Cuprizone Induces Similar Demyelination in Male and Female C57BL/6 Mice and Results in Disruption of the Estrous Cycle

Lorelei C. Taylor,^{1,2} Wendy Gilmore,³ Jenny P.-Y. Ting,^{1,2,4} and Glenn K. Matsushima^{1,2,4,5★}

¹Curriculum in Neurobiology, University of North Carolina-Chapel Hill, Chapel Hill, North Carolina

²UNC Neuroscience Center, University of North Carolina-Chapel Hill, Chapel Hill, North Carolina

³Department of Neurology, University of Southern California, Los Angeles, California

⁴Department of Microbiology and Immunology, University of North Carolina-Chapel Hill, Chapel Hill, North Carolina

⁵Program for Molecular Biology and Biotechnology, University of North Carolina-Chapel Hill, Chapel Hill, North Carolina

Multiple sclerosis is a demyelinating neurological disease that is influenced by gender, primarily reflected in greater susceptibility to disease development in women than in men. Cuprizone intoxication, an animal model that is used to study demyelination and remyelination, has been extensively characterized in male C57BL/6 mice. Here, 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 them with age-matched male mice. We find that the pattern of demyelination and remyelination is similar between genders and that there is little or no difference in the loss or repopulation of mature oligodendrocytes or accumulation of reactive glia. Furthermore, examination of α ERKO and β ERKO mice suggests that estrogen receptors do not affect the outcome for demyelination or remyelination. 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. © 2009 Wiley-Liss, Inc.

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Destruction of the myelin sheath and subsequent neurological impairments are hallmark characteristics of the demyelinating degenerative disease multiple sclerosis (MS). Women are two to three times more susceptible to developing MS than men. However, gender may also have subtle effects on disease severity, as illustrated by a natural history study in which females showed an association with slower progression of disease (Confavreux et al., 2003). Also, MRI scans of MS patients revealed that men had a higher number of “black hole” lesions,

which mark axonal loss (Pozzilli et al., 2003). 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 (Confavreux et al., 1998). 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 (Bebo et al., 1996, 1998a,b; Voskuhl et al., 1996; Papenfuss et al., 2004). In contrast, other strains exhibit increased severity in males (B10.PL and PL/J) or no sex differences (NOD/It and C57BL/6; Papenfuss et al., 2004). Another autoimmune model of MS, intracerebral infection 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 (Kappel et al., 1990), and male SJL mice exhibit more severe neurological deficits than females

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★Correspondence to: Glenn K. Matsushima, University of North Carolina, 115 Mason Farm Rd., CB 7250, Chapel Hill, NC 27599.
E-mail: gkmats@med.unc.edu

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(Alley et al., 2003). These studies indicate that the influence of gender on disease susceptibility and severity is strain-specific and dependent on the disease model.

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 (Lucchinetti et al., 2000). These models are also very useful in the study of remyelination and recovery from oligodendrocyte loss. With the ethidium bromide toxin model, it was shown that aged female rats remyelinated more efficiently than male counterparts (Li et al., 2006).

Another toxin model that produces a well-defined and highly reproducible pattern of demyelination and remyelination is the cuprizone intoxication model (Blakemore, 1973, 1974; Ludwin, 1978). Exposure of C57BL/6 mice to a low dose of the copper chelator cuprizone induces death of oligodendrocytes and loss of myelin in the corpus callosum fiber tract (Hiremath et al., 1998; Mason et al., 2000; Matsushima and Morell, 2001) as well as some degeneration and axonal loss, especially in older mice (Irvine and Blakemore, 2006). Most earlier studies used male mice, in part because it was thought that female mice do not demyelinate (Ludwin, 1978). We recently demonstrated that SJL mice not only showed differences in demyelination between male and female mice but the time course differed from that of C57BL/6 mice (Taylor et al., 2009). Although demyelination in female C57BL/6 mice had been noted (Matsushima and Morell, 2001), and at least one group has noted unpublished data indicating a lack of gender effect in C57BL/6 mice (Binder et al., 2008), no comprehensive gender comparison has been reported for this strain. Because more investigators are beginning to use female mice in cuprizone studies (Petratos et al., 2004; Selvaraju et al., 2004; Irvine and Blakemore, 2006, 2008; Liebetanz and Merkler, 2006; Wu et al., 2006), it is important to have a similar knowledge base of the processes occurring in female mice during cuprizone treatment. In this paper, we compare morphological and cellular changes in male and female C57BL/6 mice during demyelination and remyelination. In addition, the role of the estrogen receptor in demyelination/remyelination was investigated in mice deficient for either the alpha or the beta estrogen receptor (α ERKO and β ERKO). We found no differences in measures of demyelination or remyelination between knockout and wild-type mice. Furthermore, we observed that cuprizone intoxication disrupts estrous cyclicity in female mice and leads to diminished wet weights of male and female sex organs. Thus, cuprizone may interfere with potential endogenous hormone influences on demyelination and remyelination in this strain, perhaps masking potential sex differences.

