate mediators was measured by semi-quantitative PCR analysis. Due to the small amount of tissue of isolated corpus callosum and variability of E2 to produce protection from demyelination (Figure 4.2B), corpus callosi of 5 mice per group were pooled for RNA analysis. TNFα is an inflammatory cytokine that has been associated both with exacerbation of demyelinating disease [91, 165, 224] as well as having an important beneficial role in remyelination [165]. Furthermore, estrogen is known to regulate expression of TNFα [90, 211, 225]. Therefore, we sought to determine whether TNFα expression was altered by E2 administration during cuprizone-induced demyelination or remyelination.

Previous studies have shown that TNFα is upregulated during cuprizone-induced demyelination, and is primarily expressed by microglia, and occasionally by astrocytes [165]. Analysis of mRNA expression by semi-quantitative PCR indicated that in placebo-treated mice an expected increase of TNFα occurred during demyelination (70-fold compared to untreated placebo at 3 weeks and 90-fold compared to untreated placebo at 5 weeks of cuprizone treatment (Figure 4.7A). During remyelination, TNFα levels were reduced, which corresponds to the reduction in microglia and astrocytes at this time point (Figure 4.5B). Interestingly, E2 treatment reduced the upregulation of TNFα during demyelination by one third at 3 weeks and two thirds compared to placebo at 5 weeks of cuprizone treatment. This reduction in TNFα correlated with the reduced mature oligodendrocyte death from E2-treated mice.

IGF-1 is a growth factor which is expressed by most astrocytes and a sub-population of microglia during cuprizone-induced demyelination [189], and has been demonstrated to promote oligodendrocyte survival and promote remyelination [164, 167]. Therefore, we wanted to investigate the expression of IGF-1 in E2-treated mice during demyelination and remyelination. Results of semi-quantitative PCR indicated that levels of IGF-1 mRNA increase during demyelination in placebo-treated mice (Figure 4.7B) and decrease during remyelination, similar to previous results in unmanipulated mice subjected to cuprizone intoxication [162]. When mice were exposed to E2 administration, IGF-1 mRNA was substantially attenuated at both 3 and 5 weeks of demyelination (Figure 4.7B). This suggested that IGF-1 may be diminished by E2 or less IGF-1 may be needed to protect mature oligodendrocytes.
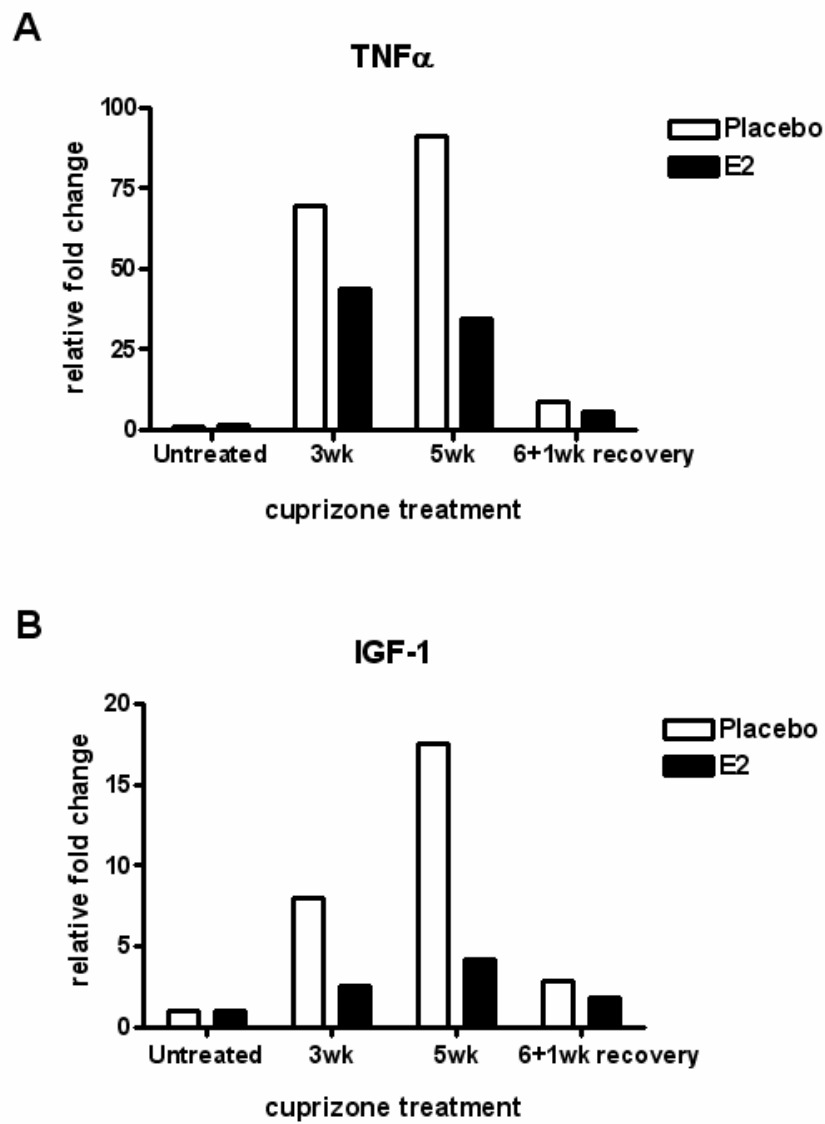
Figure 4.7. mRNA expression of TNFα and IGF-1 during demyelination and remyelination in placebo and E2 treated mice.

