White Matter Alterations in *Fmr1* Knockout Mice during Early Postnatal Brain Development

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Keywords

Fragile X syndrome · *Fmr1* knockout · Magnetization transfer · T₂ relaxation · Myelination

Abstract

Fragile X syndrome (FXS) is the most commonly inherited form of intellectual disability ascribed to the autism spectrum disorder. Studies with FXS patients have reported altered white matter volume compared to controls. The *Fmr1* knockout (KO) mouse, a model for FXS, showed evidence of delayed myelination during postnatal brain development. In this study, we examined several white matter regions in the male *Fmr1* KO mouse brain compared to male wild-type (WT) mice at postnatal days (PND) 18, 21, 30, and 60, which coincide with critical stages of myelination and postnatal brain development. White matter volume, T₂ relaxation time, and magnetization transfer ratio (MTR) were measured using magnetic resonance imaging and myelin content was determined with histological staining of myelin. Differences in the developmental accumulation of white matter and myelin between *Fmr1* KO and WT mice were observed in the corpus callosum, external and internal capsules, cerebral peduncle, and fimbria. Alterations were more predominant in the external and internal capsules and fimbria of *Fmr1* KO mice,

where the MTR was lower at PND 18, then elevated at PND 30, and again lower at PND 60 compared to the corresponding regions in WT mice. The pattern of changes in MTR were similar to those observed in myelin staining and could be related to the altered protein synthesis that is a hallmark of FXS. While no significant changes in white matter volumes and T₂ relaxation time between the *Fmr1* KO and WT mice were observed, the altered pattern of myelin staining and MTR, particularly in the external capsule, reflecting the abnormalities associated with myelin content is suggestive of a developmental delay in the white matter of *Fmr1* KO mouse brain. These early differences in white matter during critical developmental stages may contribute to altered brain networks in the *Fmr1* KO mice.

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Introduction

Fragile X syndrome (FXS) is the most commonly inherited form of sex-linked intellectual disability affecting 1 in 5,000 males [1]. FXS causes intellectual disability and behaviors commonly seen in the autism spectrum disorder, including hyperactivity, social anxiety, and attentional problems [1, 2]. FXS is caused by the mutational

insertion of CGG trinucleotide repeats, which leads to the epigenetic silencing of the *FMR1* gene [3]. The silencing results in the loss of the mRNA binding protein fragile X mental retardation protein (FMRP) [4, 5]. FMRP regulates the translation of mRNAs for proteins necessary for synaptic function and myelin integrity [6]. Most notably, the loss of FMRP leads to elevated levels of protein synthesis in the brain [7], impaired regulation of neuronal signaling and synaptic plasticity [8–12], and elevated levels of immature dendritic spines in the cortex and hippocampus [13–17]. These alterations may contribute to symptoms associated with FXS [2, 18]. A preclinical model of FXS, the *Fmr1* knockout (KO) mouse [19] is commonly used to study FXS and models many of the clinical manifestations of FXS.

Much of the research in FXS focuses on neuronal changes and neurodevelopmental symptoms [10, 20]. However, it has been reported that astrocyte and oligodendrocyte dysfunction may contribute to alterations in brain function associated with the *Fmr1* KO mouse phenotype [21–23]. Astrocytes from *Fmr1* KO mouse brain co-cultured with neurons from normal wild-type (WT) mouse brain caused *Fmr1* KO-like neuronal morphology including increased axonal branching and immature dendritic spine density in WT neurons, suggesting astrocytes may contribute to the neuronal phenotype [21, 24, 25]. FMRP binds to mRNAs of myelin proteins including myelin basic protein (MBP) [6, 23], 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP), proteolipid protein, and myelin-associated glycoprotein [6]. Although the role of FMRP in oligodendrocyte maturation is still unknown, the finding that pre- and immature oligodendrocytes contain FMRP and levels are greatly reduced in mature oligodendrocytes suggests a developmental role for FMRP [23, 26]. MBP is an abundant protein that ensures the stability of myelin sheaths [27]. Evidence of delayed myelination has been reported in the cerebellar white matter of postnatal *Fmr1* KO mice, including reduced myelin thickness and reduced concentrations of MBP and CNP at postnatal day (PND) 7, all of which were normalized at PND 30 compared to WT mice [22]. Altered MBP levels at different postnatal ages suggest that the loss

of FMRP regulation of MBP mRNA may have long-term consequences with respect to myelin integrity in *Fmr1* KO mouse brain [22].

Imaging studies of *Fmr1* KO mouse brain revealed differences in regional brain volume, including an increase in hippocampal [28] and a decrease in cerebellar [22] volumes, as well as changes in brain metabolite concentrations that may influence myelination [28, 29]. In children with FXS, greater overall white matter volume and alterations of regional white matter volumes during brain development have been reported [30, 31]. Taken together, these studies provide evidence that alterations in white matter development may contribute to altered neurodevelopment associated with FXS. However, very little has been done to characterize the underlying alterations associated with changes in brain volume using advanced imaging techniques in FXS patients and *Fmr1* KO mouse brain.