MATERIALS AND METHODS

Animals and Cuprizone Treatment

Adult male and female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). α ERKO mice

and β ERKO mice were kindly provided by Dr. Kenneth Korach (NIEHS, Research Triangle Park, NC). α ERKO mice were generated by targeted disruption of the *Esr1* gene, and β ERKO mice were produced by targeted disruption of the *Esr2* gene as previously described (Lubahn et al., 1993; Kregel et al., 1998) and back-crossed to C57BL/6 mice for greater than 10 generations. Homozygous α ERKO and β ERKO mice as well as wild-type littermate controls (α ERWT and β ERWT) were generated by mating heterozygotes in the animal facility at UNC-Chapel Hill. At 8 weeks of age, mice were fed ad libitum 0.2% cuprizone [oxalic bis (cyclohexylidenehydrazide); Sigma-Aldrich, St. Louis, MO] 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 (Mason et al., 2000, 2001; Arnett et al., 2001), remyelination was assessed by returning mice to a diet of normal Purina pellet chow for 1 or 2 weeks, after 6 full weeks of cuprizone treatment. Untreated control mice were fed ground or pelleted Purina chow during the time when experimental mice were being treated with cuprizone. All animal use was performed in compliance with the NIH *Guide for care and use of laboratory animals* and was approved by the UNC-CH Institutional Animal Care and Use Committee.

Tissue Preparation

For light microscopy, mice were deeply anesthetized and intracardially perfused with 0.15 M phosphate buffer, followed by 4% paraformaldehyde (PFA) solution. For paraffin embedding, tissue was postfixed overnight in PFA, then processed through a series of ethanol dilutions and xylene and embedded in paraffin wax at the UNC Lineberger Animal Histopathology Core Facility. For NG2 immunohistochemistry, brains were postfixed for 4 hr in PFA and then transferred to 30% sucrose for at least 24 hr prior to freezing on a bed of dry ice. Five-micrometer coronal brain sections were cut at the fornix region of the corpus callosum (approximately Bregma -0.5 mm to -0.7 mm) and corresponding to Figure 37 of *The mouse brain in stereotaxic coordinates* (Franklin, 2001). For electron microscopy, mice were deeply anesthetized and intracardially perfused with 0.15 M phosphate buffer, followed by a 4% glutaraldehyde/2% PFA solution. Brains were removed, postfixed for 2 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), which stains myelin blue, and periodic acid-Schiff (Sigma), 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 was midline, directly

above the fornix in a coronal section at approximately Bregma -0.5 mm to -0.7 mm. See Mason and colleagues (2000) for a cartoon of the region analyzed.

Immunohistochemistry

For immunohistochemistry, 5- μ m paraffin sections were deparaffinized and rehydrated, or frozen sections were warmed to room temperature and rinsed in phosphate-buffered saline (PBS). 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* agglutinin-1 (RCA-1; Vector Laboratories, Burlingame, CA), was used to detect microglia/macrophages. Astrocytes were detected with antibody to glial fibrillary acidic protein (GFAP; Invitrogen, Carlsbad, CA). Oligodendrocyte precursor cells were detected with a rabbit antibody to NG2, generously supplied by Dr. W.B. Stallcup (Genomic Institute of Novartis Research Foundation).

