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14. ABSTRACT

This project explores the potential windows for optimal use of dystrophin replacement strategies on neurodevelopment outcomes. The prolonged developmental process of myelination provides a therapeutic window of opportunity, but important unanswered questions remain. It remains unclear if postnatal dystrophin replacement would restore appropriate white matter development, and whether a critical window for therapeutic intervention exists. And, given the complexity of dystrophin expression in the brain, it may be that dystrophin replacement interfere with developmental trajectories that are regulated by endogenous dystrophins that are still present. The smaller dystrophin isoforms, which predominate in the postnatal brain, frequently remain expressed in DMD as their expression is driven by internal promoters that lie downstream of the more common DMD mutation hotspots. We are currently working to establish the cellular, temporal, and isoform requirements for potential dystrophin-replacing therapies, as well as for the capacity of dystrophin-replacement strategies to correct or disrupt brain developmental trajectories.

15. SUBJECT TERMS
None listed.

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INTRODUCTION

Our discovery that mouse models of Duchenne muscular dystrophy (DMD) have abnormalities in postnatal oligodendroglial development and myelination [1] provides an opportunity to understand if postnatal dystrophin replacement therapies may be appropriately timed to maximize the chance of preventing or attenuating neurological abnormalities. Our preliminary studies revealed that smaller dystrophin isoforms (Dp71, Dp140) predominate in the postnatal V/SVZ neural stem cell niche, the site of origin for most forebrain oligodendrocytes, as well as in developing oligodendroglia themselves [1]. We therefore hypothesize that dystrophins, *in particular the small dystrophin isoforms whose loss is correlated with increasing neurological disability* [2], may be required for appropriate V/SVZ niche function during postnatal development. We propose to generate a function map of when dystrophin loss matters to postnatal V/SVZ-driven neurodevelopment, as well as determine which particular DMD mutations and their associated patterns of isoform loss influence this process. Understanding the temporal basis for effective dystrophin restoration in the brain will provide a framework in which to appropriately test dystrophin-replacement therapies, such as systemic or CNS-targeted delivery of micro-dystrophin (mDys), for the ability to improve neurological function.

In the current research project we have been exploring how and when the loss of dystrophin affects distinct stages in postnatal brain development and maturation, as well as the assessment of the potential for dystrophin replacement to be a viable therapy to restore normal brain development. In addition, we will be exploring the interplay between potential dystrophin replacement therapies and their ability to alter the function of endogenous dystrophins that remain expressed in the CNS in the majority of DMD patients.

KEYWORDS

Dystrophin, myelination, neural stem cell, oligodendrocyte, brain development

ACCOMPLISHMENTS

3a. What were the major goals of the project?

The major goals of the project were divided into the three following specific aims:

Aim 1. Create a genotype-phenotype map for the impact of dystrophin loss on postnatal brain development. Using a DMD mouse model that lacks all brain-expressed dystrophin isoforms, we will assess the development of the V/SVZ neural stem cell niche and its ability to appropriately produce committed progenitors, followed by oligodendroglial development and myelination in relevant white matter structures. In addition, using inducible Cre driven expression of dystrophin-targeted shRNAs, we will define the time window that loss of dystrophin impacts these same developmental processes.

Aim 2. Establish the "critical window" for dystrophin restoration in the brain. Dystrophin-deficient mice (*mdx*^{3cv}) will have Dp427, Dp140, or Dp71 expression plasmids containing flox-stop-flox cassettes injected into the neonatal lateral ventricle, followed by electroporation to transduce the stem cells lining the ventricle. To achieve precise temporal control of construct expression *mdx*^{3cv} mice will have been crossed onto tamoxifen-inducible nestin-Cre^{ERT2} mice to inducibly-drive expression in neural stem cells. We will monitor the degree of dystrophin restoration, as well as its impact on neural stem cell function (e.g., oligodendrocyte production) and myelination.

Aim 3. Determine whether dystrophin restoration therapies interfere with endogenous dystrophin function in the developing postnatal brain. *Mdx* mice express all small dystrophin isoforms that predominate in the brain but lack full-length dystrophin, which predominates in muscle. *mdx* mice will have a micro-dystrophin (mDys) expression plasmid containing a flox-stop-flox cassette injected into the neonatal lateral ventricle, followed by electroporation to transduce the stem cells that line the ventricle To achieve precise temporal control of construct expression *mdx* mice will have been crossed onto tamoxifen-inducible nestin-Cre^{ERT2} mice to inducibly-drive expression in neural stem cells. We will monitor the degree and cellular location of dystrophin restoration, as well as oligodendrocyte production and myelination.