A. Semi-quantitative real-time PCR analysis of TNFα mRNA indicates the expected upregulation during demyelination in placebo treated mice, which is reduced by approximately half in E2 treated mice. All samples are normalized to the untreated placebo group.

B. Semi-quantitative real-time PCR analysis of IGF-1 mRNA indicates the expected upregulation during demyelination in placebo treated mice, which is dramatically reduced in E2 treated mice. All samples are normalized to the untreated placebo group.

115

4.5   DISCUSSION

The sex hormone E2 has been demonstrated to reduce neuronal loss and demyelination in several models of CNS injury [96, 212, 226, 227], as well as a prevent oligodendrocyte cell death and promote proliferation of oligodendrocyte precursors *in vitro* [131, 133, 134]. In this study, we used a toxin model of primary oligodendrocyte death to evaluate the role of E2 in CNS demyelination and remyelination. We report that E2 administration to male mice partially ameliorated corpus callosum demyelination and reduced mature oligodendrocyte loss. This protection was accompanied by a delay in microglia accumulation in the demyelinating lesion as well as reduced IGF-1 and TNFα expression. However, there was no statistically significant effect on numbers of oligodendrocyte precursors or astrocytes that infiltrated the lesion. In addition, E2 did not alter remyelination.

The two general mechanisms by which E2 may be attenuating demyelination include a direct anti-apoptotic effect in the mature oligodendrocytes, or an anti-inflammatory mechanism mediated by microglia and astrocytes. Both of these scenarios are plausible, given that E2 has been demonstrated to prevent death of oligodendrocytes and neurons *in vivo* [134, 228-231] as well as inhibit glial activation and inflammation in the CNS [139, 232, 233]. Furthermore, immunohistochemical expression of both estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ) has been demonstrated in brain cells: neurons and astrocytes can express either ERα or ERβ *in vivo* [229, 234, 235], oligodendrocytes and the myelin sheath co-localize *in vivo* with ERβ [123], and ERα has been demonstrated in microglia *in vivo* [125]. Attempts to co-localize ERα and ERβ in oligodendrocytes,

116

microglia and astrocytes in the corpus callosum during cuprizone-induced demyelination and remyelination have been unsuccessful so far. Nonetheless, a combination of direct protection of mature oligodendrocytes by E2 and reduced noxious products from microglia such as TNFα may be contributing to the reduced demyelination.

The ability of E2 to reduce demyelination has previously been demonstrated in the EAE model, and is generally thought to produce it effects by anti-inflammatory mechanisms of the immune system and by preventing infiltration of reactive T cells into the CNS [88-90]. Using ERα or ERβ deficient mice as well as selective agonists to each receptor, the anti-inflammatory and clinical symptom reduction effects of E2 in EAE was demonstrated to be mediated by ERα, but not through ERα in T cells [93-95]. In addition, it was recently shown that E2, and both ERα and ERβ agonists have neuroprotective effects in EAE [96, 217]. Relatively little attention has been given to effects of E2 in oligodendrocytes in EAE studies. Here, we have demonstrated that E2 can also reduce demyelination and prevent loss of oligodendrocytes in a toxin model of demyelination. Studies with ERα- and ERβ-deficient mice are currently underway to determine the contribution of each of these receptors to the observed protection.

E2 has been shown to attenuate hyperoxia-mediated loss of MBP in rat pups *in vivo* as well as hyperoxia-induced apoptotic death of primary oligodendrocytes *in vitro* [134]. Using the *in vitro* model, this group demonstrated that E2 inhibited hyperoxia-induced proapoptitic Fas-upregulation and caspase-3 activation and antagonized hyperoxia-induced inactivation of extracellular signal-regulated kinase 1 and 2 (ERK1 and 2) and Akt, key kinases of the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) cell survival-promoting pathways, respectively. Therefore, a potential mechanism to

117

explain the beneficial effects of E2 in cuprizone-induced demyelination may be an inhibition of apoptosis in oligodendrocytes. Future studies to measure expression of pro- and anti-apoptotic mediators localized to mature oligodendrocytes may provide important insights into the mechanism of E2 protection in cuprizone-induced demyelination.

