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Sex Chromosome Complement Influences Functional Callosal Myelination

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Abstract

In addition to androgen differences between males and females, there are genetic differences that are caused by unequal dosage of sex chromosome genes. Using the cuprizone-induced demyelination model, we recently showed that surgical gonadectomy of adult mice resulted in decreased normal myelination and remyelination compared to gonadally intact animals, suggesting a supporting role for sex hormones in the maintenance of myelination. However, inherent sex differences in normal myelination and remyelination persisted even after gonadectomy, with males consistently remyelinating to a lesser extent relative to normal myelination as assayed by axon conduction and immunohistochemistry. This suggests a potential role for the sex chromosome complement in mediating the differential rates of remyelination observed in males and females. The present study focuses on the impact that sex chromosomes might have on these myelination differences. Making use of the four core-genotype mice and cuprizone-diet induced demyelination/remyelination paradigm, our results demonstrate sex chromosome mediated asymmetry between XX and XY mice. The rate of functional remyelination following cuprizone diet-induced callosal demyelination in four core-genotype mice is attenuated in XY compared to XX animals of both gonadal sexes. Importantly, this difference arises only in the absence of circulating sex hormones following gonadectomy and confirms the role of sex hormones in the remyelination process reported earlier by our group. Because a genotype-mediated difference only arises following gonadectomy, the chromosomal contribution to myelination and remyelination is subtle yet significant. To explain this difference, we propose a possible asymmetry in expression of myelination-related genes in XX versus XY mice that needs to be investigated in future studies.

INTRODUCTION

Autoimmune diseases like systemic lupus erythematosus, Sjogren's syndrome, primary biliary cirrhosis, Hashimoto's thyroiditis, rheumatoid arthritis, and multiple sclerosis (MS) have increased incidence and prevalence in females which has been attributed to a more robust immune response as compared to males (Whitacre, 2001). Interestingly, females with

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MS have a better prognosis than males (Bergamaschi, 2007) and are more likely to have a benign course of the disease. Sex difference in prevalence of MS can be attributed to effects of sex hormones, sex chromosomes or a combination of both on the immune system or the central nervous system. The role of circulating sex hormones on the immune system as a potential mechanism of disease discrepancy has been studied extensively (Shuster, 2008, Greer and McCombe, 2011, Voskuhl, 2011), but specifically sex difference in central nervous system demyelination and remyelination is lacking.

Corpus callosum is the largest white matter tract and sexual dimorphism in areas of the corpus callosum has been studied in rodents (Berrebi et al., 1988, Mack et al., 1995, Bishop and Wahlsten, 1997, Nunez and Juraska, 1998, Yang et al., 2008), cats (Tan, 1993), dogs (Aydinlioglu et al., 2000), primates (Holloway and Heilbroner, 1992, Franklin et al., 2000) and adult humans (DeLacoste-Utamsing and Holloway, 1982, Allen et al., 1991, Sullivan et al., 2001, Perrin et al., 2008, Ardekani et al., 2012), although there is considerable controversy over the differences observed (Weber and Weis, 1986, Byne et al., 1988, Constant and Ruther, 1996, Bishop and Wahlsten, 1997). Recently, we investigated the role of sex hormones on corpus callosum axon conduction and myelination using the cuprizone diet-induced demyelination/remyelination mouse model (Patel et al., 2013). Cuprizone (*biscyclohexanone oxaldi-hydrazone*) ingestion in mice kills mature oligodendrocytes. This results in axon demyelination of distinct brain regions including the corpus callosum, the most frequently investigated white matter tract in this animal model (Stidworthy et al., 2003, Patel et al., 2013). Replacement of cuprizone diet with normal diet resulted in an increase in oligodendrocytes and axon remyelination. As demyelination and remyelination are induced in a highly reproducible manner within this model, we compared effects of hormone deficiency and hormone replacement during normal myelination, demyelination, and remyelination (Matsushima and Morell, 2001), (Patel et al., 2013). Our study revealed distinct sex differences during the normal myelination, demyelination and remyelination phases (Patel et al., 2013) and could be attributed to circulating sex hormones. Interestingly, differences between adult male and female animals in the remyelination phase persisted after gonadectomy, when circulating sex hormones were absent. That sex differences persisted in the absence of circulating sex hormones suggests a more fundamental, intrinsic level of distinction between male and female. Apart from the organizational influence of sex hormones during development, one possible avenue of differentiation between males and females is genotype, namely the sex chromosome complements XX or XY.

Gonadectomy in the adult phase permits the study of real-time regulatory effects of sex hormones in disease models, but it cannot control for differences in male and female brain development that occur prior to gonadectomy. Fortunately, there exists a line of transgenic mice which permits the study of chromosomal variations independently of gonadal sex: the four core genotype model. The sex-determining region (SRY) is deleted from the Y chromosome and added onto an autosome (Mahadevaiah et al., 1998) in the four core-genotype model. This manipulation creates four distinct genotypes of mice: XY⁻Sry and XXSry (male), and XY⁻ and XX (female). Because sex-matched animals differ only by genotype, the four core-genotype line permits investigation of the chromosomal contribution to biological phenomena (De Vries et al., 2002).

In the present study, we employ the four core-genotype mouse line to investigate the role of the sex chromosome complement on the rate of functional remyelination within the reproducible cuprizone-diet induced demyelination model (Matsushima and Morell, 2001, Crawford et al., 2009a, Crawford et al., 2009b). The mechanisms underlying sex differences in disease incidences will help researchers design and develop novel therapeutic treatments.