3b. What was accomplished under these goals?

Aim 1: While this award had an August 1, 2021 start date, during the first ~7.5 months we were in the process of obtaining regulatory approval for mouse work. This process ended up being more protracted than we had anticipated, during which after obtaining IACUC approval we submitted the ACURO, which then went through several rounds of revisions before achieving approval on March 23, 2022. As Aim 1 (and all the aims) is largely mouse work, we were not able to proceed with much of this Aim until late March. However we were still able to test out several key antibody reagents on other tissue samples that we had on hand in the lab, as well as research and optimize experimental strategies, all in preparation for the approved mouse experiments.

Subtask 1 has been completed, that of expanding all 3 DMD model mouse colonies (*mdx*, *mdx*^{4cv}, and *mdx*^{3cv}). For *mdx*^{3cv} mice (and heterozygous and wild type littermate controls), the focus of **Subtask 3**, we have collected an n of 8 of for tissue sections for all time points (postnatal day 8, 14, 21, and 57). We are still in the process of collecting time points for protein lysates. These lysates will be used for planned western blot analysis of myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and 2,′3′-cyclic nucleotide 3′-phosphodiesterase (CNP) protein levels, which are stage-specific proteins highly enriched specifically in myelinating oligodendrocytes. We have performed pilot western blots on a complete set of lysates for P21 (not shown), and will continue to analysis lysates from the other time points as these are collected.

In addition, we have perfused mutant *mdx*^{3cv} (and heterozygous and wild type littermate controls) and prepared floating sections on a cryostat for future immunohistochemistry (IHC; **Subtask 2 and 3**). Here we have collected a complete set of postnatal days 14, 21, and 57. We have performed IHC experiments to detect cellular events in the corpus callosum **(Subtask 3**) but are still in the process of performing IHC experiments for the SVZ neural stem cell niche **(Subtask 2)**. We have fully optimized all IHC antibody dilutions, staining and imaging conditions using extra littermate control floating sections.

We have collected tissue for Transmission Electron Microscopy (TEM) analysis, in order to assess myelin ultrastructure as well as the degree of myelination at the level of the individual axons **(Subtask 4)**. We have collected an n=4 for *mdx*, *mdx*^{4cv}, and *mdx*^{3cv} mouse models (and littermate controls) at postnatal days 14, 28, and 57. We have prepared the "thick sections" of glutaraldehyde-fixed tissue in our lab using a vibratome, and the TEM core manager has performed the embedding and the