In this study, we hypothesized decreased myelin levels are found in white matter during brain postnatal development in *Fmr1* KO mice compared to the WT mice. Measurements were taken at critical time points in the course of myelination and neural network development, with PND 18 being before, and PND 21 during the peak of myelination and synaptogenesis in rodents [32]. Additionally, ages that coincide with previous reports of neural network development at PND 30 and maturation at PND 60 were also studied [32]. Using magnetization transfer and T_2 imaging, we assessed microstructural white matter development in the corpus callosum, external capsule, internal capsule, cerebral peduncle, and hippocampal fimbria ex vivo in perfused brains from *Fmr1* KO and WT mice. Histological staining was used to quantify myelin and verify results from T_2 relaxation time and magnetization transfer imaging.

Methods and Materials

Animal Procedure

Fmr1 KO mice (JAX B6.129P2-fmr1^{tm1Cgr} mice; Jackson Laboratory, Bar Harbor, ME, USA) from a C57Bl6J background were bred in the animal facility at the University of Maryland, Balti-

Fig. 1. White matter volume between *Fmr1* KO and WT mouse brains. The representative T_2 images and ROI overlay are shown in **a**. The ROIs show representative white matter regions from WT PND 30 mouse brain, with corpus callosum (red), external capsule (green), internal capsule (yellow), cerebral peduncle (purple), and fimbria (blue). Regional white matter volume (mm^3 ; mean $\pm$ standard error) in the developing WT (gray dashed line) and *Fmr1* KO

(black solid line) mouse brains are shown in the corpus callosum (**b**), external (**c**) and internal capsule (**d**), cerebral peduncle (**e**), and fimbria (**f**). The ages studied were PND 18 (*Fmr1* KO $n = 9$, WT $n = 9$), 21 (*Fmr1* KO $n = 12$, WT $n = 14$), 30 (*Fmr1* KO $n = 13$, WT $n = 12$), and 60 (*Fmr1* KO $n = 6$, WT $n = 6$). Interaction of genotype $\times$ age was not significant in white matter regions and the whole brain with respect to volume.

(For figure see next page.)

Table 1. Effects and interactions from 2-way analysis of variance

	Region	df	Mean square	F value	p value
<i>Volume</i>					
Genotype	Corpus callosum	1	0.027	0.315	0.577
	External capsule	1	0.238	1.535	0.220
	Internal capsule	1	0.001	0.048	0.827
	Cerebral peduncle	1	0.002	0.038	0.846
	Fimbria	1	0.003	0.266	0.608
Age	Corpus callosum	3	4.063	46.699	<0.001
	External capsule	3	29.407	189.351	<0.001
	Internal capsule	3	4.168	203.613	<0.001
	Cerebral peduncle	3	2.903	50.557	<0.001
	Fimbria	3	0.770	77.064	<0.001
Genotype × age	Corpus callosum	3	0.095	1.095	0.357
	External capsule	3	0.217	1.394	0.252
	Internal capsule	3	0.028	1.369	0.260
	Cerebral peduncle	3	0.016	0.273	0.845
	Fimbria	3	0.009	0.884	0.453
<i>T₂ relaxation</i>					
Genotype	Corpus callosum	1	35.060	2.091	0.153
	External capsule	1	35.425	3.050	0.085
	Internal capsule	1	4.814	0.594	0.443
	Cerebral peduncle	1	7.209	0.927	0.339
	Fimbria	1	7.209	1.393	0.242
Age	Corpus callosum	3	145.239	8.662	<0.001
	External capsule	3	71.845	6.186	<0.001
	Internal capsule	3	20.849	2.573	0.061
	Cerebral peduncle	3	16.209	2.084	0.110
	Fimbria	3	79.850	6.036	<0.001
Genotype × age	Corpus callosum	3	22.424	1.337	0.269
	External capsule	3	17.468	1.504	0.221
	Internal capsule	3	5.553	0.685	0.564
	Cerebral peduncle	3	12.900	1.659	0.184
	Fimbria	3	24.530	1.854	0.145
<i>Magnetization transfer ratio</i>					
Genotype	Corpus callosum	1	32.768	6.722	0.011
	External capsule	1	37.016	8.406	<0.001
	Internal capsule	1	35.329	9.365	<0.001
	Cerebral peduncle	1	31.867	7.511	0.008
	Fimbria	1	21.342	6.722	0.011
Age	Cerebellar white matter	1	50.883	12.007	0.001
	Corpus callosum	3	60.358	12.382	<0.001
	External capsule	3	49.900	11.332	<0.001
	Internal capsule	3	87.207	23.117	<0.001
	Cerebral peduncle	3	101.941	24.028	<0.001
Genotype × age	Fimbria	3	60.691	19.115	<0.001
	Cerebellar white matter	3	73.379	17.315	<0.001
	Corpus callosum	3	32.020	6.568	<0.001
	External capsule	3	28.590	6.492	<0.001
	Internal capsule	3	26.824	7.111	<0.001
	Cerebral peduncle	3	19.366	4.565	0.005
	Fimbria	3	20.373	6.417	<0.001
	Cerebellar white matter	3	12.343	2.913	0.040

Table 1 (continued)