For GSTpi staining, paraffin or frozen sections were first permeabilized/blocked with 0.1% Triton X-100/2% normal goat serum in PBS for 20 min at room temperature. Antigen retrieval was performed with 0.1% trypsin/0.1% calcium chloride in 0.05 M Tris, pH 7.4, for 10 min at 37°C. After being rinsed, sections were incubated with GSTpi antibody (1:1,000 in block) or isotype control overnight at 4°C. For RCA-1 and GFAP staining, paraffin or frozen sections underwent antigen retrieval with 0.025% protease, type XIV (Sigma-Aldrich) for 2 min at 43°C. 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 hr at room temperature. To detect microglia, sections were then incubated overnight at 4°C 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 4°C. For NG2 staining, frozen sections were blocked with 0.1% Triton X-100/5% normal goat serum in potassium-phosphate-buffered saline (KPBS) for 1 hr at room temperature. Sections were then incubated with anti-NG2 antibody (1:500 in blocking solution) or isotype control overnight at 4°C.

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 hr at room temperature. Sections were then rinsed and mounted with Vectasheild medium containing the nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). An Olympus (Melville, NY) BX40 microscope, Olympus DP70 digital camera and Image-ProPlus software (Media Cybernetics, Silver Spring, MD) were used to obtain images. All comparative analyses were focused in the median corpus callosum in a region of 0.21 mm wide and 0.15–0.30 mm high (the corpus callosum area becomes larger with infiltrating cells during demyelination). Positively stained cells were counted only if a nucleus was observed and were quantified per square millimeter.

Detection of Apoptotic Oligodendrocytes

Apoptosis of oligodendrocytes in the corpus callosum was detected by labeling DNA strand breaks by terminal de-

oxynucleotidyl transferase (TUNEL reaction) colocalized with GSTpi immunostaining as a marker for mature oligodendrocytes. GSTpi immunohistochemistry was performed as described above, followed by detection of apoptotic nuclei using the In Situ Cell Death Detection Kit, Fluorescein (catalog No. 11 684 795 910; Roche Diagnostics, Mannheim, Germany) with a 1-hr incubation at 37°C in TUNEL reaction mixture prepared as described in the manufacturer's protocol except for a 1:3 dilution of Enzyme solution in TUNEL Dilution Buffer (catalog No. 11 966 006 001; Roche Diagnostics).

Electron Microscopy

Ultrathin sections were cut and stained with lead citrate and uranyl acetate, and electron micrographs were obtained with a Zeiss EM910 transmission electron microscope with a digital camera at the Microscopy Services Laboratory in the UNC Department of Pathology and Laboratory Medicine. More than 500 axons (0.3 μ m or greater diameter) from each animal were counted as myelinated or unmyelinated, and the percentage of myelinated axons from among total axons is given as percentage myelination.

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 (1922), as follows: proestrus = mostly smooth nucleated epithelial cells, estrus = cornified epithelial cells, diestrus = mostly leukocytes, metestrus = all three cell types.

Serum 17 β -Estradiol Measurement

Serum was obtained from whole-blood samples by centrifugation after removal of blood clot and stored at -80°C until use. 17 β -Estradiol was measured by radioimmunoassay (RIA) using the Double Antibody Estradiol Kit (catalog No. KE2D5; Seimens, Los Angeles, CA) and following the manufacturer's protocol.

Statistical Analysis

Statistical comparisons were made by one-way or two-way ANOVA with Bonferroni posttest or by Student's *t*-test, as indicated in the figure legends. The data are expressed as mean $\pm$ SEM.

RESULTS

Female C57BL/6J Mice Demyelinate and Remyelinate to an Extent Similar to That of Male Counterparts

Five weeks of cuprizone treatment is known to produce maximum demyelination in the midline corpus callosum of male C57BL/6 mice (Matsushima and Morrell, 2001), and remyelination begins by week 6 of cuprizone treatment (Gao et al., 2000; Mason et al., 2001; Irvine and Blakemore, 2006). Remyelination continues if cuprizone treatment is withdrawn after week 6 (Arnett et al., 2001; Mason et al., 2001). Our first objective was

Fig. 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 $\times 40$. **B:** Myelination scores were obtained by blinded scoring of the 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 5 wk (as depicted in A). Mean and SEM bars are plotted for 10–16 animals per sex at each time point. Scale bar = 50 μ m.

