AWARD NUMBER: W81XWH-14-1-0282

TITLE: Developing Gene Silencing for the Study and Treatment of Dystonia

PRINCIPAL INVESTIGATOR: Pedro Gonzalez-Alegre, MD, PhD

CONTRACTING ORGANIZATION: Children's Hospital of Philadelphia
Philadelphia, PA 19104

REPORT DATE: December 2017

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
December 2017	Final	9/30/2014-9/29/2017

4. TITLE AND SUBTITLE
Developing Gene Silencing for the Study and Treatment of Dystonia

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0282

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Pedro Gonzalez-Alegre

Email: gonzalezap@email.chop.edu

5d. PROJECT NUMBER
0010495692

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Children's Hospital of Philadelphia
Research Institute
3501 Civic Center Blvd
Philadelphia, PA 19104-4318

8. PERFORMING ORGANIZATION REPORT NUMBER
001

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
and Materiel 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: Dystonia is a disabling and incurable neurological disorder characterized by twisting movements that cause significant disability. Some forms of dystonia are caused by genetic mutations and, therefore, passed on from generation to generation. The most common form of early onset genetic dystonia is a disease known as DYT1, in which children around age ten develop dystonia, usually in a leg or arm, and over 2 or 3 years spreads to affect all body parts causing substantial disability. There is no cure for DYT1. Therefore, the development of new treatments for DYT1 is a priority in dystonia research.

Here, based on prior scientific reports from our research group and other investigators, we hypothesize that we could use two complementary approaches, known as RNA interference or antisense oligonucleotide therapy, to prevent neurons from making the mutated or "toxic" protein that causes DYT1 dystonia. By doing this in the appropriate brain region, we should be able to reverse the symptoms of the disease. We have already demonstrated that this is possible but using cells growing in a dish in the laboratory, not in living animals. In this project, we aim to answer several specific questions: *Is this treatment approach helpful and safe? What is the area of the brain in which we should inject this RNA interference vector to eliminate the symptoms? Are the motor deficits in DYT1 dystonia reversible?* We propose to use a novel rat model of DYT1 dystonia and infuse antisense oligonucleotides or viral vectors mediating RNA interference to suppress expression of the mutated protein in their brain. They will target different areas of the brain, and we will measure if they are able to reverse known abnormalities that occur in the brain of DYT1 rats, including abnormal motor function. More importantly, we will check if no side effects or toxicity occurs. Successful completion of our studies will move us a step closer to design of what is known as a phase 1 safety trial in humans afflicted by this debilitating disease. This would be a giant milestone in dystonia research. In addition, these studies will provide very helpful information on the areas of the brain that are responsible, not only for DYT1, but also for other forms of dystonia.

15. SUBJECT TERMS

Nothing 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**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	25	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION

Dystonia is a debilitating neurological disease with no cure that is characterized by involuntary muscle contractions that cause abnormal twisting postures. DYT1 dystonia, an autosomal dominant disease, the most common early onset inherited dystonia. DYT1 is caused by a common deletion of a single amino acid (ΔE) in torsinA. Abundant evidence suggests that suppressing expression of torsinA(ΔE) through gene silencing would be beneficial. Our overall hypothesis is suppressing expression of torsinA(ΔE) through RNA interference (RNAi) or antisense oligos (ASO) will be a safe an effective treatment for DYT1 dystonia. In innovative proposal, we use a rat model of DYT1 to test the efficacy and safety of viral (AAV)-mediated RNAi or intraventricular delivery of ASO in vivo. Key questions on dystonia research that we will address are whether the DYT1 phenotype is reversible and what is the neuroanatomical substrate that causes motor dysfunction in dystonia.

2. KEYWORDS

adeno-associated virus (AAV); antisense oligonucleotide (ASO); RNA interference (RNAi); torsinA; DYT1; dystonia; gene silencing; cerebellum; striatum; therapy;

3. ACCOMPLISHMENTS

We will list the major goals of the project as stated in the approved SOW for the entire funding period (months 1-36) and summarize the accomplishments and status of those goals.