	Region	df	Mean square	F value	p value
<i>Black Gold II staining</i>					
Genotype	Corpus callosum	1	0.00005	1.730	0.191
	External capsule	1	0.00043	28.056	<0.001
	Internal capsule	1	0.00023	5.313	0.023
	Cerebral peduncle	1	0.00003	0.959	0.329
	Fimbria	1	0.00020	6.748	0.010
Age	Corpus callosum	3	0.00056	18.787	<0.001
	External capsule	3	0.00197	129.770	<0.001
	Internal capsule	3	0.00351	80.925	<0.001
	Cerebral peduncle	3	0.00191	60.244	<0.001
	Fimbria	3	0.00046	15.299	<0.001
Genotype × age	Corpus callosum	3	0.00004	1.469	0.226
	External capsule	3	0.00019	12.287	<0.001
	Internal capsule	3	0.00017	3.960	0.010
	Cerebral peduncle	3	0.00009	2.993	0.033
	Fimbria	3	0.00011	3.665	0.014

Summary of two-way ANOVA for whole brain and regional white matter volumes, MTR, T₂ relaxation, and myelin density by ROIs. Included in the table are the F value and p value for genotype, age, and genotype × age interaction. Statistically significant effects (p value <0.05) are indicated in bold. A post hoc was performed if there was a significant genotype × age interaction.

more. The *Fmr1* KO mice were generated by mating homozygous *Fmr1* KO female to hemizygous *Fmr1* KO males. The WT mice were generated by homozygous mating of the WT female to the WT male. The *Fmr1* KO mice were backcrossed for more than 10 generations. The Institutional Animal Care and Use Committee at the University of Maryland, Baltimore approved the protocol used in this study. All mice were weaned at PND 21. Food and water were given ad libitum.

Mice were studied at PND 18 (9 *Fmr1* KO, 9 WT), PND 21 (12 *Fmr1* KO, 14 WT), PND 30 (13 *Fmr1* KO, 12 WT), and PND 60 (6 *Fmr1* KO, 6 WT). Once animals reached the appropriate age, the male *Fmr1* KO and WT mice were anesthetized with 4% isoflurane and perfused through the left ventricle with phosphate buffered saline (PBS), followed by 4% paraformaldehyde fixative. The whole brain was removed from the skull to ensure the brain was completely fixed before imaging and immersed in fixative for 48 h. It was decided to excise the brain from the skull to ensure fixation of white matter structure and extra precaution was made to prevent damage to the forebrain and midbrain during dissection. The fixed brain was then immersed in PBS for 1 week to minimize the effect of the fixative on T₂ relaxation time and magnetization transfer ratio (MTR) [33, 34]. The fixation procedure was tightly controlled to minimize any variations associated with tissue processing. These fixed brains were used in order to detect subtle changes between white matter of *Fmr1* KO and WT mice using MRI. The fixed brains were placed in a customized conical tube filled with a proton-free susceptibility-matching fluid Fluorinert (3M, St. Paul, MN, USA) prior to MR imaging. Fluorinert does not degrade the sample because it is biologically inert [35] and has been safely used for in vivo molecular imaging [36].

Magnetic Resonance Imaging

MRI techniques used in this study are commonly used to characterize developmental and pathological changes in white matter

because they are sensitive to the chemical and physical environments of water protons [37–40]. All experiments were performed on a Bruker Biospec 7.0 Tesla 30 cm horizontal bore scanner (Bruker Biospin MRI, Ettlingen, Germany) using Paravision 5.1 software. A Bruker 72-mm linear-volume coil as the transmitter and a four-channel Bruker ¹H surface array coil as the receiver were used for imaging. Prior to imaging, magnetic field inhomogeneity was adjusted using the FASTMAP technique [41]. Anatomical images were obtained using a 3D TurboRARE sequence covering a slab of 15 mm in thickness over the whole brain, from the forebrain to the hindbrain, to obtain T₂-weighted images at a spatial resolution of 113 × 150 × 170 μm, using an echo time (TE) and repeat time (TR) of 11 ms and 2,500 ms, respectively, with two averages.

Transverse relaxation time T₂ values decrease with the extent of myelination in white matter [42]. Quantitative information on T₂ relaxation time was obtained using an eight echo, multi-echo spin echo sequence with three continuous slices centered on the hippocampus at Bregma –1.82, without any gap. The spatial resolution was 200 × 200 μm, acquired with 1 mm slice thickness, TEs of 9.3, 27.9, 46.5, 65.1, 83.7, 102.3, 120.9, and 139.5 ms at a TR of 10,000 ms, and two averages.

Changes in the relative levels of macromolecules can be detected using magnetization transfer, a technique that exploits the exchange between the bound protons in the macromolecules and the protons in free water, providing a contrast that is reflective of the concentration of macromolecules present in tissue [43–46]. In white matter, such a contrast would be indicative of myelin water content since myelin macromolecules such as lipids and proteins serve as the major pool of bound protons. Thus, magnetization transfer imaging provides an index of relative content of macromolecules in white matter and is reported as MTR [38, 45, 46].