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 were treated for 6 weeks followed by 1 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 microscopic or electron microscopic 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 (Fig. 1A, untreated), and unmyelinated fibers appear pink (Fig. 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, although this difference was not statistically significant (Fig. 1A,B). 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, at which the mice have been off the cuprizone diet for 1 week, partial remyelination

occurred similarly, with no statistical difference between males and females (Fig. 1A,B).

To obtain a more accurate and quantitative measure of myelination, we analyzed cross-sections of corpus callosum axons by electron microscopy. For each animal, more 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 2A,B confirm the light microscopic data in Figure 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.

Female C57BL/6 Exhibit No Differences in Mature Oligodendrocyte Loss Compared With Male Counterparts

Depletion of mature oligodendrocytes is a feature of cuprizone-induced demyelination in male C57BL/6 mice (Mason et al., 2000). 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 (Fig. 3A,B). 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 (Fig. 3A,B) as well as the number of apoptotic oligodendrocytes (Fig. 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 (Fig. 3A,B). In keeping with this result, no difference was found in the number of NG2-positive oligodendrocyte precursor cells between male and female mice during demyelination (data not shown).

Female C57BL/6 Exhibit No Difference in the Accumulation of Microglia Compared With Male Counterparts

Microglia accumulate in the corpus callosum, presumably to clear debris during demyelination and to provide trophic factors that support remyelination. Histochemical staining with the lectin RCA-1 (a marker for microglia/macrophage) was performed on paraffin-embedded brain sections. The numbers 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 untreated male and female mice, and the numbers of microglia are similar between

Fig. 2. Myelinated and demyelinated axons in male and female C57BL/6 mice. **A:** Representative electron micrographs of corpus callosum axons during cuprizone treatment. Magnification $\times 10,000$. **B:** Percentages of myelinated axons in corpus callosum were obtained from electron micrographs by counting greater than 500 axons per animal. Mean and SEM bars for percentage of total axons that are myelinated are plotted for 8–12 animals per sex at each time point. Scale bar = 2 μ m.

the sexes (Fig. 4A,B). 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 (Fig. 4).

Female C57BL/6 Exhibit No Difference in the Accumulation of Astrocytes Compared With Male Counterparts

Astrocytes are present at low numbers in normal adult mice and accumulate in the corpus callosum similarly 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 (Fig. 5A,B). During demyelination, astrocyte numbers increase at week 3 and reach a peak by week 5. There were no significant sex differences in

Fig. 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 $\times 40$. **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 were plotted for 12 animals per sex after 3 weeks of cuprizone administration. Scale bar = 50 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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 (Fig. 5). However, these differences did not reach statistical significance.

Estrogen Receptor-Deficient Mice Demyelinate and Remyelinate Similarly to Wild-Type Controls

Both forms of the estrogen receptor, ER α and ER β , have been detected in neuronal populations in various brain regions of rodents (Sheng et al., 2004; Mehra et al., 2005) and also in astrocytes (Azcoitia et al., 1999; Cardona-Gomez et al., 2000; Milner et al., 2001; Platania et al., 2003), oligodendrocytes (Arvanitis et al., 2004; Zhang et al., 2004; Hirahara et al., 2009), and microglia (Garcia-Ovejero et al., 2002; Takahashi et al., 2004; Sierra et al., 2008). In an effort to identify a role for these receptors in demyelination and remyelination, we administered cuprizone to both male and female mice deficient in either receptor (α ERKO and β ERKO) alongside their respective wild-type littermate controls (α ERWT and β ERWT). We found that, after 3 or 5 weeks of cuprizone treatment, for both males and females, demyelination was similar between α ERKO and α ERWT (Fig. 6A,C) and between β ERKO and

Fig. 4. Accumulation of microglia in the corpus callosum in 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 $\times 40$. **B:** Quantification of RCA-1⁺ microglia/macrophages in the corpus callosum. Mean RCA-1⁺ cells per square millimeter and SEM bars were plotted for 10–16 animals per sex at each time point. Scale bar = 50 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

β ERWT (Fig. 6B,D). Remyelination after 1 or 2 weeks off of the cuprizone diet also proceeded without significant differences between knockout and wild-type mice for both receptors, and this was true of both sexes (Fig. 6). In addition, for both males and females, the number of mature oligodendrocytes did not differ between α ERKO and α ERWT (Fig. 7A,C) or between β ERKO and β ERWT males or females (Fig. 7B,D) during demyelination or remyelination. Therefore, neither form of the estrogen receptor appears to play a major role in cuprizone-induced demyelination or remyelination.