Though E2 has been shown to increase proliferation of oligodendrocyte precursors *in vitro* [131, 236], we did not see an increase in OPCs in our E2 treated mice. In fact, in the mice in which E2 produced the most protection from demyelination, there were fewer OPCs in the lesion than in any of the other mice (Figure 4.4B, gray circles). The most likely interpretation for this observation is that OPCs were not called into the lesion, because there was less damage to the mature oligodendrocytes, and hence, there is less need for repair. This phenomenon has been seen in IGF-1 transgenic mice [164] and nNOS-/- mice [237], where there was very little loss of mature oligodendrocytes in the demyelinating lesion, and subsequently very little accumulation of OPCs. Alternatively, the reduction of TNFα in E2-treated mice shown here may be at least partly responsible for diminishing OPC numbers, given that cuprizone-treated TNFα-deficient mice display a significant reduction in accumulation and proliferation of OPCs during demyelination [165].

Quantification of microglia and astrocytes during cuprizone-induced demyelination and remyelination indicated that overall E2 resulted in a delay of microglia accumulation, but had no effect on astrocyte numbers in the corpus callosum. However, an analysis of the mice that showed the greatest protection from demyelination by E2 indicated that there was a reduction in both of these cell types, especially at the 5 week time point (Figures 4.5B and 4.6B, gray circles). This begs the difficult question: is there less gliosis because there is less demyelination and oligodendrocyte perturbation to respond to, or is there less demyelination

because of a reduction in the cells that produce pro-inflammatory cytokines which may have toxic effects to oligodendrocytes? Evidence that microglia are responsible for cuprizone-induced demyelination comes from a study which demonstrated that microglia inactivation by minocycline significantly attenuated demyelination [147]. In addition to a delay in microglia accumulation, we have demonstrated a reduction in TNFα mRNA in E2-treated mice. If one assumes a similar reduction in protein levels, this attenuation in microglial response may be a mechanism to explain the protective effect of E2, since it has previously been shown that demyelination is delayed in TNFα-deficient mice [165]. Alternatively, the reduction in TNFα may simply reflect that the microglia are less activated, due to the reduction in demyelination.

We have also demonstrated that E2-treatement attenuated demyelination-induced upregulation of IGF-1 expression. IGF-1 is a growth factor which is a survival factor for oligodendrocytes [164, 238, 239] and promotes remyelination [167]. There is evidence for cross-talk between the actions of estradiol and insulin-like growth factor –1 (IGF-1) in several neural events, such as: survival of developing neurons, neuronal differentiation, synaptic plasticity, female sexual behavior, adult neurogenesis, and neuroprotection (reviewed in [240]; [241]; [242]). However, the effects of E2 on the expression of IGF-1 are mixed, with a few reports of E2 administration resulting in increased IGF-1 expression in various tissues [243-245] and others reporting a reduction [246, 247]. Whether the reduction of IGF-1 mRNA expression during cuprizone-induced demyelination demonstrated here is directly mediated by E2 or is a result of reduced activation of microglia and astrocytes, is not known. Although in our subgroup of mice that showed protection from demyelination with E2 (Figure 4.2B), these animals had reduced numbers of microglia which may suggest a

diminished immune insult on mature oligodendrocytes. Both *in vitro* and *in vivo* studies have indicated that IGF-1 can protect oligodendrocytes from TNFα-mediated death [239, 248]. Therefore, the reduced IGF-1 expression may be due to a reduced need for protection. Future studies using mice with specific elimination of TNFR1 or IGF-1R on mature oligodendrocytes may indicate whether one or both mechanisms are employed.

Studies of brain injury indicate that the neuronal survival mediated by estradiol or IGF-1 each depend on the co-activation of ER and IGF-1 receptor ([230]; [229]). Both *in vitro* and *in vivo* studies have shown that estradiol regulates the expression of IGF-1 receptors in neural tissue ([240]; [249]). Furthermore, estradiol has been shown to regulate several downstream mediators of IGF-1 signaling, including extracellular-signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K), the kinase Akt, and glycogen synthase kinase 3β (GSK3β) [112] [250]; [251] [252]; [241]; also see [242] for review) and acts synergistically with IGF-1 to increase Akt in rat brain [251]. IGF-1 may also act synergistically with E2 to prevent SIN-1-mediated death of oligodendrocytes *in vitro* [133]. Therefore studies are planned to measure expression/phosporylation of the IGF-1 receptor and downstream signaling molecules such as those of the PI3K and MAPK pathway in these E2-treated brain samples, particularly on mature oligodendrocytes.