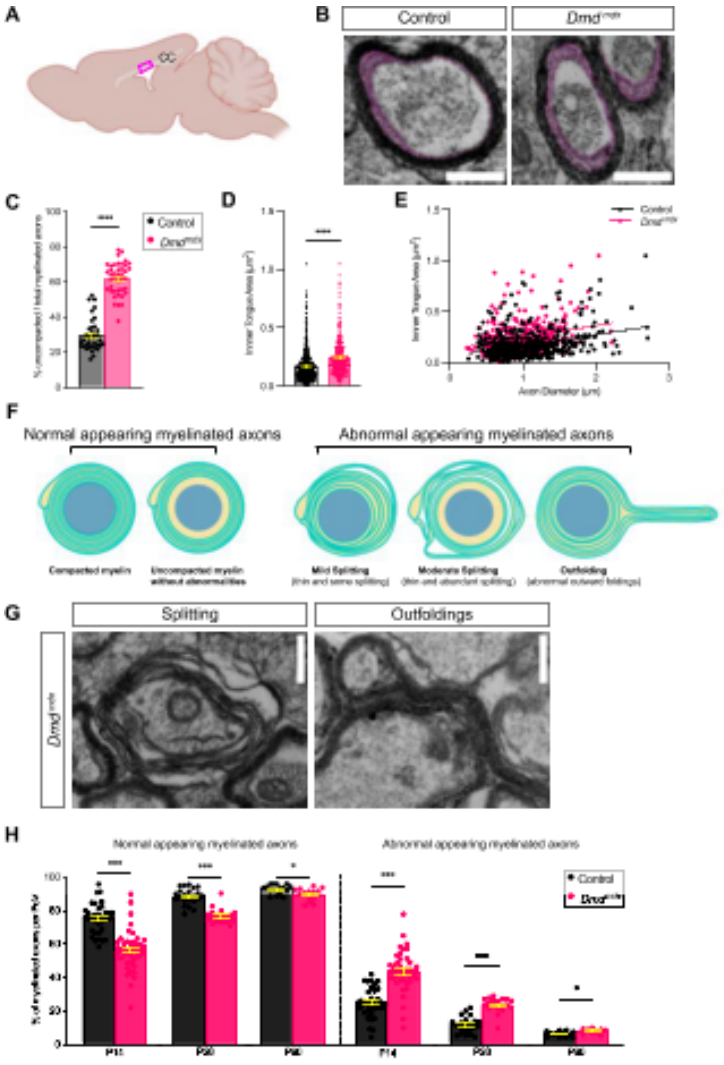

Fig. 1. Increased percentages of abnormal and uncompacted myelinated axons in *Dmd*^{mdx} mice during early myelination. (A) Coronal section schematic depicting area of the corpus callosum dissected for transmission electron microscopy analysis. (B) Electron micrographs of inner tongue (magenta). (C) Quantification of % uncompacted / total myelinated axons. *** P<0.001, unpaired two-tailed t-test. (D) Inner tongue area (μm^2) measured from electron micrographs, minimum 150 axons per mouse *** P<0.001, unpaired two-tailed t-test. (E) Inner tongue area (μm^2) vs axon diameter (μm). *** P<0.001, simple linear regression of intercepts, not significant simple linear regression of slopes. (F) Schematics of normal and abnormal appearing myelinated axons. (G). Representative electron micrographs of myelin splitting and outfoldings in *Dmd*^{mdx} mice. (H) Quantification of % of myelinated axons per FoV, normal appearing (left) and abnormal appearing (right). A minimum of 1,200 axons per genotype and on average 48 axons per FoV; 35 FoV for control and 42 FoV for *Dmd*^{mdx} mice; * P<0.05, *** P<0.001, multiple unpaired two-tailed t-test,*p<0.05; ***p<0.001. Scale bars 500nm. Error bars denote SEM. *Abbreviations: corpus callosum (CC);* field of view (FoV).

ultrathin sections for a subset of these sections. The TEM core manager has trained 2 graduate students in our lab to image the grids on the electron microscope. We have collected images and performed TEM analysis on postnatal days 14, 28, and 57 samples.

During myelination, mature oligodendrocyte processes wrap around axons many times, ultimately generating a mature, multi-layered myelin sheath of stereotypical thickness relative to axon thickness. Aside from wrapping tightly around an axon segment, myelin undergoes compaction, a process by which the oligodendrocyte cytosolic region ("inner tongue") that lies between the myelin layers and the axon cell surface largely disappears [3]. We performed ultrastructural analysis of the myelinated axons in the corpus callosum, in cross section, at P14, P28 and 2 months (P60) (Fig. 1). While a modest degree of uncompacted myelin is a normal feature of early myelination, we observed that *Dmd*^{mdx} mice at P14 had a significantly higher percentage of *uncompacted* myelinated axons (within total myelinated axons) than that in control littermates, mean 29.70% ± 1.533% in control and 61.82% ± 1.420% in *Dmd*^{mdx}. (Fig. 1B,C). To quantify the degree of compaction more precisely, we measured the area of the inner tongue as previously described [4, 5], and observed that *Dmd*^{mdx} mice had a significantly larger inner tongue area, mean area 0.1735 µm ± 0.006 µm in control and 0.2442 µm ± 0.008 µm in *Dmd*^{mdx} (Fig. 1D). Furthermore, we observed that inner tongue areas were larger in myelinated axons from *Dmd*^{mdx}