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 (Hiremath et al., 1998). 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 (Becker et al., 2005). 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 4–5-day

Fig. 5. Accumulation of astrocytes in the corpus callosum in 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 $\times 40$. **B:** Quantification of GFAP⁺ astrocytes in the corpus callosum. Mean GFAP⁺ cells per square millimeter and SEM bars were plotted for 10–16 animals per sex at each time point. Scale bar = 50 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

estrous cycle the mouse is undergoing (Fig. 8A, top). We began by monitoring the 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. Mice were included in the study only if they displayed a 4–6-day estrous cycle, which is typical for C57BL/6 mice (Lohff et al., 2005; Spencer et al., 2008).

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 with samples from untreated mice that were monitored concurrently. The data are plotted in Figure 8B, 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 normally cycling mouse, an estrus phase should occur every 4–6 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, cuprizone-treated mice exhibited a dramatic change in estrous cyclicity and appeared to enter an extended diestrus-like phase. This is demonstrated by

Fig. 6. Cuprizone-induced demyelination and remyelination in male and female α ERKO/ α ERWT and β ERKO/ β ERWT mice. Myelination scores obtained by blinded scoring of midline corpus callosum sections stained with LFB-PAS for α ERKO and α ERWT males (A) and females (C) and β ERKO and β ERWT males (B) and females (D). Mean and SEM bars were plotted for 4–12 animals per group at each time point. Two-way ANOVA indicate no significant differences.

representative vaginal cell samples in the bottom panel of Figure 8A and the drop in number of estrus phases per week as shown in Figure 8B. 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 of 13 mice, and nearly all of the samples displayed features similar to those shown in the bottom panel of Figure 8A.

Upon removal of cuprizone from the diet, female mice began displaying an estrus phase within the first 1–2 weeks, and cycles became regular by week 3 or 4 off the cuprizone diet (Fig. 8B). We presume this ability to recover estrous cycling indicates that the reproductive tissue was not irreversibly damaged by cuprizone toxicity.

Reproductive Tissues of Male and 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 (White et al., 1978; Ji et al., 2008). Furthermore, uterine weight is often used as a bioassay of estrogenic activity (Lubahn et al., 1993; Owens and Koeter, 2003). The wet weights of uteri plus 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 (Fig. 8B). 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 plus ovary weights, with average weights showing a two- to threefold reduction compared with weights in untreated females (Fig. 9A). Four weeks after cuprizone removal from the diet, uterine plus ovary wet weights have returned to levels equivalent to those of untreated mice, mirroring the return of estrous cyclicity shown in Figure 8B. An analysis of the uteri and ovaries weighed separately indicated that both the uterus (Fig. 9B) and the ovaries (Fig. 9C) of mice are significantly diminished in weight after 5 weeks of cuprizone treatment ($*P < 0.01$). These observations of disrupted estrous cyclicity and reduced uterine weight suggest that circulating hormone levels may not be normal in cuprizone-treated mice. To determine whether the male reproductive axis might also be affected by cuprizone, wet weights were

Fig. 7. Cuprizone-induced loss of oligodendrocytes and subsequent repopulation during recovery in corpus callosum of male and female α ERKO/ α ERWT and β ERKO/ β ERWT mice. Myelination scores were obtained by blinded scoring of midline corpus callosum sections stained with LFB-PAS for α ERKO and α ERWT males (A) and females (C) and β ERKO and β ERWT males (B) and females (D). Mean and SEM bars were plotted for 4–12 animals per group at each time point. Two-way ANOVA indicate no significant differences.

Fig. 8. Cuprizone treatment reduces the number of estrus phases compared with 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 $\times 40$. **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 in untreated mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with untreated by two-way ANOVA and Bonferroni posttest. Scale bars = 50 μ m.

