AWARD NUMBER: W81XWH-21-1-0562

TITLE: Can Dystrophin-Replacement Therapies Improve Cognitive Function in DMD? Development of Strategies to Maximize Effectiveness and Avoid Detrimental Effects

PRINCIPAL INVESTIGATOR: Holly Colognato

CONTRACTING ORGANIZATION: Research Foundation for the State University of New York
Stony Brook, NY

REPORT DATE: August 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2022	Annual	01Aug2021-31Jul2022

4. TITLE AND SUBTITLE

Can Dystrophin-Replacement Therapies Improve Cognitive Function in DMD? Development of Strategies to Maximize Effectiveness and Avoid Detrimental Effects

5a. CONTRACT NUMBER
W81XWH-21-1-0562

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Holly Colognato

E-Mail: holly.colognato@stonybrook.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Research Foundation for the State University of New York
West 5510 Frank Melville Memorial
Library
Stony Brook NY 11794-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	9	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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 are exploring how and when the loss of dystrophin affects distinct stages in postnatal brain development and maturation, as well as whether (and when) replacement of dystrophin has the possibility to restore normal brain developmental trajectories. In addition, we will be exploring the interplay between potential dystrophin therapeutics, such as AAV9-delivered microdystrophins, and how these exogenous dystrophins could 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, which were each further subdivided into multiple subtasks, as follows:

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.

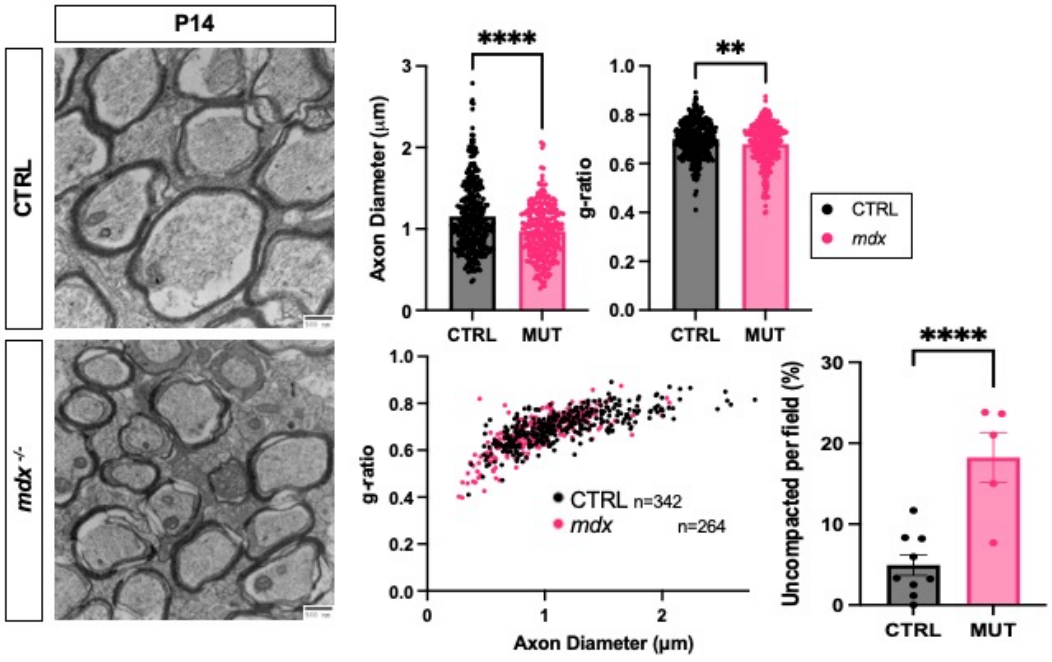
3b. What was accomplished under these goals?

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.

While awaiting regulatory approval, we were able to embark upon **Subtask 1** and appropriately expand all 3 DMD model mouse colonies (*mdx*, *mdx*^{4cv}, and *mdx*^{3cv}), and therefore by spring could start collecting tissue for analysis. For *mdx*^{3cv} mice (and heterozygous and wild type littermate controls), the focus of **Subtask 3**, we have collected an n of 6 of for both corpus callosum and cerebral cortex protein lysates for postnatal day 14 (P14), P21, and P57. We are still in the process of collecting earlier time points as well as additional n for the later time points in order to have an n of 8 for each. 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. While waiting to perform these western blots on a complete set of lysates, we have optimized our conditions for all 3 antibodies using pilot protein lysates from wildtype litters.

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 thus far collected P14 (n=3), P21 (n=8), and P57 (n=8). Once we have obtained the complete set we will embark upon IHC to detect both cellular events in the SVZ neural stem cell niche (Subtak 2) and the corpus callosum **(Subtask 3**). During this time we have fully optimized all IHC antibody dilutions, staining and imaging conditions using extra littermate control floating sections.