What were the major goals of the project?

Task 1. Regulatory review and approval processes for animal studies (2-4 months): completed at month 5.

Task 2. Dose-finding experiments with ASO in DYT1 knock in rats. There are two genotypes (DYT1 and WT), two different ASO (#3 and MIS) and three doses of each. Therefore, there are a total of 12 experimental groups, each comprised of 12 rats. This

experiment will require 72 DYT1 KI rats and 72 wild type littermates. DYT1 rats reproduce with mendelian frequency. We will need to generate 144 total mice (72 WT and 72 DYT1). We will complete this study with four separate cohorts (4 weeks apart) due to the required number of animals. Once tissues are collected, they will be processed simultaneously (both histology and molecular studies).

2a. Animal cohort 1:

1) Generation of animal cohort 1 (months 1-4). We will generate 2 month-old rats (18 DYT1 and 18 WT): completed by month 5.

2) Baseline behavior (month 4): completed by month 5.

3) Infusion of ASO during 2 weeks (month 4-5): completed by month 6.

4) Post-infusion behavioral testing/sacrifice (month 5): completed by month 6

5) Molecular and histological analyses (months 8-14): completed by month 20.

2b. Animal cohort 2:

1) Generation of animal cohort 2 (months 2-5). We will generate 2 month-old rats (18 DYT1 and 18 WT): completed by month 6.

2) Baseline behavior (month 5): completed by month 6.

3) Infusion of ASO during 2 weeks (month 5-6): completed by month 7.

4) Post-infusion behavioral testing/sacrifice (month 6): completed by month 7.

5) Molecular and histological analyses (months 8-14): completed by month 20.

2c. Animal cohort 3:

1) Generation of animal cohort 3 (months 3-6). We will generate 2 month-old rats (18 DYT1 and 18 WT): completed by month 8.

2) Baseline behavior (month 6): completed by month 8.

3) Infusion of ASO during 2 weeks (month 6-7): completed by month 9.

4) Post-infusion behavioral testing/sacrifice (month 7): completed by month 9.

5) Molecular and histological analyses (months 8-14): completed by month 20.

2d. Animal cohort 4:

1) Generation of animal cohort 4 (months 4-7). We will generate 2 month-old rats (18 DYT1 and 18 WT): completed at month 12.

2) Baseline behavior (month 7): completed at month 12.

3) Infusion of ASO during 2 weeks (month 7-8): completed by month 13.

4) Post-infusion behavioral testing/sacrifice (month 8): completed by month 13.

5) Molecular and histological analyses (months 8-14 completed by month 20.

Task 3. Therapeutic trial with ASO in DYT1 rats. Once a dose has been selected from previous studies, a therapeutic study will be conducted in DYT1 transgenic males and control littermates. As the primary outcome of this experiment is behavior, we will use only males. This experiment includes two genotypes (DYT1 and WT) and two ASO (#3 and MIS) at a single dose. Each experimental group includes 16 animals. Therefore, this experiment will require 32 DYT1 KI and 32 wild type littermates. DYT1 mice reproduce with mendelian frequency. Expecting a 50:50 male:female ratio, we will need to generate 128 total rats (64 WT and 64 DYT1). Because these animals will be used once they reach 12 months of age, and due to the inherent variability in genotypes and sex of animals obtained, we expect to generate a cohort of ~175 rats. Females will be sacrificed early and males of both genotypes will be maintained to reach the experimental age. We will complete this study with two separate cohorts (4 weeks apart) due to the required number of animals.

3a. Animal cohort 1:

1) Generation of animal cohort 1 (months 6-18). We will generate 12 month-old male rats (16 DYT1 and 16 WT): cohort was bred between months 6-10 and started the process of aging to the target age of 12 months as planned. However, due to the findings in Task#3 explained below, the planned therapeutic trial with ASO was no longer sacientifically reasonable and was not completed. This was reported in our Progress Report in Year 2 and approved by the scientific program officers.