The magnetization transfer images were obtained using a fast low shot (FLASH) T₁ technique. Acquisition parameters were slice

Table 2. Whole brain and normalized white matter volumes in *Fmr1* KO and WT mice

Whole brain volume	2-way ANOVA			
	df	Mean square	F value	p value
Genotype	1	0.421	0.701	0.405
Age	3	143.306	238.515	<0.0001
Genotype × age	3	1.118	1.861	0.144
	Means			
	WT volume, mm ³	SE	<i>Fmr1</i> KO volume, mm ³	SE
Age				
PND 18	352.09	22.64	341.82	5.94
PND 21	342.47	9.58	376.16	14.71
PND 30	360.60	5.13	302.66	37.65
PND 60	387.02	4.72	412.60	15.97
Normalized white matter volume	2-way ANOVA			
	df	Mean square	F value	p value
Corpus callosum				
Genotype	1	0.000001	0.002	<0.0001
Age	3	0.01280	21.69	0.9634
Genotype × age	3	0.00004	0.072	0.9749
External capsule				
Genotype	1	0.00014	0.1191	0.7310
Age	3	0.00107	0.9054	0.1130
Genotype × age	3	0.00024	0.2049	0.8926
Internal capsule				
Genotype	1	0.00006	0.2948	0.5889
Age	3	0.01075	53.49	<0.0001
Genotype × age	3	0.00020	0.9854	0.4048
Cerebral peduncle				
Genotype	1	0.00001	0.0275	0.8687
Age	3	0.00003	0.0843	0.9684
Genotype × age	3	0.00011	0.3016	0.8241
Fimbria				
Genotype	1	0.000003	0.0315	0.8597
Age	3	0.00035	3.810	0.0137
Genotype × age	3	0.00002	0.2360	0.8710

thickness 0.5 mm, 0.5 mm gap, 16 slices that covered the cerebral cortex and the cerebellum, spatial resolution of $200 \times 200 \mu\text{m}$ over a field of view of $1.5 \times 1.5 \text{ cm}$, TE/TR = 3.6/280 ms, and 32 averages. For the magnetization transfer images (M_s), a Gaussian off-resonance saturation pulse was used with duration of 12.6 ms and flip angle of 180 degrees at an off-resonance frequency of 5,000 Hz from the center frequency of water protons. The reference magnetization transfer image (M_0) was scanned using the same parameters except without the off-resonance pulse.

Image Processing

Images were transferred offline to an independent database for further processing. Medical Image Processing, Analysis and Visualization tool (MIPAV v5.3.1, CIT; NIH, Bethesda, MD, USA) was

used to obtain volumes of different regions and to extract information for computing the MTR. Regions of interest (ROIs) were drawn manually on both the 3D T_2 anatomical and magnetization transfer images by comparison with an anatomical reference atlas [47]. The image contrast was not clear enough for automatic segmentation at early ages; therefore, the ROIs were drawn by one operator for consistency. ROIs included the whole brain, corpus callosum, external capsule, internal capsule, cerebral peduncle, and fimbria. Both absolute and normalized white matter volume was obtained by dividing each regional white matter volume by whole brain volume. MTR was calculated as $([M_0 - M_s]/M_0) \times 100$, where M_0 is the signal intensity from images obtained without the off-resonance pulse and M_s is the signal intensity from images where the saturation pulse was applied [45]. T_2 relaxation time was

Table 2 (continued)

	Means			
	WT normalized volume	SE	<i>Fmr1</i> KO normalized volume	SE
Age and region				
PND 18				
Corpus callosum	0.294	0.002	0.290	0.014
External capsule	0.444	0.005	0.452	0.028
Internal capsule	0.050	0.003	0.047	0.006
Cerebral peduncle	0.137	0.006	0.135	0.006
Fimbria	0.076	0.003	0.072	0.004
PND 21				
Corpus callosum	0.291	0.005	0.288	0.007
External capsule	0.435	0.007	0.430	0.007
Internal capsule	0.055	0.003	0.066	0.004
Cerebral peduncle	0.138	0.005	0.135	0.006
Fimbria	0.082	0.002	0.082	0.004
PND 30				
Corpus callosum	0.264	0.010	0.266	0.007
External capsule	0.443	0.010	0.435	0.006
Internal capsule	0.076	0.005	0.076	0.004
Cerebral peduncle	0.135	0.003	0.142	0.004
Fimbria	0.081	0.003	0.082	0.002
PND 60				
Corpus callosum	0.227	0.001	0.230	0.008
External capsule	0.450	0.007	0.444	0.010
Internal capsule	0.112	0.007	0.112	0.005
Cerebral peduncle	0.138	0.012	0.139	0.013
Fimbria	0.074	0.002	0.075	0.003

Normalized volume was calculated by dividing regional white matter volume (mm³) by its respective whole brain volume (mm³). The mean normalized volume and standard error for each white matter region in the *Fmr1* KO and WT mouse brain are presented at PND 18, 21, 30, and 60. Also included is a summary of two-way ANOVA for whole brain volume and normalized white matter volume. The normalized volume was calculated by dividing the regional white matter volume by the whole brain volume for each mouse. The *F* value and *p* value for genotype, age, and interaction of genotype × age are presented.

measured using an in-house Matlab program [48]. The data from all TEs for each ROI were fitted to a mono-exponential decay curve. The first echo at 9.3 ms was ignored to minimize potential contamination from the stimulated echo. The volumes, T₂ relaxation times, and MTR values from each ROI were obtained and analyzed for statistically significant differences.