EXPERIMENTAL PROCEDURES

Animals

C57Bl/6 four core genotype (4CG) mice (12–16 per genotype, per treatment group) ages 7–9 weeks were either surgically gonadectomized or given sham surgery (Figure 1A). MF1Y⁻*Sry* mice are outbred mice that have a mutation in the *Sry* gene on the Y chromosome, and to which a transgenic *Sry* gene has been added back on an autosome. MF1Y⁻*Sry* mice were bred and back crossed onto the C57Bl/6 background (Smith-Bouvier et al., 2008). Gonadectomies were carried out in 8 wks old mice. Ovariectomy and castration were performed as described in (Palaszynski et al., 2005). All procedures were conducted in accordance with the National Institutes of Health (NIH) and were approved by the Animal Care and Use Committee of UCLA.

Genotyping

four core-genotype mice were genotyped according to Palaszynski et al (Palaszynski et al., 2005). Tail samples were obtained from mice and genomic DNA was isolated using the DNeasy tissue kit (QIAGEN, Valencia, CA). The *Ssty1* multicopy gene on the Y chromosome was amplified (primers: YMTFP1, CTG GAG CTC TAC AGT GAT GA; YMTRP1, CAG TTA CCA ATC AAC ACA TCA C) to test for the presence of the Y⁻ chromosome; the autosomal gene *Myogenin* [primers MYOF (forward): TTA CGT CCA TCG TGG ACA GCA T; MYOR (reverse): TGG GCT GGG TGT TAG TCT TAT] served as an amplification control. Amplification of DNA from XY⁻ females and XY⁻*Sry* males mice yielded the 245-bp *myogenin* product and the 342-bp *Ssty1* product, whereas amplification of DNA from XX females and XX*Sry* males yielded only the 245-bp *myogenin* product.

Cuprizone diet-treatment groups

Intact and gonadectomized mice were randomized into one of two groups: one group (normal myelination, NM) received three weeks of *ad libitum* chow diet and water; the other group (demyelination, DM) received three weeks of *ad libitum* 0.2% cuprizone diet and water. At the conclusion of three weeks, half of the DM group was sacrificed for assays and the other half returned to *ad libitum* chow diet (remyelination, RM) (Figure 1B).

Electrophysiological Compound Action Potential (CAP) recording procedures

As described previously (Crawford et al., 2009a, Crawford et al., 2009b), mice were deeply anesthetized using isoflurane and decapitated. The brain was removed and vibratome (Leica, Germany) sectioned into 400 µm coronal sections while submerged in ice-cold carbogen (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF, in mM NaCl 124, KCl 5, NaH₂PO₄ 1.25, NaHCO₃ 26, MgSO₄ 1.3, CaCl₂ 2, glucose 10; adjusted to pH 7). Slices were incubated for at least one hour in room-temperature ACSF. Two slices per animal containing the hippocampus (plates 35–48, Paxinos and Franklin mouse brain atlas) were used for electrophysiology recordings of CAP, conduction velocity, and refractoriness. The corpus callosum was stimulated at 1mm from midline using a tungsten bipolar electrode; responses were measured 2mm (CAP and refractoriness; distances varies for velocity recordings) from the stimulating electrode with a 3M NaCl-filled glass micropipette recording electrode. Field potentials recorded through the recording electrode were routed through an Axopatch 2a amplifier (Axon Instruments) to Clampex software. Waveforms were recorded and analyzed offline with Microcal Origin software (Figure 1C).

Histopathology, Immunohistochemistry and Electron Microscopy

(a) Perfused fixed brain slices: Mice were deeply anesthetized with isoflurane and intracardially perfused with phosphate-buffered saline (PBS) followed by either 4% paraformaldehyde solution (for immunohistochemistry-IHC) or 2% paraformaldehyde + 2% glutaraldehyde solution (for electron microscopy-EM) (Crawford et al., 2009b). The brain and spinal cord were removed, post-fixed in paraformaldehyde, cryoprotected in a 30% sucrose solution and embedded in gelatin (which was also subsequently post-fixed and cryoprotected). 40 μ m coronal brain sections were cut at the fornix region of the corpus callosum as previously described and placed in 24-well plates in PBS. (b) Post-fixed CAP-recorded brain slices: After recording, these slices were placed directly in 4% paraformaldehyde for 12 hours and then transferred to sucrose. The slices were carefully embedded in gelatin and post-fixed, cryoprotected, and cut. Corpus callosum blocks from a few animals were also post-fixed in 2% paraformaldehyde + 2% glutaraldehyde solution for electron microscopy studies. Paraformaldehyde -fixed slices were washed and permeabilized with 1–2 % TritonX-100 detergent in the presence of 2% NGS and blocked with 15–20% NGS. Consecutive sections were examined by immunohistochemistry (Crawford et al., 2009) and various series of cell type-specific antibodies were used by double or triple immunolabeling. White matter immunostaining was enhanced by treating sections with 95% ethanol/5% acetic acid for 30 minutes prior to permeabilization and blocking. To detect axons: anti-neurofilament-NF200 (Sigma), proliferating oligodendrocyte progenitors: anti-olig2 + anti-Ki67 (Millipore), mature oligodendrocytes : anti-CC1 (adenomatous polyposis coli gene) (Genetex), and myelin: anti-myelin basic protein-MBP (Sigma) were used. The secondary antibody step was performed by labeling with antibodies conjugated to TRITC and Cy5 (Vector Labs and Millipore). IgG-control experiments were performed for all primary antibodies, and no staining was observed under these conditions. To assess the number of cells, a nuclear stain 4',6-Diamidino-2-phenylindole, DAPI (2ng/ml; Molecular Probes) was added for 15 minutes prior to final washes after secondary antibody addition. Sections were mounted on slides, allowed to dry, and coverslipped using fluoromount G (Fisher Scientific). For electron microscopy studies, brains were cut sagittally, the genu area of the corpus callosum according to Paxinos and Franklin was identified under a dissecting microscope, and 4 mm² blocks were carefully dissected. These blocks were further cut in 1 mm² sections for Epon embedding and processed according to Crawford et al (2009a). Animals intended for electron microscopy analysis were treated simultaneously with animals intended for immunohistochemistry.