2) Baseline behavior (months 18)

3) Infusion of ASO during 4 weeks (months 19)

4) Post-infusion behavioral testing all animals (months 19-20).

5) Sacrifice of half of animals in each group (month 19-20)

6) Behavioral testing in all surviving animals (months 21-22).

7) Sacrifice of all surviving animals (month 21-22)

8) Molecular and histological analyses (months 22-28)

3b. Animal cohort 2:

1) Generation of animal cohort 2 (months 7-19). We will generate 12 month-old male rats: cohort was bred between months 6-10 and started the process of aging to the target age of 12 months as planned. However, due to the findings in Task#3 explained below, the planned therapeutic trial with ASO was no longer sacientifically reasonable and was not completed. This was reported in our Progress Report in Year 2 and approved by the scientific program officers.

2) Baseline behavior (months 19)

3) Infusion of ASO during 4 weeks (months 20)

4) Post-infusion behavioral testing all animals (months 20-21).

5) Sacrifice of half of animals in each group (month 20-21)

6) Behavioral testing in all surviving animals (months 22-23).

7) Sacrifice of all surviving animals (month 22-23)

8) Molecular and histological analyses (months 23-28)

The findings observed upon completion of tasks 1 and 2 led us to conclude that ASO#3 does not reduce expression of torsinA(ΔE) transgenic in rats *in vivo*. Furthermore, we found evidence of toxicity that could be even more prominent in transgenic rats over wild type littermates. As a consequence, it was not scientifically reasonable to complete the proposed Task 3. Task #3 was proposed as a therapeutic trial of ASO#3 in aged rats using the dose identified as efficacious in Task#2. As we found no efficacy with any dose, and detected potential toxicity (as potentially predicted in the Alternative Outcomes and Strategies section of the initial application), we decided to move on to Tasks #4 and #5. This was approved after submission of the Progress Report for Year 2.

Task 4. To generate AAV2/1.CMVGFP.miRNA. We have shuttle plasmids to generate these viruses. We anticipate requiring only one prep from each virus (active and control) for the proposed experiments. We will give the shuttle plasmids to the Vector Core Facility in month 6, and expect to receive the viruses in month 7. We will do a pilot study in cultured HEK cells monitoring for GFP expression by fluorescence microscopy to verify expression (month 7): Completed at month 18.

Task 5. To evaluate the efficacy and safety of AAV-RNAi in striatum and cerebellum of DYT1 rats. As the primary outcome of this experiment is behavior, we will use only males. This experiment includes two genotypes (DYT1 and WT), two AAV (miTorA and miMIS) and two target tissues (cerebellum and striatum) at a single dose. Each experimental group (8 total) includes 12 animals. Therefore, this experiment will require

48 DYT1 KI and 48 wild type littermates. DYT1 mice reproduce with mendelian frequency. Expecting a 50:50 male:female ratio, we will need to generate 192 total rats (96 WT and 96 DYT1). Because these animals will be used once they reach 12 months of age, and due to the inherent variability in genotypes and sex of animals obtained, we expect to generate a cohort of ~250 rats. Females will be sacrificed early and males of both genotypes will be maintained to reach the experimental age. We will complete this study with two separate cohorts (4 weeks apart) due to the required number of animals.

5a. Animal cohort 1 (targeting striatum):

1) Generation of animal cohort 1 (months 12-24). We will generate 12 month-old male rats (24 DYT1 and 24 WT): completed at month 20

2) Baseline behavior (months 24): completed at month 24.

3) Injection of AAV into striatum (month 25): completed al month 26

4) Post-injection behavioral testing#1 (month 26): completed at month 28

5) Post-injection behavioral testing#2/sacrifice (months 28-29): completed at month 30

6) Molecular and histological analyses (months 30-36): completed at month 36.