Fig. 9. Cuprizone treatment results in diminished weights of sex organs. **A:** Wet weights of uteri plus ovaries obtained from untreated (5 wk UT, circles) and cuprizone-treated female mice at 5 weeks (5 wk cuprizone, squares) as well as 10 weeks untreated (10 wk UT, circles) or mice that were treated with cuprizone for 6 weeks followed by 4 weeks of recovery with a normal diet (6 + 4 wk recov, triangles). Sex organ weights are significantly diminished by 5 weeks of cuprizone treatment but return to untreated weights 4 weeks after removal of cuprizone from the diet ($*P < 0.001$ by one-way ANOVA and Bonferroni's posttest; nsd, no significant difference, $P > 0.05$). Wet weights of uteri (**B**) or ovaries (**C**) by themselves both

indicate a significant difference between untreated and 5 weeks of cuprizone treatment by Student's *t*-test ($*P < 0.01$). **D:** Wet weights of testes from 5 week untreated (5 wk UT, circles), 5 week cuprizone-treated (squares), and remyelinating (6 + 1 wk recov, triangles) mice, which were treated with cuprizone for 6 weeks followed by 1 week of normal diet. Similarly to the female gonads, testes weights were diminished by cuprizone treatment but recovered after removal of cuprizone from the diet (one-way ANOVA and Bonferroni's posttest, $*P < 0.01$). Seminal vesicles (**E**) and coagulating glands (**F**) were also diminished in weight by cuprizone administration (Student's *t*-test, $*P < 0.001$).

obtained for testes, prostate, seminal vesicles, coagulating gland, and epididymis. Testes weights were diminished after 5 weeks of cuprizone administration compared with the age-matched control that did not receive cuprizone (Fig. 9D). Similar to the recovery seen in female mice, testes weights were not significantly different from untreated just 1 week after removal of cuprizone from the diet. In addition, it was found that seminal vesicle and coagulating gland weights were diminished by cuprizone administration (Fig. 9E,F). Prostate and epididymis weights were not significantly altered by cuprizone (data not shown).

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 (Ludwin, 1978; Matsushima and Morell, 2001). 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 documented. We report here that, similarly to male C57BL/6 mice, females develop extensive demyelination by week 5 of cuprizone treatment (Fig. 1). Although there was a trend for less severe demyelination at week 3 in female mice, as detected by LFB-PAS staining, the difference was not statistically significant. In addition, analyses at the electron microscopic level indicated no differences in demyelination or remyelination between female and male mice (Fig. 2). Similarly, mature oligodendrocyte depletion and recovery were the same between females and males (Fig. 3), and this was true of oligodendrocyte precursor populations that accumulated during demyelination. 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. In addition, sex organ weights were diminished in both male and female mice. Thus, the measures of demyelination and remyelination used in this study do not reveal differences between males and females in the C57BL/6 mouse strain.

The lack of sex difference in cuprizone-induced demyelination in the C57BL/6 strain is in contrast to the SJL strain, insofar as we have recently shown that female SJL mice display less severe demyelination and loss of oligodendrocytes compared with male counterparts (Taylor et al., 2009). Sex differences have been characterized in other rodent models of demyelinating disease, particularly EAE. Most studies of sexual dimorphism in EAE are carried out in the SJL mouse strain, in which females show an increase in susceptibility and severity of disease (Bebo et al., 1996; Voskuhl et al., 1996; Papenfuss et al., 2004; Teuscher et al., 2004). However, in other strains, males exhibit increased susceptibility (B10.PL and PL/J) or no sex differences (NOD/lt and C57BL/6) (Papenfuss et al., 2004). Likewise, the TMEV model displays sex differences that vary by strain. An early study in the C57L/J mouse strain demonstrated that female mice are completely resistant to TMEV-induced demyelinating disease (Kappel et al., 1990). This was later shown to be due to a more robust antiviral response to the initial infection by female mice (Fuller et al., 2005). In the SJL mouse strain, both males and females are susceptible, but male mice demonstrate more severe neurological deficits (Alley et al., 2003).

Sex may also play a role in remyelination, as demonstrated by Li and colleagues (2006). In that study, ethidium bromide was used to induce death of oligodendrocytes in young and old male and female adult rats. Remyelination was less efficient in older animals. Whereas 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 whether sex differences in cuprizone-induced disease occur in older mice.