We have also begun to collect 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)**. Thus far we have collected an n=2 for *mdx*, *mdx*^{4cv}, and *mdx*^{3cv} mouse models (and littermate controls) at P14, and n=2 for mdx at P28 and P57. While we prepare the so-called thick sections of glutaraldehyde-fixed tissue in our lab using a vibratome, the TEM core manager has performed the embedding and the ultrathin sections. She has gone on to train a graduate student in our lab to image the grids on the electron microscope. We have therefore recently begun TEM analysis on P14 and P28 samples only but should be completing the analysis of these sets, at an n=2, in a few weeks (we have an additional 2n for each genotype and control that have been fixed and embedded, but are awaiting thin sections) We have started to analyze the g-ratio (axon diameter divided by the diameter of the myelin sheath; **Figure 1**). However, we quickly noted that many of the myelinated axons in the mdx mutant sample images were uncompacted (with a large zone of cytosol in between the axon and the myelin) and thus the g-ratio was smaller (i.e., typically indicative of more myelin) in those axons despite having the appearance of less myelin. The unusually large degree of uncompacted versus compacted myelin sheaths is shown in the graph to the right (n<0.0001). We are now working on optimizing a different method for assessing myelin thickness by tracing the inner and outer myelin



boundaries to obtain an area, which will be plotted relative to the axon diameter. While we have not analyzed P28 as extensively yet, we do note that P28 also has unusual structures including unusual levels of uncompacted myelin (not shown), indicating that this alteration may not simply reflect a delay in myelination. Over the coming 6 months we will collect additional samples for TEM and complete their analysis, enabling us to achieve a more complete picture of myelin abnormalities in DMD mouse models.

Aim 2: As with Aim1, we were not able to begin mouse work on this aim until March 23, 2022. However out of the 3 dystrophin isoforms we hope to express in mdx3cv mice for this aim, we now have a working Dp71 plasmid (Subtask 7 and 8). We will soon be gearing up to validate Dp71 plasmid in cell cultures, and if successful gene expression is obtained, we will proceed to neonatal electroporation to deliver this plasmid to the developing V-SVZ.

In addition, we have been working to establish a better culture model for assessing dystrophin plasmids in cultures, including *mdx*^{3cv} "rescue" cultures. Here, the V-SVZ neurospheres have been difficult (high variability) to work with so we have piloted doing mixed glial cultures from neonatal forebrains, including those from *mdx*, *mdx*^{4cv}, and *mdx*^{3cv} mice. These cultures are now optimally working, giving us consistent oligodendrocyte progenitor expansion followed by oligodendrocyte differentiation upon switching from a serum-containing growth medium to a defined medium with pro-differentiation factors such as T3.

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 now up and running and we plan to test them for this Aim in 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 one of them in immunohistochemistry, confocal image acquisition, and protein lysate preparation and analysis. In addition, the TEM core facility manager has been training one graduate student to prepare samples and acquire images on the electron microscope. As a result several graduate students has been making substantial contributions to the project during the past 12 months.

3d. How were the results disseminated to communities of interest?
We have not yet presented this work at an external conference, however a graduate student who is working on the projected has presented the preliminary findings at a graduate student symposium this year.

3e. What do you plan to do during the next reporting period to accomplish the goals?
We will continue to design and construct the remaining plasmids for experiments in Aims 2 and 3, and will test out each one, first in cell culture using our newly established and optimized glial culture system, followed by in vivo analysis using nasal electroporation. We will continue to *mdx*^{3cv} phenotypes (stem, progenitor, and oligodendrocyte cellular phenotypes including proliferation, differentiation, and survival, myelination timing, and myelination ultrastructure).

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, beginning to map the points 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
We have performed some pilot experiments using V-SVZ neurosphere cultures and had difficulty with their consistency and viability. Therefore we piloted a modified form of mixed glial cultures that are highly enriched for oligodendrocyte progenitor cells in which we can transfect with plasmids or infect with viral vectors and then track oligodendrocyte construct expression as the progenitors differentate.

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 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 used that time to optimize many of our assays and reagents, which should speed analysis going forward.

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 supervised the project.*
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 assessments, and helped expand colonies. In addition Ms. Azmi has been working on optimizing methods to assess myelin proteins and mRNA in western blots and qRT-PCR, respectively.*
Funding Support:	

Name:	*Mohanllal Narine*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*5*

Contribution to Project:	*Mr. Narine has worked on optimizing methods to assess myelin proteins and mRNA in western blots from oligodendrocyte cultures, as well as performed culture preparations.*
Funding Support:	

Name:	*Erika Deppenschmidt*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	*Ms. Deppenschmidt has helped optimize mRNA analysis using qRT-PCR, as well as assisted with culture preparation and maintenance. In addition, she has performed plasmid preparation and analysis.*
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.

REFERENCES.

1. Aranmolate, A., N. Tse, and H. Colognato, *Myelination is delayed during postnatal brain development in the mdx mouse model of Duchenne muscular dystrophy.* BMC Neurosci, 2017. **18**(1): p. 63.
2. Doorenweerd, N., *Combining genetics, neuropsychology and neuroimaging to improve understanding of brain involvement in Duchenne muscular dystrophy - a narrative review.* Neuromuscul Disord, 2020. **30**(6): p. 437-442.