5b. Animal cohort 2 (targeting cerebellum):

1) Generation of animal cohort 2 (months 13-25). We will generate 12 month-old male rats (24 DYT1 and 24 WT): see deviation from protocol detailed below.

2) Baseline behavior (months 25)

3) Injection of AAV into cerebellum (month 26)

4) Post-injection behavioral testing#1 (month 27).

5) Post-injection behavioral testing#2/sacrifice (months 29-30)

6) Molecular and histological analyses (months 30-36)

Since the beginning of this project, there have been additional data reported from different laboratories indicating that, rather than targeting the striatum and cerebellum in isolation, both might need to be targeted simultaneously. In light of that data, we felt it made more sense to develop a protocol to target both simultaneously. In fact, this is what we initially planned to achieve with ASOs, although their lack of efficacy and toxicity precluded us from completing that experiment. Based on mouse data, we hypothesized that intracerebroventricular injections of AAV into neonatal rat brain (P0-P1), would achieve widespread neuronal transduction. We completed these pilot experiments and optimized the protocol. Although this required more time and the

experiment could not be completed, this data has been used as preliminary data in a submission of an R21 grant proposal to NIH/NINDS in October 2017 (study section review pending).

Deviations from Initial Plan:

There are two deviations from the plan based on results obtained, as described in Annual Progress Reports and noted above:

1) As described below, the findings observed upon completion of tasks 1 and 2 led us to conclude that ASO#3 does not reduce expression of torsinA(ΔE) transgenic in rats *in vivo*. Furthermore, we found evidence of toxicity that could be even more prominent in transgenic rats over wild type littermates. As a consequence, it was not scientifically reasonable to complete the proposed Task 3. Task #3 was proposed as a therapeutic trial of ASO#3 in aged rats using the dose identified as efficacious in Task#2. As we found no efficacy with any dose, and detected potential toxicity (as potentially predicted in the Alternative Outcomes and Strategies section of the initial application), we decided to move on to Tasks #4 and #5. This was approved after submission of the Progress Report for Year 2.

2) Cerebellar injections: since the beginning of this project, there have been additional data reported from different laboratories indicating that, rather than targeting the striatum and cerebellum in isolation, both might need to be targeted simultaneously. In light of that data, we felt it made more sense to develop a protocol to target both simultaneously. In fact, this is what we initially planned to achieve with ASOs, although their lack of efficacy and toxicity precluded us from completing that experiment. Based on mouse data, we hypothesized that intracerebroventricular injections of AAV into neonatal rat brain (P0-P1), would achieve widespread neuronal transduction. We completed these pilot experiments and optimized the protocol. Although this required more time and the experiment could not be completed, this data has been used as preliminary data in a submission of an R21 grant proposal to NIH/NINDS in October 2017 (study section review pending).

What was accomplished under these goals?

A) <u>Tasks 1-3: Evaluation of the therapeutic potential of antisense oligonucleotides (ASOs) in DYT1 transgenic rats</u>.

Initial experiments were based on infusing different doses of the ASOs into the lateral ventricle of 2-month-old DYT1 and control rats to identify efficacy and toxicity thresholds. At this age, most torsinA(ΔE) rats don't exhibit motor dysfunction. Behavioral evaluation was completed at baseline. Subsequently, an osmotic pump with ASO (active or missense control) was inserted in the mid-scapular subcutaneous space and its tip placed into the lateral ventricle. We used 3 different doses (50, 100 or 200 µg/day). The infusion lasted for 14 days. The rats underwent repeated behavioral testing after the infusion and were sacrificed. Their brains were extracted for mRNA, protein and histological analyses. To determine silencing efficacy, we measured levels

of the target mRNA and protein (torsinA) with RTPCR and western blotting from multiple brain regions, and histological analysis was completed for markers of torsinA expression, glial reaction and neuronal loss. These analyses disclosed the following information:

- Behavioral analysis: baseline analysis showed abnormal clasping and gait in DYT1 transgenic rats when compared to controls, but no differences on performance on the rotarod and open field (**figure 1** and not shown). After the injections, the following was observed: there was no statistically significant effect of any of the treatment groups. However, there as a trend towards a dose-dependent worsening of the clasping phenotype in the groups that received either ASO (Missense or ASO#3), more obvious for DYT1 transgenic rats than controls (**figure 2** and not shown). This suggested the possibility of toxicity by the ASOs, and possible increased susceptibility for this toxic effect in DYT1 rats.