Microscopy and Quantification

Stained sections were examined and photographed using a spin disc confocal fluorescent microscope (BX62 DSU; Olympus, Tokyo, Japan) equipped with Plan Fluor objectives connected to a camera (Hamamatsu Orca). Digital images were collected and analyzed using SlideBook and Image J (NIH). Image intensities were adjusted evenly for each set and were rotated, cropped and assembled using Adobe Photoshop (Adobe Systems, San Jose, CA). Images were captured under the microscope (10 $\times$ or 40 $\times$) with identical light intensity and exposure times from each experimental set. Images from the same areas of corpus callosum were acquired separately from delineated whole gray and white matter regions. All images were converted to grayscale and then analyzed by density measurement with ImageJ v1.29 (the Windows version of NIH ImageJ), downloaded from <http://rsb.info.nih.gov/ij>. A fixed threshold range of 0 to 160 was chosen to highlight the staining signals in normal corpus callosum, and the total area within this range was measured, averaged, compared and presented per mm², except where data was subsequently normalized.

Statistical Analysis

To quantify immunostaining results, we used 2 sections (about 400 μ m apart)/ mouse from electrophysiology-recorded brains and 2 sections / mouse from perfused-fixed brains. There were n=4 mice from electrophysiology + n=4 mice that were perfused fixed, with a total of n=8 mice/per treatment group, for a total of 16 sections per treatment group that were immunostained. To quantify electrophysiology results from each treatment group: recordings from 2 caudal slices $\times$ 4 animals $\times$ 2 experiments = a total of 16 recordings were analyzed. For electron microscopy, 10 random caudal area fields/animal at $\times$ 4,800 and $\times$ 14,000 were used to quantify the “g-ratio”. Values are expressed mean $\pm$ SEM. Statistical analysis of mean values was carried out using 2 way ANOVA (Sex $\times$ Genotype) Bonferroni posttests. Differences were considered significant at the * $p < 0.05$ level, unless stated otherwise. The group sizes used for all statistical comparisons of morphometric determinations were n=8 subjects per experimental group/experiment and the experiment was repeated. For electron microscopy analysis, more than 300 axons from n=4 subjects/ experimental group from 2 separate experiments were measured. Statistics were performed using Microcal Origin or Prism 4 (Prism®, GraphPad San Diego, CA) program for Windows.

RESULTS

Callosal axon myelination and conduction in gonadally intact four core-genotype mice: Similar level of demyelination followed by remyelination in XX and XY mice

To characterize normal myelination, cuprizone-induced demyelination, and the effectiveness of remyelination during recovery from cuprizone diet in gonadally intact four core-genotype mice groups, corpus callosum electrophysiology compound action potential (CAP) recordings were performed. CAP recordings of gonadally intact males (XXSry, XY-Sry) and females (XX, XY-) under the normal myelinating condition presented two downward phases of the compound action potentials, 'N1' and 'N2' (Figure 2A, (Crawford et al., 2009a)). The N1 CAP amplitudes represent fast depolarization from mostly large, myelinated axons and N2 CAP amplitudes represent slower depolarization from non-myelinated axons. Axon dysfunction can be estimated by assaying changes in axon refractoriness, which is indicative of reduced excitability following an action potential. In addition, conduction velocity measurements are functional indicators of myelination-induced changes in axon conduction (Crawford et al., 2009a, Crawford et al., 2009b). No differences in N1 CAP amplitude, axon refractoriness, or conduction velocity were observed between XX and XY- females. XXSry males showed a small but significant increase in N1 CAP amplitude as compared to XY-Sry males ($p < 0.05$; 2 way ANOVA (Sex $\times$ Genotype) Bonferroni posttests, n=8; Figure 2Ai-iv). However, no differences in axon refractoriness or conduction velocity were observed between XXSry and XY-Sry male groups (Figure 2Av-vi and Table 1).

Following 3 weeks of *ad libitum* 0.2% cuprizone diet, extensive corpus callosum demyelination was observed. As a consequence, there was a significant decrease in male and female N1 CAP amplitudes to a similar level (Figure 2Bi-ii). Because of the negligible N1 amplitude axon refractoriness and conduction, velocity measurements were not possible in the DM groups. A small but significant recovery in N1 amplitude of males and females was observed under remyelinating condition induced by 3 weeks of *ad libitum* 0.2% cuprizone diet-induced demyelination followed by 3 weeks of the normal diet (Figure 2Ci-iv). Similarly, no differences in axon refractoriness or conduction velocity were observed between genotypes in males or females (Figure 2Cv-vi).