Histology

Following MRI, six of the perfused brains from each genotype and age were placed in 30% sucrose solution for cryoprotection. Brains were then frozen and coronal sections of 40 μm in thickness were prepared with a cryostat at −20 °C. One section was collected from every 12 sections, approximately 0.50 mm apart. Sections were stained for myelin with Black Gold II (EMD Millipore, Billerica, MA, USA) by the following procedure described by Schmued et al. [49]. Mounted slides were rehydrated with two washes of de-ionized water, followed by staining with 0.3% Black Gold II in saline solution at 60 °C. After staining was complete, the slides were washed in 1% sodium thiosulfate solution at 60 °C

followed by three washes in water at room temperature [49]. Images were taken on a brightfield Olympus BX53 microscope at 10× magnification and analyzed using Bioquant Life Sciences 2013 v13.5.6 software (Bioquant Image Analysis Inc., Nashville, TN, USA). A white field correction was applied to all images prior to obtaining optical density. The red channel was used to determine density because Black Gold II stains myelinated fibers red-brown. The intensity of myelin staining in white matter regions was measured as optical density. The optical density was calculated as the log of the intensity of the ROI divided by the intensity of the background [50]. The optical density value was normalized to the area (in μm²) of the ROI. Optical densities were measured between Bregma 0.50 to −2.46 mm, which included 5–6 sections of the corpus callosum and external capsule, 1–2 sections with the internal capsule, 2–3 sections for cerebral peduncle, and 3–4 sections for the fimbria. The ROIs selected for histology were matched with anatomical references by Franklin and Paxinos [47] during ROI selection and number of ROIs were adjusted to with age to cover similar white matter regions through-

Fig. 2. T₂ relaxation time in white matter regions of the *Fmr1* KO and WT mouse brain. The representative images at TE = 9.3 are shown for *Fmr1* KO and WT mouse brains at each age in **a**. T₂ relaxation time (ms ± standard error) changes in WT (gray dashed line) and *Fmr1* KO (black solid line) mice from PND 18 (*Fmr1* KO *n* = 9, WT *n* = 9), 21 (*Fmr1* KO *n* = 12, WT *n* = 14), 30 (*Fmr1* KO

n = 12, WT *n* = 12), and 60 (*Fmr1* KO *n* = 6, WT *n* = 6). Relaxation times were measured in white matter regions of the corpus callosum (**b**), external (**c**) and internal capsule (**d**), cerebral peduncle (**e**), and fimbria (**f**). No significant genotype × age interaction was found in T₂ relaxation.

out the forebrain and midbrain. While the number of brains used for histology was less than the number of brains imaged, each brain was randomly selected among the litter group to avoid litter effect and bias.

Statistical Analysis

All statistical analyses were performed in SPSS (IBM Corp., 2013, IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY, USA). A separate two-way analysis of variance (ANOVA) was performed for each region in each parameter, including volume, normalized volume, MTR, T_2 relaxation, and optical density. Each two-way ANOVA compared the effect of genotype, age, and the interaction of genotype $\times$ age. Genotype and age effects were analyzed by Student's t tests if the interaction between factors was statistically significant ($p < 0.05$). To control for multiple pairwise comparisons, a false discovery rate with threshold of $\alpha = 0.05$ was performed on the p values. The threshold assumes 5% of significant p values may be false positives, and if a significant p value does not pass false discovery rate then that p value may be a false positive.

Results

White Matter Volumes

Representative images of the fixed brain are shown in Figure 1a for each age and genotype. Additionally, Figure 1a shows a representative image of the drawn white matter ROIs from WT PND 30 mouse brain. No differences in regional white matter or whole brain volumes were found between *Fmr1* KO and WT mice (Fig. 1; Table 1). The white matter volume normalized to whole brain volume was also not significantly different between *Fmr1* KO and WT mice (Table 2). Significant effect of age was observed in all white matter regions studied and the white matter volume increased with age (Fig. 1; Table 1). The genotype $\times$ age interaction was not statistically significant in the whole brain and regional white matter volumes (Table 1), as well as with normalized regional white matter volumes (Table 2), indicating that the developmental courses of change in white matter volumes were similar between the two genotypes.

T_2 Relaxation Time

T_2 relaxation time can provide insights into white matter environment and generally decreases following development of white matter [42]. Representative T_2 images at TE = 9.3 ms are shown in Figure 2a for each age and genotype. T_2 relaxation time was measured in the corpus callosum, external capsule, internal capsule, cerebral peduncle, and fimbria of brains from *Fmr1* KO and WT mice (Fig. 2b–f, respectively). The genotype $\times$ age interaction was not statistically significant in the white matter

regions studied (Table 1) indicating that changes in T_2 relaxation time over the course of development were not different between the two genotypes. The significant effect of age in the corpus callosum, external capsule, and fimbria is indicative of developmental decrease in T_2 relaxation times [42] (Table 1). These trends in white matter regions of WT mice showed decreased T_2 relaxation times between PND 18 and PND 30 as expected over the course of myelination. In *Fmr1* KO mice, however, T_2 relaxation time decreased more sharply in the corpus callosum, external capsule, and fimbria between PND 21 and PND 30, suggesting delayed patterns in myelination. It should be noted that poor signal to noise prevented analysis of one *Fmr1* KO mouse brain at PND 30.