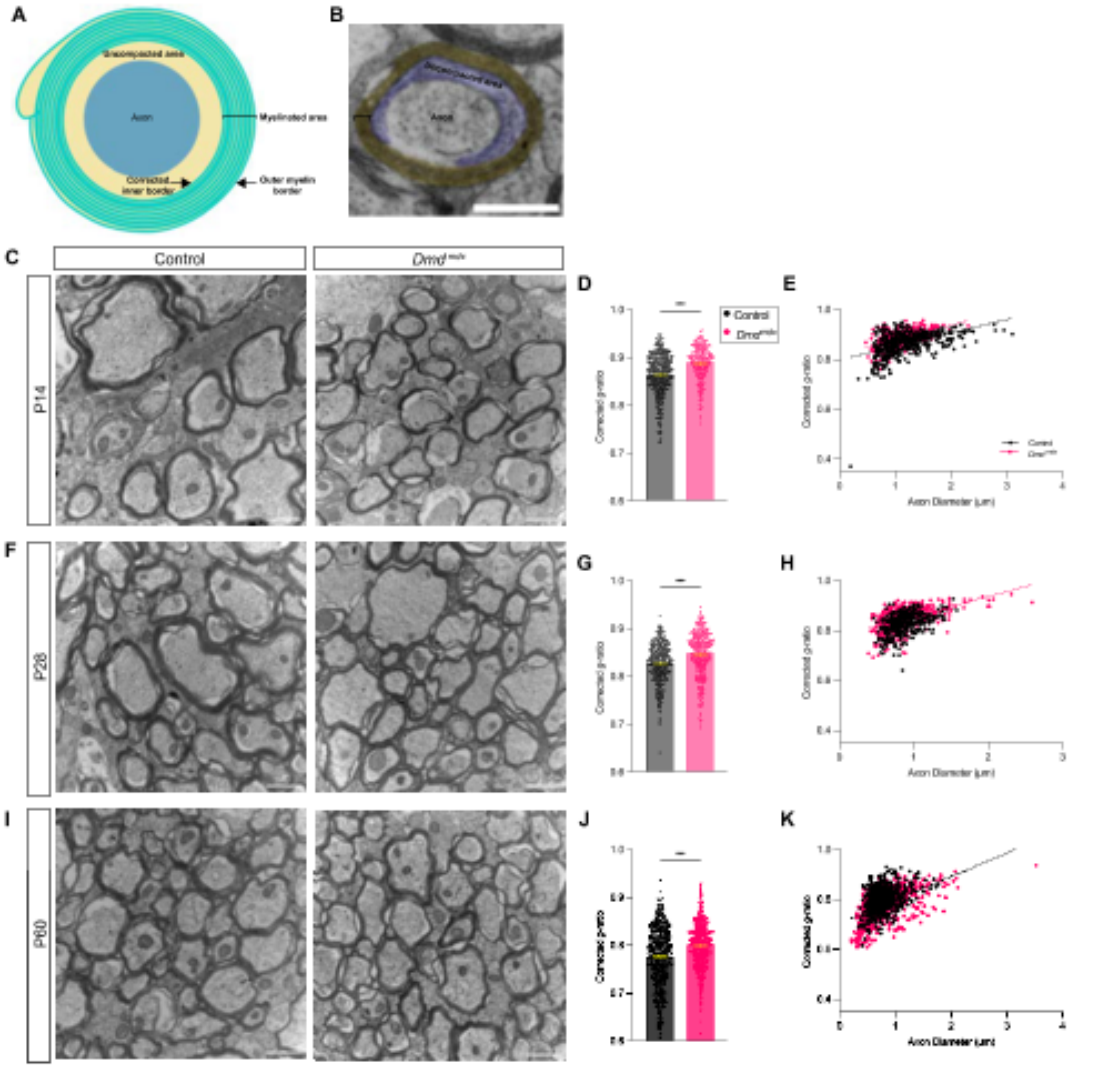

Fig. 2. *Dmd*^{mdx} mice have thinner myelin at all ages assessed.
(A) Schematic representation of an uncompacted myelinated axon. The corrected inner border is shown to illustrate how the myelin area is determined for the corrected *g*-ratio analysis. (B). Electron micrograph with an example of an uncompacted area (blue) and myelin (yellow). (C) Representative electron micrographs from the corpus callosum at P14 (left, control; right, *Dmd*^{mdx}). (D) Corrected *g*-ratio measurements from P14 control and *Dmd*^{mdx} mice. *** *P*<0.001, unpaired two-tailed t-test. (E) Scatterplot depicting corrected *g*-ratio vs. axon diameter (µm) at P14. *** *P*<0.001 simple linear regression of intercepts, not significant simple linear regression of slopes. (F) Representative electron micrographs from the corpus callosum at P28 (left, control; right, *Dmd*^{mdx} mice). (G) Corrected *g*-ratio measurements from P28 control and *Dmd*^{mdx} mice. *** *P*<0.001, unpaired two-tailed t-test. (H) Scatterplot depicting corrected *g*-ratio vs. axon diameter (µm) at P28. *** *P*<0.001 simple linear regression of intercepts, not significant simple linear regression of slopes. (I) Representative electron micrographs from the corpus callosum at P60 (left, control; right, *Dmd*^{mdx} mice). (J) Corrected *g*-ratio measurements from P60 control and *Dmd*^{mdx} mice. *** *P*<0.001, unpaired two-tailed t-test. (K) Scatterplot depicting corrected *g*-ratio vs. axon diameter (µm) at P60. *** *P*<0.001 simple linear regression of intercepts, not significant simple linear regression of slopes. Scale bar 1µm (C, F, H). A minimum of 150 axons per mouse. Error bars denote SEM.