In conclusion, we have demonstrated that E2 administration to male mice provides a partial protection from oligodendrocyte loss and demyelination. This protection may be due to a combination of a delay in microglia accumulation and a reduction in TNFα and IGF-1 mRNA expression or a direct effect on mature oligodendrocyte survival.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The goal of this dissertation work was to provide insights into sex differences and the potential therapeutic benefit of estrogen in demyelinating disease. These issues are currently the focus of active research in many disciplines including autoimmune diseases and neurological disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), stroke, epilepsy and traumatic brain injury. Use of the cuprizone model to explore the role of sex and sex hormones may provide information applicable to other conditions in addition to MS which display some oligodendrocyte death, dysfunction, or demyelination such as AD, Huntington's disease, spinal cord injury, and the irradiated CNS [253-261]. We have demonstrated that cuprizone-induced demyelination is characterized by strain dependant sex differences, and that administration of a pregnancy level of 17β-estradiol (E2) is effective in partially reducing oligodendrocyte loss and demyelination in male mice. Future studies to expose potential mechanisms of these sex differences, E2 effects, and therapeutic potential are described in this final chapter of the dissertation.

Female SJL exhibit less severe cuprizone-induced demyelination.

Sex differences in cuprizone-induced demyelination were analyzed in the SJL mouse strain, which has previously demonstrated sex differences in other models of demyelination. We have demonstrated that SJL females exhibit a modest protection from demyelination and loss of oligodendrocytes at several points during a time course of cuprizone administration. However, there was no sex difference in the numbers of OPCs, microglia, or astrocytes that accumulated in the corpus callosum lesions. Furthermore, a preliminary study of remyelination indicated that there was no difference in remyelination at the particular time point tested.

In order to determine whether circulating sex hormones are responsible for this sex difference, gonadectomies/adrenalectomies could be performed. One would want to test both sexes, since either male or female sex hormones could mediate disease, as has been demonstrated in EAE. Furthermore, replacement of individual hormones after gonadectomy/adrenalectomy would provide more conclusive evidence for the role of that particular hormone. If a particular hormone was found to mediate the sex difference in demyelination severity, further studies would be needed to determine it's mechanism of action. One potential method is to use gene microarray analysis to compare gene expression between male and female corpus callosum lesions.

The role of sex chromosomes could be tested by using XX and XY$^-$ ovary bearing females, and XY$^-$*Sry* and XX *Sry* testes bearing males which give one the ability to study the effect of the sex chromosome makeup on the background of both female and male hormones. Use of this model in SJL mice demonstrated that the XX chromosome makeup lead to more severe EAE, and was associated with reduced expression of Th2 cytokines IL-4, IL-10 and IL-13 [38].

Temporal and regional differences in demyelination patterns in SJL mice.

An interesting finding that came out of the study of SJL sex differences is that this strain displays temporal and severity differences in demyelination compared to C57BL/6 mice. In beginning to work with the SJL, we first performed a cuprizone dose titration, from which it was determined that 0.2% cuprizone produced the most demyelination without overt systemic toxicity. The dose titration was analyzed after 5 weeks of cuprizone administration, since this is the time point at which maximal demyelination occurs in the corpus callosum of

C57BL/6 mice. We found that at this time point, SJL mice did not display maximal demyelination. Therefore, we next performed a time course of continuous cuprizone exposure, analyzing demyelination at weekly intervals from 3 to 10 weeks. Both male and female SJL gradually demyelinated during this time period never achieving full demyelination. There was also less accumulation of microglia and astrocytes in the SJL demyelinating lesions than is typical for C57BL/6. We found that even after 13 weeks of continuous cuprizone exposure, demyelination was not significantly different than at the 10 week time point (data not shown). Maximum loss of oligodendrocytes occurred after 7 weeks of cuprizone exposure, compared to 5 weeks in C57BL/6 mice. Furthermore, a preliminary study of remyelination suggests that SJL are slower to remyelinate than C57BL/6 mice. This strain difference in demyelination severity indicates that there might be genetic differences between SJL and C57BL/6 mice which partially protect SJL from cuprizone-induced death of oligodendrocytes. One way to analyze a genetic influence on cuprizone-induced demyelination severity would be to use recombinant inbreeding between SJL and C57BL/6 and analyze the crossed offspring to identify quantitative trait loci (QTL) controlling demyelination severity. This approach has been used in the EAE model, in crosses between the highly susceptible SJL/J and resistant C57BL/10.2 mice [262-266] and was successful in identifying several QTL that regulate parameters such as disease onset, severity, and cell infiltration during EAE in mice.