Magnetization Transfer Ratio

MTR is considered to be an index of macromolecular content in white matter, including myelin proteins and lipids that contribute to the structural integrity of white matter [38]. MTR detected differences in macromolecular content, indicative of differences in white matter maturation of *Fmr1* KO mouse brain compared to WT brain (Fig. 3). Figure 3a shows representative images at the center frequency of water protons for *Fmr1* KO and WT mouse brains at PNDs 18, 21, 30, and 60. Significant genotype $\times$ age interaction was found in MTR data for all white matter regions studied (Table 1). In WT mice, MTR tended to be fairly level between PND 18 and PND 30 and increased thereafter (Fig. 3). At PND 60, when the brain networks are more mature [32], MTR in all regions examined was higher in WT compared to *Fmr1* KO mice (Fig. 3). Before the peak of myelination at PND 18, MTR in the white matter regions of *Fmr1* KO mice was lower in the internal capsule ($t[2.56]$, $p < 0.05$), cerebral peduncle ($t[2.41]$, $p < 0.05$), fimbria ($t[2.42]$, $p < 0.05$), and cerebellar white matter ($t[2.58]$, $p < 0.05$) (Fig. 3d, e, f, and g, respectively) compared to WT mice. MTR in *Fmr1* KO mouse brain showed a steep increase between PND 18 and PND 30 followed by a decrease or leveling off between PND 30 and PND 60 (Fig. 3). No differences were found at PND 21. At PND 30, during a period when the neural networks begin to establish along with continued myelination in the mouse brain [32], the internal capsule ($t[2.21]$, $p < 0.05$) and fimbria ($t[2.33]$, $p < 0.05$) showed significantly higher MTR in *Fmr1* KO mouse brains compared to the WT (Fig. 3d, f, respectively). At PND 60, when the brain networks are more mature [32], lower MTR was observed in the corpus callosum ($t[2.29]$, $p < 0.05$), external capsule ($t[2.54]$, $p < 0.05$), and internal capsule ($t[2.27]$, $p < 0.05$) in *Fmr1* KO mouse brains

Fig. 3. Magnetization transfer ratio of white matter regions between *Fmr1* KO and WT mouse brains. The representative images at the center frequency of water protons are shown in **a** for *Fmr1* KO and WT mouse brains. Magnetization transfer ratio (mean $\pm$ standard error) from regional white matter between WT (gray dashed line) and *Fmr1* KO mice (black solid line) in the corpus callosum (**b**), external (**c**) and internal capsule (**d**), cerebral pe-

duncle (**e**), fimbria (**f**), and cerebellar white matter (**g**) at PND 18 (*Fmr1* KO $n = 9$, WT $n = 9$), 21 (*Fmr1* KO $n = 12$, WT $n = 14$), 30 (*Fmr1* KO $n = 13$, WT $n = 12$), and 60 (*Fmr1* KO $n = 6$, WT $n = 6$). A significant genotype $\times$ age interaction was found in all white matter regions. The significant genotype differences in MTR between WT and *Fmr1* KO mice are marked with * $p < 0.05$ and # $p < 0.10$.

Fig. 4. Myelin density of *Fmr1* KO and WT mouse brains. **a** Representative images of myelin staining with Black Gold II in WT and *Fmr1* KO mouse brains. Approximate ROIs are drawn with corpus callosum (CC) in red, external capsule (EC) in green, internal capsule (IC) in yellow, and fimbria (FI) in blue. The cerebral peduncle is not shown but is posterior to the internal capsule. The images were taken between Bregma -1.70 and -2.30 at PND 18 (*Fmr1* KO $n = 6$, WT $n = 6$), 21 (*Fmr1* KO $n = 6$, WT $n = 6$), 30 (*Fmr1* KO

$n = 6$, WT $n = 6$), and 60 (*Fmr1* KO $n = 6$, WT $n = 6$). The scale bar represents 500 μ m and is shown in the top left figure. Myelin density was measured by optical density normalized to the area of the ROI. Normalized optical density (mean $\pm$ standard error) changes are shown for each ROI including corpus callosum (**b**), external capsule (**c**), internal capsule (**d**), cerebral peduncle (**e**), and fimbria (**f**) for both genotypes. Significant differences between *Fmr1* KO and WT myelin density are indicated with * $p < 0.05$ and # $p < 0.10$.

compared to the WT (Fig. 3b–d, respectively). The significant p values from multiple pairwise comparisons passed false discovery rate with a threshold of $\alpha = 0.05$. The developmental differences in MTR between *Fmr1* KO and WT mouse brains suggested a lower content of myelin proteins and/or lipids at PND 18 and 60 in the white matter of *Fmr1* KO mouse brain. Overall, alterations in MTR are suggestive of fluctuation in macromolecule content during development in the *Fmr1* KO mouse brain that may have an impact on stable myelin structure formation.