An important finding in this study is that cuprizone intoxication disrupts estrous cyclicity and leads to diminished weights of sex organs in male and female mice. To our knowledge, this is the first indication that the estrous cycle or sex organs are affected during cuprizone treatment and would suggest that circulating sex hormone levels are not normal in treated female or male mice. We do not know whether the circulating estrogen levels were in fact altered by cuprizone treatment, because we were unable to detect estradiol by RIA in serum from normal untreated females or in cuprizone-treated females. It appears, based on wet weight measurements of sex organs in male mice, that cuprizone may also have a detrimental effect on male reproductive tissue. These effects are consistent with reduced androgen levels (Zenick et al., 1994). It is plausible that cuprizone directly affects gonadal tissue, the neuroendocrine centers

that regulate gonadal tissue, or it may alter steroid hormone biosynthesis or metabolism. Alternatively, the weight loss that occurs during cuprizone administration may be responsible for the loss of estrous cyclicity. It has been shown that dietary restriction can cause acyclicity in female C57BL/6 mice (Nelson et al., 1985). In this study, diet-restricted mice had body weights 80–90% compared with *ad libitum* fed controls, and 30% of the mice stopped cycling after 1 month of restricted diet and 80% after 2 months. In our study, five weeks of cuprizone administration led to body weights approximately 67% of untreated control weights (data not shown); however, the majority of mice stopped cycling within 2 weeks, at which point body weight data were not obtained. Therefore, it is likely that weight loss contributes to the disruption of the estrous cycle during cuprizone administration, but there may be additional toxic effects of the cuprizone itself. In addition, male mice treated with cuprizone for 5 weeks had body weights approximately 78% of untreated control weights, so it is possible that this weight loss/lack of weight gain could also be contributing to the effect on male sex organs.

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 up-regulated after brain injury (for review see Garcia-Ovejero et al., 2005). In the future, it would be of interest to measure the levels of circulating steroid and trophic hormones to assess hormone status more completely and, if specific changes are detected, to determine whether they vary with mouse strain related to cuprizone treatment. This is especially important in view of our findings that sex differences occur in cuprizone-induced demyelination in SJL mice (Taylor et al., 2009). Interestingly, strain differences in estrogen responsiveness have been reported to occur in reproductive tissues, indicating a genetic basis for strain-specific variation in hormone responsiveness (Griffith et al., 1997; Roper et al., 1999). These data suggest that future studies of sex differences in demyelination and remyelination using the C57BL/6 mouse strain may require hormone replacement strategies and/or analysis of mechanisms of hormone metabolism.

Because 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. The treatment of estrogen receptor knockout mice with cuprizone also indicates that estrogen receptor signaling does not play a major role in demyelination or remyelination, insofar as there were no significant differences between knockouts and wild-type controls of either sex. However, in light of the evidence that cuprizone disrupts estrous cyclicity and alters ovarian and uterine weights, it is possible that estrogen receptors do not have access to normal concentrations of circulating estrogen. It is possible that administration of estrogens or other

estrogen receptor ligands might reveal a role for estrogen receptors in the pathogenesis of cuprizone-induced demyelination or remyelination. In support of this idea, ongoing studies from our laboratory suggest that administering high doses of estrogen can lead to protection from cuprizone-induced demyelination, which may be dependent on estrogen receptor function (unpublished data). This point is also relevant to the potential use of tamoxifen for inducible gene expression in transgenic mouse lines. As an estrogen receptor antagonist/partial agonist, tamoxifen may have effects on demyelination/remyelination. Thus, it would be important to include the proper controls in comparisons of tamoxifen treated with untreated transgenic mice in cuprizone studies. Our findings also show that developmental loss of ER action does not influence the number of oligodendrocytes present in the corpus callosum, insofar as no difference was observed in the untreated knockout mice compared with wild-type littermates. One potential caveat is a possible compensation of one receptor for the other in these studies. Unfortunately, the need to breed these mice as heterozygotes makes it quite difficult to generate adequate numbers of double knockout mice for experiments.

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 oligodendrocyte precursors, microglia, and astrocytes in female mice suggest a lack of inherent differences in these cells in comparison with 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 an influence on reproductive tissue, manifested as reduced reproductive tissue weights and, in females, disrupted estrous cycling. Furthermore, the administration of cuprizone to estrogen receptor knockout mice indicates that there is not a significant role for either ER α or ER β under the condition of endogenous (potentially disrupted) hormone levels in this model of demyelination and remyelination.

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