Immunohistological analysis of myelination in gonadally intact four core-genotype mice: No sex genotype-mediated difference following demyelination and remyelination

Brain slices were immunostained to identify the status of myelin basic protein (MBP), mature oligodendrocyte, and oligodendrocyte progenitors in the four core-genotype mice (Figure 3A–B). Paraformaldehyde-fixed coronal brain slices were treated with primary and secondary antibody and imaged for fluorescence intensity or cell counting of the corpus callosum by confocal microscopy. In gonadally intact normal myelinating groups, myelin basic protein (MBP) staining intensity was significantly lower in XX females compared to XY- females as well as in XXSry males compared to XY-Sry males ($p < 0.05$, 2 way ANOVA (Sex $\times$ Genotype) Bonferroni posttests; $n=8$). Overall average MBP intensity significantly decreased in demyelinating conditions compared to normal myelinating conditions, but with no difference between XX and XY in males or females. Overall average intensity in the remyelinating condition significantly recovered compared to demyelination, but not to normal myelinating levels, with no differences between genotypes (Figure 3A–B).

Mature myelinating oligodendrocytes were immunostained with CC1 (aenomatous polyposis coli gene) antibody (Figure 3C-inset). Even though it may weakly stain other cell types such as astrocytes and Schwann cells (Bhat et al., 1996), CC1 staining is specific for mature oligodendrocytes. This was confirmed by co-staining with another mature oligodendrocyte marker, GST-pi. Nearly 98% of GST-pi cells stained with CC1. No differences were observed in the number of CC1-positive mature oligodendrocyte cells among genotypes under normal myelinating conditions. There was a significant decrease in the numbers of oligodendrocytes under demyelinating conditions that recovered significantly during remyelination. There was no difference in number of cells among genotypes under all conditions (Figure 3C).

To identify proliferating oligodendrocyte progenitors, brain sections were immunostained with olig2 and Ki67 antibody and imaged to count double positive (olig2⁺+Ki67⁺) oligodendrocyte progenitors (Figure 3D-inset). *Olig2* is a bHLH transcription factor that is strongly up-regulated in oligodendrocyte progenitors (Fancy et al., 2004). Ki-67, a proliferation marker is a protein present during all active phases of the cell cycle (G₁, S, G₂, and mitosis) but is absent from resting (G₀) cells (Scholzen and Gerdes, 2000). In the gonadally intact normal myelinating group, XY-Sry and XY- both showed elevated oligodendrocyte progenitor counts compared to XXSry and XX, respectively. During demyelination, oligodendrocyte progenitor numbers declined on average compared to normal myelination, but there were no differences between genotypes. During remyelinating conditions, oligodendrocyte progenitor numbers recovered significantly above demyelination but again with no significant differences between groups (Figure 3D).

Callosal axon myelination and conduction in the four core-genotype mice: Gonadectomized four core-genotype mice unveil influence of sex chromosome complement during remyelination

To investigate the possible influence of sex chromosome complement in axon conduction during normal myelinating, demyelinating, and remyelinating conditions, age and sex matched four core-genotype mice were gonadectomized and subjected to cuprizone diet induced demyelination followed by remyelination. A lower N1 amplitude under normal myelinating condition for gonadectomized XXSry and XY-Sry males was observed as compared to intact XXSry and XY-Sry males (Figure 4Ai, iii, Figure 2Ai, iii, Table 1). Similar to gonadally intact animals, significant increase in N1 amplitude of XXSry males over XY-Sry males persisted in gonadectomized mice ($p < 0.05$, 2 way ANOVA (Sex $\times$ Genotype) Bonferroni posttests; $n=8$). In contrast N1 amplitudes of XX and XY- females under normal myelinating conditions did not show a significant difference between

gonadectomized and intact groups (Figure 4Aii, iv and Figure 2Aii, iv). There was also no difference in the level of N1 amplitudes between gonadectomized XX and XY- females (Figure 4Aii, iv). No differences in axon refractoriness or conduction velocity were observed under normal myelinating conditions between males (XXSry and XY-Sry) and females (XX and XY-) (Figure 4A and Table 1).

N1 components were significantly suppressed after cuprizone-diet induced demyelination in both male and female groups. No differences were observed in N1 CAP amplitude, axon refractoriness, or conduction velocity in males or females under demyelinating condition. Under RM condition, gonadectomized XXSry males had significantly larger N1 amplitudes as compared to XY-Sry males. In addition, gonadectomized XX females had significantly larger N1 amplitudes as compared to XY- females (Figure 4Ci-iv and Table 1). A small but significant difference in conduction velocity was observed between males and females, but only males showed differences in refractoriness (Figure 4Ci-iv and Table 1).

Immunohistological analysis of myelination in the four core-genotype mice: Gonadectomized four core-genotype mice unveil influence of sex chromosome complement during remyelination

MBP staining intensity was not significantly different in XX females compared to XY- females, but XXSry males had significantly less staining intensity compared to XY-Sry males under normal myelination condition ($p < 0.05$, 2 way ANOVA (Sex $\times$ Genotype) Bonferroni posttests; $n=8$). Overall average MBP intensity significantly decreased in demyelinating conditions compared to normal myelinating conditions, but with no difference between males or females. Significant recovery in MBP intensity was observed in all the groups under RM condition.