Myelin Staining

To directly determine myelin content in brain, we stained tissue with Black Gold II, a histological stain for myelin [49]. Figure 4a shows the myelin staining of a representative section from *Fmr1* KO mouse brain and WT mouse brain at each age. Myelin density measured from Black Gold II staining showed significant interaction of genotype $\times$ age in the external capsule, internal capsule, cerebral peduncle, and fimbria (Table 1). The myelin density was lower in *Fmr1* KO mouse brain at PND 18 in the external capsule ($t[6.88]$, $p < 0.001$), internal capsule ($t[3.50]$, $p < 0.001$), and fimbria ($t[2.01]$, $p < 0.05$) compared to the corresponding regions of WT mouse brain (Fig. 4c, d, and f, respectively). Myelin density at PND 21 was elevated in the cerebral peduncle of *Fmr1* KO mouse brain compared to WT ($t[2.10]$, $p < 0.05$) (Fig. 4e). By PND 30, the myelin density was elevated in many white matter regions of the *Fmr1* KO mouse brain, including the external capsule ($t[4.82]$, $p < 0.001$), internal capsule ($t[2.11]$, $p < 0.05$), and fimbria ($t[4.61]$, $p < 0.001$) when compared to the corresponding regions of WT mouse brain (Fig. 4c, d, and f, respectively). At PND 60, the myelin density in *Fmr1* KO mouse brain was lower in the external capsule ($t[6.61]$, $p < 0.001$) compared to the WT brain (Fig. 4c). The significant p values passed false discovery rate ($\alpha = 0.05$) for multiple pairwise comparisons.

The myelin density in the white matter regions of *Fmr1* KO mice showed different developmental time courses than the WT mice (Fig. 4). In WT brain, myelin density increased steeply between PND 30 and PND 60 in the corpus callosum and external capsule, whereas in the corresponding regions of *Fmr1* KO brain, the increase in myelin density occurred between PND 18 and PND 30 and tended to level off thereafter. The myelin densities of internal capsule, cerebral peduncle, and hippocampal fimbria in WT brain increased steadily from PND 18 to 60, whereas in *Fmr1* KO brain, the myelin density in-

creased more gradually from PND 18 to 60. The myelin density in the external and internal capsules showed similar trends as the MTR results, where *Fmr1* KO mouse showed lowered myelin density at PND 18 followed by elevated levels at PND 30, which was reduced at PND 60 compared to the corresponding regions of WT mouse brain (Fig. 4).

Discussion

Myelination Delay in White Matter in the Developing Fmr1 KO Mouse

Myelination of axons in normally developing human brains starts prenatally and undergoes conspicuous growth during the first 2 years of life [51], then continues to increase at a lower rate from childhood into early adulthood, and declines in late adulthood [52, 53]. In comparison, the peak of myelination in the normally developing rodent brain occurs between PND 20 and 21 [32], grows steadily at a reduced rate throughout adulthood, and declines at around 18 months of age [32, 52, 53]. Moreover, the developmental process of myelination varies regionally [54].

Our results demonstrate an abnormal pattern of accumulation of macromolecules determined by MTR and myelin staining during early postnatal development in brains of *Fmr1* KO mice compared to brains of WT mice (Fig. 3, 4). The results of MTR and the myelin density measurements show similar trends in the *Fmr1* KO mouse brain, suggesting that histological changes in myelin density correspond closely with MTR in the fixed brain. In the present study, the differences in myelin density and MTR at PND 18, 30, and 60 in the *Fmr1* KO mice compared to WT suggests that alterations in forebrain and midbrain white matter, most notably in the external and internal capsules, occur throughout postnatal brain development including before the peak of myelination at PND 18 and during development and maturation of the neural networks at PND 30 and PND 60, respectively.

Reduced myelin thickness has been reported in the cerebellar white matter of *Fmr1* KO mice at PND 7, although differences were not observed at PND 15 or older ages [22]. However, reduced rates of myelination or delayed myelination may also be present at older ages, which could explain lowered MTR and myelin density at PND 18 and PND 60 found in white matter regions of the current study. Magnetization transfer imaging is sensitive to the macromolecules in white matter, particularly the proteins and lipids in myelin [55]. Previous in-depth research

demonstrated that cholesterol and myelin lipids contribute significantly to the magnetization transfer effect [55, 56]. The MTR from the present study suggests altered levels of white matter macromolecules, including lipids and proteins, at different stages of brain development that could contribute to altered myelin composition in *Fmr1* KO mice. When placed in context with myelin staining, the most prominent differences were alterations during the developmental phase of the internal capsule and later in the external capsule in the maturing *Fmr1* KO mice brains. The alteration in myelin composition and density during brain development suggests that the process of myelination is dysregulated during development in *Fmr1* KO brain, which could adversely influence neuronal function and potentially have a negative impact on the formation and maturation of brain networks. Future studies in the mouse model will incorporate in vivo functional imaging methods in *Fmr1* KO mice at the critical developmental ages revealed from the current studies.

Reduced MTR has been associated with pathological states of demyelination and axonal degeneration in patients with and in preclinical models of multiple sclerosis [37, 39, 40]. In an animal model of multiple sclerosis, lower MTR was found in the corpus callosum and external capsule of mice treated with cuprizone, an agent that caused demyelination [39]. It is important to note that similar MTR values were found for in vivo and ex vivo imaging of the mouse model of multiple sclerosis by Thiessen et al. [39].