In addition to the difference in time course and severity of demyelination in the SJL strain, there was an interesting difference in regional demyelination within the corpus callosum. Whereas C57BL/6 display robust demyelination in the medial region of a coronal section of corpus callosum, SJL mice demonstrate a preservation of myelinated fibers at the

midline, and in the lower part of the more lateral regions, just above the fornix (see Figure 2.1). The reason for this heterogeneity of demyelination is far from clear, but speculation results in the following potential mechanisms. One potential explanation is that axons coming from different regions of the cortex may provide different levels of trophic signals to the oligodendrocytes myelinating them. Although the literature does not reveal any evidence for a pro-survival signal from axons to oligodendrocytes which are already fully mature and myelinating, there is certainly evidence that axons provide pro-survival cues to immature oligodendrocytes, such as through neuregulin [267-269]. Alternatively, different neuronal populations may be more or less perturbed by the cuprizone insult and hence producing different levels of toxic factors which affect the oligodendrocytes myelinating them. It is known that cuprizone intoxication results in some axonal pathology [145, 174, 270] but whether this occurs before or after the axon has become demyelinated is not known. Another possibility is that there are different levels of secreted factors of a protective or exacerbatory nature coming into the corpus callosum from the neighboring brain regions. Alternatively, there may be regional differences in penetration of the cuprizone toxin.

A synthesis of the findings from demyelination in C57BL/6 versus SJL mice indicate that there are genetic differences which mediate sex effects as well as demyelination patterns and severity. These strain differences may have important implications for genetic influences on demyelination in an outbred human population and may mediate some of the heterogeneity of MS pathology.

Lack of sex differences in demyelination/remyelination in C57BL/6 mice.

The results from the work of chapter 3 of this dissertation demonstrate that female C57BL/6 female mice exhibit an equivalent pattern of cuprizone-induced demyelination and remyelination as their male counterparts. The possible exception to this was that LFB-PAS histological staining at the 3 week time point indicated a trend for decreased demyelination in the female mice. However, quantification of myelinated axons from electron micrographs at this time point indicated no significant differences between males and females. Furthermore, there were no significant differences in the numbers of oligodendrocytes in the demyelinating or remyelinating corpus callosum lesion at any time point. Accordingly, the numbers of microglia and astrocytes which accumulate in the lesion were equivalent. The results of this work are closely aligned with that of the EAE model, whereby it has been demonstrated that C57BL/6 mice do not exhibit sex differences in susceptibility or severity of demyelinating disease [44, 46].

Interestingly, it was determined that cuprizone intoxication disrupted the estrous cycle in these mice, beginning in the first two weeks. This disruption was reversible, as females resumed a normal estrous cyclicity within 4 weeks of discontinuation of cuprizone administration. Because estrous cyclicity and uterine weight is an excellent bioassay for sex hormone function [108, 205-208], it is assumed that circulating levels of sex hormones are altered in cuprizone treated female mice. However, this was not directly measured, and would be an important step in any future studies of gender or the role of sex hormones in the cuprizone model. Furthermore, a disruption in circulating hormone levels could possibly be attenuated in the brain, since many CNS cells have the ability to produce sex [77, 271]

steroids. The mechanism of cuprizone's effect on estrous cyclicity is not known. Possible explanations include: a toxic effect to ovarian cells which produce the hormones that control estrous cyclicity; a disruption of the steroid biosynthesis pathway; or a general effect of the stress and weight loss that occurs in cuprizone-intoxicated mice.

In an effort to determine whether reproductive functions of male mice might be similarly perturbed, testes weights were analyzed. There was no significant difference in the weights of testes between cuprizone treated and age-matched controls. However, this method of analysis may not be as sensitive a measure of sex hormone function as is the vaginal lavage and uterine weight assays used in the female mice. Therefore, further analysis would have to be performed to conclude without a doubt that sex hormone function was not perturbed in the male mice. This could be done by measuring serum levels of sex hormones and performing histological analysis of the testes tissue.

Overall, this finding that cuprizone disrupts estrous cyclicity in C57BL/6 mice presents a limitation to the use of the cuprizone model in the study of sex differences. Based on the findings of the previous chapter, the glaringly obvious question is: was estrous cyclicity disrupted in female SJL mice? Unfortunately, this question remains to be answered. The cuprizone studies in SJL mice were performed before the estrous cyclicity assays were performed in C57BL/6 mice. However, measures of estrous cyclicity and sex hormone function are at the top of the list for future studies of sex differences in SJL mice.

E2 partially prevents oligodendrocyte loss and demyelination.

In order to ascertain whether E2 has protection functions during demyelination *in vivo*, we administered pregnancy levels of E2 to male mice during cuprizone administration, in the form of subcutaneously implanted continuous release pellets. We have demonstrated that E2 was effective in reducing oligodendrocyte loss and demyelination. This protection was accompanied by a delay in microglia accumulation as well as a significant reduction in TNFα and IGF-1 mRNA. Treatment with E2 did not significantly alter numbers of OPCs or astrocytes which accumulated in the lesion, nor was remyelination significantly different at the 7 week time point.