regardless of axon diameter (Fig. 1E). These data suggest that myelin compaction is impaired during early myelination.

In addition to alterations in myelin compaction in *Dmd*^{mdx} mice, we also noted a higher frequency of abnormal appearing myelin structures such as myelin splitting and outfoldings (Fig. 1F). Myelin splitting and outfoldings were seen throughout all ages (P14, P28 and P60), and were observed at a significantly higher percentage in *Dmd*^{mdx} than in control littermates (Fig. 1H). This difference was the greatest at P14, where 43.64% ± 1.514% of axons per field of view (FoV) in the *Dmd*^{mdx} mice were abnormal appearing, compared to only 24.77% ± 1.827% in control littermates, and these findings were still substantial at P28 (23.42% ± 1.016% in *Dmd*^{mdx} vs 11.57% ± 1.035% in control) (Fig. 1H). By 2 months (P60), abnormal structures were rare, but a small yet significant difference was still observed between genotypes (7.878% ± 0.5027% in *Dmd*^{mdx} vs 6.092% ± 0.4497% in control) (Fig. 1H). Of note, most abnormal structures present in both genotypes were varying degrees of myelin splitting, whereas outfoldings were rare. The high percentage of these abnormal myelin structures in the *Dmd*^{mdx} mouse, particularly early in myelination, indicates that Dp427 might be involved in early phases of myelin compaction.

We also determined the thickness of the myelin by measuring the *g*-ratio, which determines the thickness of the compact myelin relative to the thickness of the axon. To adequately do this measurement, we calculated a "corrected *g*-ratio" [4]. As illustrated in Fig. 2A, the "corrected *g*-ratio" follows the inner myelin border rather than the axon border, to avoid incorrectly including the inner tongue area as myelin. We observed that at all ages evaluated (P14, P28 and P60), *Dmd*^{mdx} mice had significantly thinner myelin (higher *g*-ratio) compared to control littermates. (Fig. 2C-K). We additionally binned *g*-ratios based on axon diameter, and found that at P21, regardless of axon diameter, the myelin was thinner (not shown). By P21 and 2 months (P60), a pattern emerged in which axons with a diameter of smaller than 1.5μm had significantly thinner myelin, whereas axons greater than 1.5μm did not have a significant difference in myelin thickness (not shown). This finding is consistent with the observation of less MBP expression in the corpus callosum, particularly at younger timepoints (P14 and P21).

Over the coming 6 months we will complete the analysis for the other DMD models, enabling us to achieve a more complete picture of myelin abnormalities that accrue with increasing loss of DMD isoforms. Our initial analysis indicates that all three DMD models have significant myelin abnormalities, to varying degrees (not shown).

Aim 2: As with Aim1, we were not able to begin mouse work on this aim until March 23, 2022. We have thus far successfully expressed a Dp71 plasmid in cultures (Subtask 7 and 8). We will proceed to neonatal electroporation to deliver this plasmid to the developing V-SVZ in the coming year. In addition, we have started to analyze the different Dp71 isoforms in developing oligodendrocytes, as we noted that these cells express at least two distinct isoforms. This more detailed view of the isoform distribution will enable us to better target replacement constructs (for instance, Dp71d versus Dp71f, which have differential cytosolic-nuclear distribution patters). Our pilot work on this indicates that immature oligodendrocytes favor Dp71f expression while mature oligodendrocytes switch their Dp71 expression to favor Dp71d expression (not shown).

Aim 3: As with Aims1 and 2, we were not able to begin mouse work on this aim until March 23, 2022. However, we have been working on the mixed glial cultures as in Aim 2, as a good way to assess microdystrophin effects on *mdx* cultures. These cultures are in use for testing expression over the next 6 months **(Subtask 10 and 11).**

3c. What opportunities for training and professional development has the project provided?