There was a significant decrease in the number of CC1+ mature oligodendrocytes in XX, XY- females, and XXSry, XY-Sry males of gonadectomized animals as compared to gonadally intact counterparts during normal myelinating condition (Figure 3C and 5C). Gonadectomy revealed an increase in mature oligodendrocyte numbers in XXSry as compared to XY-Sry males within the corpus callosum. There was a significant decrease in the numbers of oligodendrocytes under demyelinating conditions that recovered significantly during RM condition in all groups. There was no difference in number of cells among genotypes under normal myelinating, demyelinating and RM conditions (Figure 5C).

Gonadectomy caused a significant overall decrease in number of proliferating oligodendrocyte progenitors as compared to gonadally intact groups (Figure 3D, 5D). Under normal myelinating condition within the gonadectomized group, there was a trend for more proliferating oligodendrocyte progenitors in the corpus callosum of XX females as compared to XY- females and XXSry males compared to XY-Sry males. During demyelination, oligodendrocyte progenitor numbers declined on average compared to normal myelinating, but there were no differences between genotypes. Oligodendrocyte progenitor numbers recovered significantly during remyelination. Proliferating oligodendrocyte progenitors in XX females were significantly more numerous than XY- females. In addition, XXSry males had significantly more proliferating oligodendrocyte progenitors than XY-Sry males ($*p < 0.05$, 2 way ANOVA (Sex $\times$ Genotype) Bonferroni posttests; $n=8$, Figure 5D).

XX genotype shows better axon remyelination than XY- genotype

To investigate the genotype-mediated differences observed during remyelination in the gonadectomized animals, ultrastructure electron microscopy analysis of female callosal axons was performed (Figure 6A–B). G-ratio was calculated from axon diameters and fiber

(axon+myelin) diameters. Average g-ratios for the female gonadectomized normal myelinating groups did not show a significant difference and the g-ratios for XX and XY– were 0.82 ± 0.006 and 0.81 ± 0.002 respectively. However, consistent with the trend of sex chromosome complement-mediated differences arising following gonadectomy, under remyelinating conditions, gonadectomized XX animals had more remyelination and a g-ratio of 0.87 ± 0.01 as compared to the XY– g-ratio of 0.90 ± 0.006 (* $p < 0.05$, 2 way ANOVA (Sex $\times$ Genotype) Bonferroni posttests; $n=4$). A closer analysis of axons by plotting G-ratio versus axon diameter revealed a higher population of un-myelinated axons in XY– genotypes that were middle-larger diameter axons as compared to XX axons (Figure 6Ci–ii). Quantification of myelinated and un-myelinated axons showed a higher percentage of myelinated axons (~60%) in XX animals after remyelination as compared to less than 45% myelinated axons in XY– genotype corpus callosum (Figure 6Ciii).

DISCUSSION

Using gonadectomized four core-genotype mice, we demonstrate sex chromosome-mediated asymmetry between XX and XY mice. The rate of functional callosal remyelination and number of proliferating oligodendrocytes is accelerated in XX compared to XY animals of both gonadal sexes, following cuprizone diet-induced demyelination. Importantly, this difference arises only in the absence of circulating sex hormones following gonadectomy, confirming the role of sex hormones in the remyelination process, reported earlier by our lab (Patel et al., 2013). Because a genotype-mediated difference only arises following gonadectomy, the chromosomal contribution to myelination and remyelination is subtle, yet important. To explain this difference, we propose a possible asymmetry in expression of myelination-related genes in XX versus XY mice. The asymmetry is masked by the activating influences of sex hormones in gonadally intact mice.

Mammalian sex chromosomes show an asymmetric pattern of inheritance (XY, male; XX female), a phenomenon that has profound implications for gene expression throughout bodily tissues. Before the discovery of gene imprinting and epigenetics, it was hypothesized that gene expression from the second X chromosome in XX individuals was silenced in order to maintain an equal level of X-related gene expression in XX and XY mice (Disteche, 1999). However, there exists the possibility of asymmetric sex-related gene expression due to imprinting, in which certain X-related genes are epigenetically marked based on either paternal or maternal inheritance (Davies et al., 2005, Lentini et al., 2012).

Chen's group found, using the four core-genotype model, that XX mice of either gonadal sex show elevated expression of the prodynorphin gene (*Pdyn*) relative to sex-matched XY cohorts (Chen et al., 2009). Their proposed mechanism for elevated *Pdyn* expression in XX animals is that some X-chromosome related genes escape inactivation and contribute to a dose-dependent expression of *Pdyn*. Another study using four core-genotype mice revealed that mice with two X-chromosomes have higher calbindin mRNA expression in the cerebellum and frontal cortex (Abel et al., 2011). Such findings constitute a precedent for differential gene expression in the central nervous system between XX and XY animals.

We propose a similar argument in which there is a dose-dependent effect of X chromosome copy number on gene expression for genes related to myelination. It is well established that the maturation of oligodendrocyte progenitors to myelinating oligodendrocytes requires the synchronized expression of numerous genes that coordinate each phase of lineage progression (Baumann and Pham-Dinh, 2001, Arnett et al., 2004, Rowitch, 2004). A number of transcription factors essential for one or more of these phases have been identified. These stem from transcription factor families with known roles in many developmental processes, such as the basic helix–loop–helix and homeodomain, and high-mobility-group(HMG)-

containing transcription factors like the oligodendrocyte transcription factor (*Olig*), homolog gene family NKx (*Nkx*), and Sry-related HMG box proteins (*Sox*) (Wegner, 2008). Interestingly, the gene for proteolipid protein (*PLP*), an important structural protein for myelin, resides on the X chromosome (Fahim and Riordan, 1986, Hobson et al., 2002). Pelizaeus-Merzbacher disease, an X chromosome-linked disorder in humans, demonstrates a striking failure of oligodendrocyte differentiation and is genetically similar to the dysmyelinating mouse mutant *jimpy*, which contains a point mutation in the gene encoding proteolipid protein (Hudson et al., 1989). It occurs most exclusively in males.