Currently, several experiments are under way that should help elucidate a mechanism for this protection by E2. In order to determine which estrogen receptor is involved, ERα and ERβ deficient mice (designated αERKO, and βERKO) were implanted with placebo or E2 pellets, and treated with cuprizone for 5 weeks.   If one or the other receptor is solely responsible for the protection by E2, then we will not see any protection by E2 in those knock-out mice. Two potential caveats exist which may prevent this experiment from conclusively determining the role of these receptors. First, if they compensate for each other, we may see protection in both knock outs. In this case, we will have to conclude that either there is compensation, or that E2 is working through an estrogen receptor-independent mechanism, such as through its antioxidant actions (reviewed in [212]). Since cuprizone-intoxication is associated with oxidative stress [142, 147, 272, 273], a potential antioxidative effect of estradiol is intriguing. In order to evaluate whether the receptors are compensating for each other, double knockout mice could be generated. We have tried this in the past, however due to the fact that these mice must be bred as heterozygotes, since the monozygous

knockouts are infertile, it requires very large numbers of breeding pairs to obtain enough double knockouts of similar age to use for an experiment. Alternatively, administration of the ER antagonist ICI 182 780 (selective for both ER subtypes) at the same time as the E2 treatment could be performed. The other caveat is that we have seen a frustrating amount of variability in the response of individual mice to the E2 treatment, such that only 6 of 15 mice were significantly protected from demyelination at the 5 week time point. Therefore, only if significant numbers of the wild type mice show protection in this experiment will we be able to make a conclusion about the knockout mice. We have included 7-8 placebo treated and 7-8 E2 treated male mice of each genotype (wild type C57BL/6, αERKO, and βERKO) for this analysis.

Determining the expression of ERα and ERβ in the corpus callosum and during cuprizone-induced demyelination is an important step in elucidating the mechanism of action of E2 in this model. I have made several attempts at immunohistochemical analysis of expression of these receptors, but so far have been unsuccessful. Currently, we are in the process of testing additional tissue processing methods, and perhaps additional antibodies, and hope to be able to obtain co-localization data in the near future. In the meantime, analysis of mRNA expression by RT-PCR and protein expression by western blot will be performed. In this way we will have an idea of the relative levels of expression of each receptor in an untreated corpus callosum and during demyelination and remyelination.

In addition, further analysis using the mRNA and protein collected from these experiments will be used to measure the expression levels of various candidates potentially mediating the effects of E2 in this system. We have already demonstrated that TNFα expression is reduced in E2 treated mice, and this may have important consequences, since

129

TNFα deficient mice have been shown to exhibit less severe EAE [91], and a delay in cuprizone-induced demyelination [165]. Therefore, a downregulation of TNFα by E2 may lead to reduced oligodendrocyte death. Next, we will determine whether expression of TNF receptor 1 (TNFR1) or TNFR2 are altered by E2 administration. TNFR1 is a death receptor which mediates cell death, or potentially cell survival [274-276], whereas TNFR2 can enhance cell death through TNFR1 or promote survival [277, 278] but has also been demonstrated to be important for proliferation of oligodendrocytes and remyelination [165]. Estrogen administration has been shown to decrease TNFR1 in PC12 cells [226], but increased expression of TNFR1 and decreased TNFR2 in a rodent ischemia model [279]. It should be noted that a decrease in TNFα signaling presents potential inhibition to remyelination. It was demonstrated by Arnett and colleagues that in TNFα and TNFR2 deficient mice, proliferation of OPCs and repopulation of mature oligodendrocytes was decreased [165]. Though we did not detect a significant decrease in OPC numbers, or remyelination, this potential inhibition of remyelination should remain a concern during future studies.

We have also demonstrated that E2 administration led to a reduction in IGF-1 expression. The significance of this reduction is not clear, and a review of the literature regarding E2/IGF-1 crosstalk in neuroprotection suggests that regulation of IGF-1 receptor or downstream signaling may be a better measure of the potential benefits of E2 administration. We hypothesize that IGF-1R will be increased in mature oligodendrocytes with E2 treatment and may participate in the protection from death mediated by E2. We plan to measure this by Western blot and immunochemical co-localization with mature oligodendrocytes. In the future, we hope to assess the activation state of the receptor by assessing levels of the

phosphorylated protein. Futhermore, our lab already possess some of the reagents necessary to perform western blot analysis of the expression of phosphorylated downstream mediators of IGF-1R signaling, namely ERK and Akt.

In addition, we would like to further characterize the potential role of E2 in preventing apoptosis of oligodendrocytes by looking at mediators of apoptosis that have been demonstrated to be regulated by E2 in models of neuroprotection, such as Bcl-2, Bcl-xL, and Bax, [280-283].