A previous study with the MBP-deficient Shiverer mouse revealed no differences in Black Gold II staining compared to controls, suggesting that Black Gold II staining of myelin may have greater specificity to lipids [57]. Therefore, altered MTR and myelin staining in *Fmr1* KO mouse brain likely reflects differences in lipid content because myelin composition is composed of 70–85% lipids [54]. Thus, the similar trends between MTR and myelin density data from the present study suggest alterations in lipid levels in the developing *Fmr1* KO mouse brain, in addition to altered myelin protein levels reported by Pac-ey et al. [22]. A recent study reported reduced plasma cholesterol and reduced low- and high-density lipoproteins in male FXS patients (4–20+ years) compared to the normal control population [58]. Although it has not been determined whether brain cholesterol levels might be altered in FXS patients or *Fmr1* KO mice, cholesterol is known to be heavily incorporated into myelin particularly during the peak of myelination in the normal brain [59] and the cholesterol utilized for myelination is mainly synthesized in the brain [60]. Furthermore, an overall

cholesterol deficiency could contribute to impaired neurotransmission and has been associated with neurodegenerative diseases [61]. Thus, the lowered MTR and myelin density found in white matter of *Fmr1* KO mice may be due to decreased lipids and also the lack of FMRP regulation on the mRNA levels and synthesis of myelin proteins [6], which may lead to delayed myelination as observed in the present study.

White Matter Volume in the FXS Brain

Numerous MRI studies have reported alterations in brain volumes of young and adolescent FXS patients, including changes in regional gray and white matter volumes of cortical and subcortical regions, compared to normally developing children [30, 31, 62, 63]. Although the current findings do not show consistent differences in white matter volume between *Fmr1* KO and WT mouse brains, our data agree with a recent study that reported no difference in white matter volumes of the corpus callosum, internal capsule, and fimbria in fixed C57Bl6J *Fmr1* KO mouse brain compared to WT at PND 60 [64]. An earlier in vivo study from our group found increased volume of the hippocampus in *Fmr1* KO brains compared to WT brains [28]. The C57Bl6J strain of *Fmr1* KO mouse was chosen for this study because it is a widely used model of FXS [15, 16, 65] and a previous study demonstrating delayed myelination also used this model [22]. Although white matter volumes were not changed, the magnetization transfer imaging technique used in this study showed evidence of alterations in myelin and white matter in developing *Fmr1* KO mice.

Imaging the Fixed Brain

The imaging findings along with results from myelin staining suggest that magnetization transfer imaging may be particularly useful to better understand the microstructure of white matter in FXS brain in vivo. However, the current findings have to be taken in context of the limitations of the study. It is likely that the T_2 and MTR values measured in vivo may be different from those measured in a fixed tissue sample because the fixative solution has been shown to affect the T_2 and MTR values compared to in vivo measurements [33, 34]. In the present study precautions were taken to minimize the effect of fixative in T_2 relaxation and MTR in the fixed brain, based on published studies of fixed and in vivo brain samples [33, 34]. In addition, it has been shown that the data trends from fixed samples are similar to in vivo with T_2 [34] and with MTR following immersion of PFA fixed brain tissue in PBS [39].

Our goal in this study was to obtain an accurate assessment of any subtle changes in white matter development by obtaining high-resolution volumetric, T_2 , and MTR data. Thus, we elected to perform these measurements on fixed brains to stabilize brain structure, allow longer imaging durations, and eliminate confounds of any motion artifacts as in previous studies [22, 29, 64]. The findings of the current study regarding the utility of MTR in revealing developmental changes in *Fmr1* KO mouse are compelling. Future studies should focus on using MTR in conjunction with other advanced MRI techniques in vivo to assess their utility as imaging markers for FXS and also to assess whether such changes alter brain connectivity in *Fmr1* KO mouse.

Conclusions

In this study, we found altered levels of myelin and macromolecules in the white matter of *Fmr1* KO mouse brain at key developmental ages compared to the corresponding regions of WT mouse brain. We observed delayed myelination before the peak of myelination at PND 18, and then increased myelin density at PND 30 suggesting compensation for the delayed myelination, followed by lowered myelin levels in adult *Fmr1* KO mouse brain compared to WT mouse brain. Differences in white matter and myelin between *Fmr1* KO and WT mouse brains were found at several ages during development and could potentially contribute to changes in the brain networks of *Fmr1* KO mouse. Alterations in white matter and myelin that coincided with key stages of brain development including early myelination, the peak of myelination and synaptogenesis, neural network development, and maturation in mouse brain suggest dysregulation of myelination and white matter maturation in *Fmr1* KO mouse brain during development and suggest complex differences in white matter development which may ultimately result in altered brain function.

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Acknowledgements

The authors thank Dr. Jaylyn Waddell, Dr. Shiyu Tang, and Dr. Carolyn B. Smith for their advice and assistance with statistics. Thank also to the Core for Translational Imaging @ Maryland (C-TRIM), which is part of the University of Maryland School of Medicine Center for Innovative Biomedical Resources in Baltimore for providing the imaging services.

Statement of Ethics

The Institutional Animal Care and Use Committee at the University of Maryland, Baltimore approved the protocol used in this study.

Disclosure Statement

The authors have no conflicts of interests to disclose.

Funding Sources

This study was supported by the FRAXA Research Foundation, and NIH P01 grant HD016596 (M.C.M.).

Author Contributions

D.S. performed histological and MRI experiments, data analysis, and writing of manuscript. S.X. contributed technical and conceptual expertise to MRI and assisted in writing and proofreading of manuscript. J.Z. contributed technical and analytical expertise to MRI and assisted in proofreading of manuscript. M.C.M. contributed to design of the experiments, histological expertise, and preparation of manuscript. R.P.G. contributed to design of the experiments, MRI expertise, and preparation of manuscript.

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