Another X-linked gene, methyl-CpG binding protein 2 (MeCP2) (Vacca et al., 2001, Bourdon et al., 2003) has also been found to play a role in neuronal maturation and maintenance, including dendritic arborization (Kishi and Macklis, 2010). MeCP2 gene mutations have been identified in several disorders such as Rett syndrome, autism, and ADHD (Nagarajan et al., 2006). Recently, it has also been shown to be present in oligodendrocytes and regulate myelin specific gene expression in transgenic mice (Vora et al., 2010). Does the expression of transcription factors that play an essential role in oligodendrocyte differentiation, maturation, and the expression of various myelin proteins differ in XX and XY animals during remyelination and repair? Screening these related transcription factors in gonadectomized four core-genotype mice is one aim of our future studies and may explain the dose-dependent effect of X chromosome on remyelination.

While every attempt was made in the present studies to ascribe functional myelination differences to the influence of genotype, animals were gonadectomized well into adulthood (8 weeks of age) to control for activating effects of sex hormones on myelination. This does not account for the potentially permanent organizing effects of sex hormones *in utero* and during puberty, before gonadectomy. Indeed, sex hormones may play an important organizational role on the epigenome. Schwarz's group (Schwarz et al., 2010) found that neonatal rodent exposure to estradiol significantly increased DNA methylation that persisted into adulthood. Nugent and McCarthy (Nugent and McCarthy, 2011) highlight numerous examples of sex differences in DNA methylation, especially during the hormone-sensitive period of brain development *in utero*. To reduce the organizational influence of sex hormones, we are performing future studies in mice that have been gonadectomized prior to adulthood. Additionally, we are planning genetic studies using mice carrying XO and XXY chromosome complements that may confirm whether the difference in remyelination is attributable to X chromosome dosage rather than effects of the Y chromosome (Chen et al., 2012).

In conclusion, using four core-genotype mice, we find that the XX genotype enhances the number of proliferating oligodendrocyte progenitors and the rate of functional remyelination in the mouse corpus callosum following a cuprizone-diet induced demyelinating insult.

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Research highlights

- ▶ Potential role for sex chromosome complement in mediating differential rates of remyelination.
- ▶ Four core-genotype mice and cuprizone-diet induced demyelination/remyelination model was used.
- ▶ Rate of functional remyelination is attenuated in XY compared to XX animals of both gonadal sexes.
- ▶ A genotype-mediated difference arises only following gonadectomy.
- ▶ Our results demonstrate sex chromosome mediated asymmetry between XX and XY mice.

Figure 1. Animal Breeding Strategy, Experimental Groups, and Callosal Conduction

(A) Backcrossed C57Bl/6–*Sry* males are bred with female C57Bl/6 mice. The breeding yields the informative four core genotypes to dissociate sex chromosomes from gonadal sex. Two are genotypic males (XY-Sry and XY[−]; blue boxes), and two are genotypic females (XX and XXSry; red boxes). The phenotypic males both carry the *Sry* transgene, which determines testis formation, but differ in the number of X and Y chromosomes that they carry.

(B) *Treatment groups*: Intact and gonadectomized mice were randomized into one of two groups: one group (normal myelination, NM) received three weeks of *ad libitum* chow diet

and water; the other group (demyelination, DM) received three weeks of *ad libitum* 0.2% cuprizone diet and water. At the conclusion of three weeks, half of the demyelination group was sacrificed for assays and the other half returned to *ad libitum* chow diet (remyelination, RM).

(C) Compound action potential (CAP) recordings were obtained from acute brain slices containing the hippocampus (plates 35–48, Paxinos and Franklin mouse brain atlas). The corpus callosum was stimulated at 1mm from midline using a tungsten bipolar stimulating electrode (sti); responses were measured 2mm (CAP and refractoriness; distances varies for velocity recordings) from the stimulating electrode with a 3M NaCl-filled glass micropipette recording electrode (rec). CAP stimulation results in a large stimulus artifact followed by two depolarizing phases of the compound action potentials, 'N1' and 'N2' (Crawford et al., 2009a). The stimulus artifact generally lasts less than 0.2 ms and is blocked beyond the dashed line and is disregarded for the purpose of compound action potential analysis(Crawford et al., 2009a). The N1 compound action potential amplitudes represent fast depolarization from mostly large, myelinated axons and N2 compound action potential amplitudes represent slower depolarization from non-myelinated axons.

Figure 2. Electrophysiology Analysis of corpus callosum axons: Similar effects of demyelination and remyelination in gonadally intact four core-genotype mice

(A) Representative compound action potential (CAP) recordings were obtained from acute brain slices containing corpus callosum of gonadally intact, normal-myelinating (NM) animals. Representative traces of 4.0mA compound action potential recordings from male (i) and female (ii). Quantification of N1 (myelinated fiber component) compound action potential amplitude in male (iii) and female (iv), showing no differences at 4.0mA stimulus between males (XXSry=0.74±0.07mV vs. XY⁻Sry=0.7±0.03mV) or females (XX=0.76±0.04mV and XY⁻= 0.75±0.04mV). N1 refractoriness of in male (v) and female (vi) animals, showing no difference between males or females.