Working Model

The work presented here regarding the potential therapeutic benefit of E2 in demyelinating disease leads to a working model with two alternative (or potentially additive) mechanisms (Figure 5.1). First, E2 may be preventing the accumulation and activation of microglia and astrocytes in the corpus callosum, and preventing the production of pro-inflammatory mediators which exacerbate oligodendrocyte death. This model is supported by the finding that oligodendrocytes in culture are not killed by the addition of cuprizone unless TNFα is also added [147]. Furthermore, mice that are deficient for TNFα experience a delay in cuprizone-induced demyelination [165]. Second, E2 may have a pro-survival effect in oligodendrocytes, thus directly preventing cuprizone-induced death and subsequent demyelination. In the absence of dying oligodendrocytes and myelin debris, fewer astrocytes and microglia are activated to accumulate in the lesion and thus there is a reduction in production of mediators such as TNFα and IGF-1. In support of this mechanism, E2 has been demonstrated to protect oligodendrocytes from oxygen-mediated and toxin-induced death *in vitro*, through anti-apoptotic mechanisms [133, 134].

Astrocytes, microglia, and oligodendrocytes have been shown to express estrogen receptors in several other systems (see section 1.5), however, an analysis of their expression in the corpus callosum in response to cuprizone-induced demyelination may shed further light on which of the proposed models is more likely. Assuming all of these cell types do express the estrogen receptors in this scenario, one way to determine whether E2 is acting primarily through an anti-inflammatory or pro-survival mechanism would be to engineer conditional knockout mice in which the estrogen receptors are ablated from either oligodendrocytes or microglia and astrocytes. This could be accomplished with the cre-lox system using cell type specific promoters (PLP for oligodendrocytes, GFAP for astrocytes, CD11b for microglia). Ideally, one would also like to make the ablation inducible, so that developmental effects do not complicate the ability to draw conclusions during injury and repair in the adult mice.
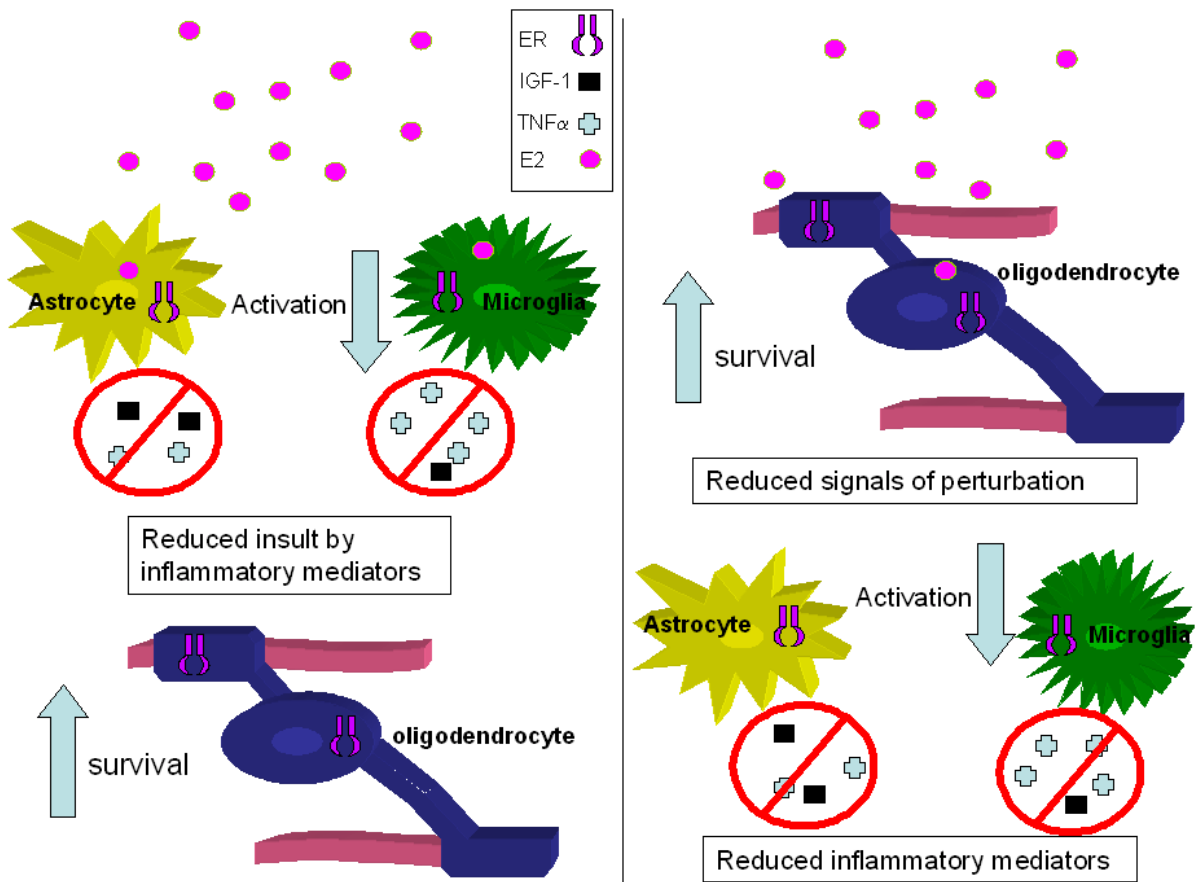
Figure 5.1. Two potential models to explain E2 reduction of cuprizone-induced oligodendrocyte loss, demyelination, microglia and astrocyte accumulation and expression of TNFα and IGF-1.