There have been extensive training opportunities during the project. The PI has been training three graduate students in oligodendrocyte purifications and cell culture assay techniques, as well as for immunohistochemistry, confocal image acquisition, and protein lysate preparation and analysis. In addition, the TEM core facility manager has been training two graduate students to prepare samples and acquire images on the electron microscope. As a result, two graduate students have made substantial contributions to the project during the past 12 months, with both of them being authors on a manuscript that is now in revisions.

3d. How were the results disseminated to communities of interest?

A graduate student who is working on the projected presented the findings at a graduate student symposium as well as at an international conference. In addition, a manuscript was submitted to the journal "Disease

Models and Mechanisms", and we are currently working on the revisions prior to resubmitting this in September.

3e. What do you plan to do during the next reporting period to accomplish the goals?
We will continue to work on establishing appropriate plasmids and experiments in Aims 2 and 3. We will continue to determine the full extent of *mdx*^{3cv} phenotypes (stem, progenitor, and oligodendrocyte cellular phenotypes including proliferation, differentiation, and survival, myelination timing, and myelination ultrastructure) and prepare a manuscript to submit to a journal on these findings in early 2024.

IMPACT

4a. What was the impact on the development of the principal discipline(s) of the project?
Given that neurological function is frequently compromised in DMD, it is important to also determine whether dystrophin replacement can be successfully achieved to mitigate or even correct neurological dysfunction in DMD patients. In addition, determining the appropriate timing for therapeutic intervention in the CNS is critical to develop successful approaches. Thus far we have established particular windows when neurodevelopmental disturbances are present in mouse models, thus revealing the timing of when therapies would be beneficial.

4b. What was the impact on other disciplines?
Given that myelination disturbances are a feature of many neurodevelopmental disorders (e.g., leukodystrophies, autism), establishing whether gene restoration before, during, and after myelination can be successful in modulating aberrant myelination trajectories, may prove useful more broadly in those conditions.

4c. What was the impact on technology transfer?
Nothing to report.

4d. What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS:

5a. Changes in approach and reasons for change
Due to technical issues with V-SVZ neurosphere cultures that affected their consistency and viability, we optimized a mixed glial culture that is highly enriched for oligodendrocyte progenitor cells for use with plasmids or viral particles. This allows us to track construct expression in emerging oligodendrocytes as the progenitors differentiate.

5b. Actual or anticipated problems or delays and actions or plans to resolve them.
While this award had an August 1, 2021 start date, during the first ~7.5 months we were in the process of obtaining regulatory approval for mouse work. This process ended up being more protracted than we had anticipated, during which after obtaining IACUC approval we submitted the ACURO, which then went through several rounds of revisions before achieving approval on March 23, 2022. As all Aims are largely mouse work, we were not able to proceed with much of the project until late March of 2022.

5c. Changes that had a significant impact on expenditures.
Due to the delay in awaiting ACURO approval, we were not able to use funds to support the animal work on this project for much of the first year.

5d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Nothing to report.

5e. Significant changes in use or care of human subjects.
Not applicable.

5f. Significant changes in use or care of vertebrate animals.
Nothing to report.

5g. Significant changes in use of biohazards and/or select agents.
Not applicable.

PRODUCTS:

Not applicable.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Holly Colognato*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.8*
Contribution to Project:	*Dr. Colognato has designed the project, supervised the project personnel, and analyzed data sets.*
Funding Support:	

Name:	*Maryam Aziz*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Ms. Azmi has optimized culture conditions, characterized immunohistochemistry experiments, and helped expand and maintain mouse colonies. Ms. Azmi has also optimized methods to assess myelin proteins and mRNA in western blots and qRT-PCR, and has analyzed myelin levels in tissue sections.*

Funding Support:	

Name:	*Zijian Shao*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	*Mr. Shao has optimized mRNA analysis using qRT-PCR, performed culture experiments, and optimized and analyzed Transmission Electron Microscopy experiments. In addition, he has performed plasmid preparation and analysis of dystrophin isoforms.*
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Yes; see attached for revised Support document.

What other organizations were involved as partners?
Nothing to report.

SPECIAL REPORTING REQUIREMENTS

Not applicable.

APPENDICES

Not applicable.

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