(B) Compound action potential recordings from intact, demyelinating (DM) animals. Representative traces of 4.0mA compound action potentials from male (i) and female (ii). Quantification of N1 compound action potential amplitude in male (iii) and female (iv) animals, showing a loss of N1 signal due to robust demyelination.

(C) Compound action potential recordings from intact, remyelinating (RM) animals. Representative traces of 4.0mA compound action potentials from male (i) and female (ii). Quantification of N1 compound action potential amplitude in male (iii) and female (iv) animals shows no differences between males ($XXSry=0.4\pm0.02mV$ vs. $XY-Sry=0.4\pm0.1mV$) or females ($XX=0.4\pm0.02mV$ vs. $XY^-=0.4\pm0.02mV$). There was a significant recovery compared to demyelinating condition in both groups. N1 refractoriness in male (v) and female (vi) animals, with no differences between males or females. Values are expressed mean $\pm$ SEM, 2 way ANOVA (Sex $\times$ Genotype) Bonferroni posttests $*p<0.05$; n=8.

Figure 3. Immunohistochemistry analysis: similar levels of demyelination and remyelination in gonadally intact four core-genotype mice

(A) Myelin basic protein (MBP, red) staining of corpus callosum counterstained with DAPI (nuclei, blue) during normal myelination (NM, left), demyelination (DM, center), and remyelination (RM, right) of the four core genotypes.

(B) MBP fluorescence intensity during normal myelinating condition showed an increase in phenotypic males (XY-Sry and XX-Sry) compared to phenotypic females (XX and XY⁻). MBP fluorescence intensity decreases significantly during demyelination and recovers during RM with no significant differences between genotypes. Values are expressed mean $\pm$ SEM, ANOVA (Sex $\times$ Genotype) Bonferroni posttests * $p < 0.05$; $n = 8$.

(C) Representative XX corpus callosum containing confocal images from brain sections immunostained with anti-CC1 antibody (red) to label mature oligodendrocytes (OL) and co-stained with DAPI (blue) to label all nuclei. White square dashed box is enlarged to show a few cells with unmerged staining of CC1 and DAPI. Quantification of oligodendrocytes showed a significant decrease during demyelination (DM) as compared to normal myelination (NM) conditions. A significant increase in oligodendrocytes was observed during remyelination (RM), but with no significant differences between genotypes.

(D) Representative XX corpus callosum containing confocal images from brain sections immunostained with anti-olig2 antibody (red) to label oligodendrocyte progenitors (OLP),

anti-Ki67 (green) to label proliferating cells, and co-stained with DAPI (blue) to label all nuclei. White square dashed box is enlarged to show a few cells with unmerged staining of olig2 and Ki67. Quantification of doubly positive olig2 and Ki67 proliferating oligodendrocyte progenitor cell counts showed a significant decrease during demyelination followed by significant recovery during remyelination, with no significant differences between genotypes.

Figure 4. Electrophysiology Analysis of corpus callosum axons of gonadectomized four core-genotype mice: XX genotypes show improved remyelination-induced recovery as compared to XY genotypes

(A) Representative traces of 4.0mA evoked compound action potentials (CAP) recordings from gonadectomized male (i) and gonadectomized female (ii) brain slices. N1 compound action potential amplitude in male (iii) and female (iv), showing that XX genotype promotes enhanced N1 response in males (XXSry=0.6±0.02mV vs. XY-Sry=0.4±0.03mV, $p<0.05$; $n=8$) but not females (XX=0.8±0.13mV vs. XY=0.8±0.07mV). N1 refractoriness (v) and female (vi) animals showing similar results between XX and XY male and female genotypes.

(B) Compound action potentials from gonadectomized animals undergoing demyelination. Representative traces of 4.0mA compound action potential recordings from male (i) and female (ii) brain slices. N1 compound action potential amplitude in male (iii) and female (iv) animals showing a loss of N1 response indicative of robust demyelination. Refractoriness and velocity measurements were not calculated due to low N1s.

(C) Compound action potentials from gonadectomized undergoing remyelination. Representative traces of 4.0mA compound action potential recordings from male (i) and female (ii). N1 compound action potential amplitude in male (iii) and female (iv) animals, showing XX genotype compared to XY promotes accelerated remyelination and N1 compound action potential amplitudes in both males (XXSry=0.5±0.04mV vs. XY⁻Sry=0.3±0.03mV) and females (XX=0.5±0.04mV vs. XY⁻=0.3±0.04mV). N1 refractoriness in males (v) showed a small but significant difference (XXSry=3.8±0.02ms vs. XY⁻Sry=4.4±0.04ms) but females (vi) refractoriness values were not significant (XX=4.2±0.06ms vs. XY⁻=4.5±0.04ms). Values are expressed mean ± SEM; 2 way ANOVA (Sex × Genotype) Bonferroni posttests *p<0.05; n=8.

Figure 5. Immunohistochemistry analysis of gonadectomized four core-genotype mice: XX genotypes have more oligodendrocyte progenitors during remyelination

(A) Myelin basic protein (MBP, red) staining of corpus callosum counterstained with DAPI (nuclei, blue) during normal myelination (NM-left), demyelination (DM-center), and remyelination (RM-right) of gonadectomized (GDX) four core genotypes.

(B) MBP fluorescence quantification shows significant loss of intensity during demyelination and recovery during remyelination, with no significant differences between genotypes.