The left panel describes the potential anti-inflammatory affect of E2, and the subsequent reduction in inflammatory mediators such as TNFα which may contribute to oligodendrocyte death and demyelination. The right panel illustrates the potential pro-survival affect of E2 in oligodendrocytes, which would limit demyelination and subsequent activation of microglia and astrocytes. Since oligodendrocytes, astrocytes and microglia have all been shown to express estrogen receptors, either or both of these scenarios are a plausible mechanism.

Future directions for sex hormone therapy.

The study of sex hormones such as estrogen has great potential for treatment of

neurodegenerative diseases, due to their anti-inflammatory and neuroprotective effects.

Results from EAE studies indicate that administration of physiologic doses of testosterone

can ameliorate disease [98] and a recent clinical trial in male MS patients suggests that

133

testosterone therapy has neuroprotective effects, but may not be effective in preventing inflammation [85]. Therefore, testosterone treatment in combination with other anti-inflammatory drugs may represent a good treatment option for male MS patients. Testosterone therapy in females is probably not feasible due to masculinizing side effects. Our work, combined with that of others, has shown that administration of estradiol is effective at reducing demyelination severity in male and female mice. However, the use of estrogen as a treatment in male patients presents the complication of feminization and is therefore probably not feasible. Clinical trials in female MS patients suggests that administration of a pregnancy level dose of estriol is effective at reducing gadolinium-enhancing lesions [79]. Although this treatment was well tolerated for the 6 month period of the study, longer treatment periods would require the combined administration of progesterone, to prevent uterine endometrial hyperplasia [79]. A potential concern with using estrogen as a treatment (especially if given in high doses or for prolonged periods) is that there could be serious unwanted side effects such as an increased risk of chronic heart disease, stroke and breast cancer [284, 285]. Combination therapies with currently used immunomodulatory drugs may be beneficial if synergistic or additive effects allowed for the use of lower doses of estrogen, which could minimize unwanted side effects.

Furthermore, the ERβ-selective agonist DPN was shown to reduce EAE symptoms in the chronic phase of the disease, and produce significant neuroprotective effects [96]. Although this compound did not seem to have anti-inflammatory effects or prevent the onset of EAE, it may have therapeutic potential in the prevention of axonal and neuronal loss. Given in combination with standard anti-inflammatory treatments, ERβ ligands may

represent an attractive therapeutic candidate, since many of the growth-stimulatory effects of estrogens in breast cancer have been linked to ERα [286].

The development of therapies that produce the positive effects of estrogen while reducing systemic side effects are in high demand. Selective estrogen receptor modulators (SERMs) are molecules with mixed ER agonist/antagonist properties in different tissues and represent a promising therapeutic potential. Several classes of SERMs including phytoestroges, tamoxifene, and raloxifene have demonstrated neuroprotective properties *in vitro* (reviewed in [287]). Raloxifene is a nonsteroidal benzothiophene that inhibits the growth of estrogen receptor-dependent mammary tumors in rats. It has been classified as a SERM on the basis of studies in which it prevented bone loss and lowered serum cholesterol levels without stimulating the endometrium [288]. Recently, raloxifene and a raloxifene analog, WAY-138923, were shown to reduce incidence, delay onset, and reduce clinical symptoms in EAE [93]. These compounds did not alter splenocyte cytokine production from EAE mice, but did reduce proliferation of T cells. Effects on brain cells were not characterized in this study. Raloxifene was also shown to have neuroprotective effects in a mouse model of Parkinson's disease in two out of three studies [289-291] and in an excitotoxic injury model [292]. An exciting new avenue of SERM development is underway in which in silico techniques are being employed in an effort to develop NeuroSERMS, which will target the brain, cross the blood brain barrier, and lack feminizing properties [287].

Finally, another potential therapeutic strategy may be to increase production of estrogen in the brain. Aromatase, the enzyme responsible for the conversion of testosterone to estradiol, is expressed in subpopulations of neurons in the mammalian brain, and is

135

induced in reactive astrocytes by different forms of brain lesion [229, 293]. Aromatase

activity reduces neuronal death in neurodegeneratice models in vivo [107, 229, 294-298].

The aromatase gene is regulated by tissue-specific promoters [299] and therefore it is

conceivable that selective aromatase modulators could be developed to enhance expression in

the brain but not other tissues.

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