(C) Representative XXSry and XY-Sry corpus callosum containing confocal images from brain sections immunostained with anti-CC1 antibody (red) to label mature oligodendrocytes (OL) and co-stained with DAPI (blue) to label all nuclei. White square dashed box in XXSry is enlarged to show a few cells with unmerged staining of CC1 and DAPI. Quantification of CC1⁺ mature oligodendrocytes (OLs) staining showed significant differences between male genotypes only with XXSry having more mature OLs than XY-Sry corpus callosum (* $p < 0.05$; $n = 8$).

(D) Representative XXSry and XY-Sry corpus callosum containing confocal images from brain sections immunostained with anti-olig2 antibody (red) to label oligodendrocyte progenitors (OLP), anti-Ki67 (green) to label proliferating cells, and co-stained with DAPI (blue) to label all nuclei. White square dashed box in XXSry is enlarged to show a few cells with unmerged staining of olig2 and Ki67. Quantification of doubly positive olig2⁺ and

Ki67⁺ oligodendrocyte progenitors (OLPs) show significantly more cells in XXSry compared to XY-Sry during normal myelination. In addition, XXSry and XX genotypes have more oligodendrocyte progenitors during remyelination (RM) condition as compared to XY-Sry and XY⁻ genotypes. Values are expressed mean $\pm$ SEM, 2 way ANOVA (Sex $\times$ Genotype) Bonferroni posttests * $p < 0.05$; $n = 8$.

Figure 6. Electron microscopy analysis: more efficient axon remyelination in gonadectomized female mice

(A–B) Representative electron micrograph images of callosal axons from gonadectomized XX and XY- mice during normal myelination (NM, Ai–ii) and remyelination (RM, right Bi–ii) are shown. No difference was observed during normal myelination, but increased number of myelinated axons was observed in XX mice as compared to XY- mice. Quantification of average g-ratio measurements (calculated as diameter of axon divided by diameter of fiber) revealed similar g-ratios in XX and XY- groups during normal myelination ($p>0.4$; $n=4$). In contrast, during remyelinating condition, XX genotype had a small but significant decrease in g-ratio of 0.87 ± 0.006 as compared to XY- = 0.90 ± 0.007 indicative of more efficient

remyelination. Scale bar is 1 μ m. (Values are expressed mean $\pm$ SEM, 2 way ANOVA (Sex $\times$ Genotype) Bonferroni posttests * $p < 0.05$; $n = 4$).

(C) Scatter plots of axon diameter versus g-ratio indicated remyelination-induced increase in myelinated axons in XX callosal axons (i) as compared to XY- callosal axons (ii) that showed fewer myelinated axons and increased number of un-myelinated axons with g-ratio closer to 1. The number of all myelinated and all non-myelinated axons were counted and divided by total number of axons to get the % myelinated and un-myelinated axons (iii). XX callosum showed more myelinated axons versus XY-callosum that had more un-myelinated axons during remyelination.

Table 1

Intact and gonadectomized brain slices under normal myelination (NM), demyelinating (DM) and remyelinating (RM) conditions were subjected to various electrophysiology protocols (compound action potential-CAP amplitude assessment, axon refractoriness and conduction velocity-(see Experimental Procedures). The averages of only the myelinated component (N1) under various conditions were calculated from n=8 mice from each group. Demyelination group had insignificant N1s and were not calculated.

Genotype	Protocol	Units	INTACT			GDX		
			NM	DM	RM	NM	DM	RM
XX	N1 CAP							
	Amplitude	N1 4.0mA, mV	0.76±.04	NA	0.40±.02	0.8±0.06	NA	0.50±0.04
	Refractoriness	IPI V50, ms	3.76±.10	NA	3.60±.2	3.6±.23	NA	4.2±0.04
XY-	Velocity	Slope (ms/mm)	0.7 ± 0.03	NA	0.55 ± 0.08	0.6 ± 0.05	NA	0.52 ± 0.08
	N1 CAP							
	Amplitude	N1 4.0mA, mV	0.75±0.04	NA	0.41±.02	0.8±0.13	NA	*0.34±0.3
XXSry	Refractoriness	IPI V50, ms	3.90±.12	NA	3.4±.25	4.0±0.16	NA	4.5±0.10
	Velocity	Slope (ms/mm)	0.71 ± 0.06	NA	1.1 ± 0.50	0.7 ± 0.06	NA	*1.2 ± 0.40
	N1 CAP							
XXSry	Amplitude	N1 4.0mA, mV	0.74±.07	NA	0.42±.02	0.6±.02	NA	0.54±.05
	Refractoriness	IPI V50, ms	3.35±.36	NA	3.4±.26	4.1±.20	NA	3.8±.02
	Velocity	Slope (ms/mm)	0.61 ± 0.05	NA	0.7 ± 0.05	0.7± 0.03	NA	0.56 ± 0.04
XY-Sry	N1 CAP							
	Amplitude	N1 4.0mA, mV	0.70±0.02	NA	0.4±.02	*0.4±0.03	NA	*0.30±0.04
	Refractoriness	IPI V50, ms	4.1±.15	NA	3.4±.14	4.0±0.13	NA	*4.4±0.04
XY-Sry	Velocity	Slope (ms/mm)	0.66 ± 0.03	NA	0.8 ± 0.20	0.70 ± 0.08	NA	*0.7 ± 0.07

Comparison between genotypic females (XX and XY-) or genotypic males (XXSry and XY-Sry) are shown with values expressed as mean ± SEM, 2 way ANOVA (Sex × Genotype) Bonferroni posttests *p<0.05; n=8.