- Protein analysis of different brain regions by western blotting showed no evidence of silencing by ASO#3 when compared to control missense or vehicle (**figure 3** and not shown).

- mRNA analysis of different brain regions by QPCR showed no evidence of silencing by ASO#3 when compared to controls missense or vehicle (**figure 4** and not shown).

- Neurohistological analysis of efficacy: blinded quantification of optical density did not show significant changes in torsinA immunostaining. However, we found a trend toward increased astroglial reaction (increase staining by the marker of activated astrocytes GFAP) in both ASOs (missense and #3) (**figure 5** and not shown). Furthermore, this seemed to be more pronounced in DYT1 transgenics rats than controls, suggesting this might not be a valid therapeutic option for DYT1.

Upon the conclusion of these experiments, we conclude that ASO#3 is not a viable therapeutic option for DYT1. As a result, we decided to not complete Task #3 (therapeutic trial of ASO#3 in aged rats) and proceed to Tasks #4-5 as requested and agreed upon submission of the Progress Report from Year 2.

B) <u>Tasks 4-5: Evaluation of the therapeutic potential of AAV.miRNA targeting torsinA(ΔE) in DYT1 transgenic rats</u>.

Using previously designed shuttle plasmids, we generated AAV1 encoding microRNAs targeting human torsinA(ΔE) and a missense sequence. As we did not complete Task#3, we decided to generate AAV vectors with two different microRNAs targeting human torsinA(ΔE) (AAV1.U6miRNA-TOR1A(ΔGAG) vectors (A and B)) and a missense control (M) with no selective target. All vectors encode GFP as a reporter of transduction. We generated one prep for each virus in the Vector Core Facility and completed a pilot study in cultured HEK cells monitoring for GFP expression by fluorescence microscopy and measuring levels of co-transfected human torsinA(ΔE) to verify expression and silencing, confirming reduction in torsinA(ΔE) expression (**figure 6**). Next, we evaluated the efficacy and safety of the 3 constructs *in vivo*. Nine-month

old wildtype and DYT1 transgenic rats (n=4 per group) received striatal injections of 10 µl of 1E12 or 2E12 viral genomes (vg) per ml, with miRNA targeting torsinA(ΔE) or control missense. Animals were sacrificed 3 weeks later and their brains extracted for protein analysis. Using western blotting, we found that both AAV1.U6miRNA-TOR1A(ΔGAG) vectors efficiently reduced torsinA(ΔE) levels when compared with the non-injected contralateral striatum in a dose-dependent manner (**Figure 7**). Microscopic analysis showed no evidence of toxicity (data not shown). These experiments demonstrated that we have generated AAV vectors to efficiently and safely suppress expression of human mutant torsinA in DYT1 transgenic rats, making a therapeutic trial in this model feasible.

Nest, we investigated the effects of torsinA(ΔE) silencing in the adult striatum of DYT1 transgenic rats on motor phenotype. Three independent cohorts of 12 to 14-month-old wildtype and DYT1 transgenic rats were assessed (a total of 12 animals per experimental group) by infusing 10µl of 5E12 of AAV1.U6miRNA-TOR1A(ΔGAG)(B) or missense viral vectors bilaterally into the striatum. Behavioral tests were performed at baseline (before injection) and 2.5 months post-injection before being euthanized. We first confirmed efficient RNAi-mediated knockdown of torsinA(ΔE) in the striatum (**Figure 8A, B**) with no effect on endogenous rat endogenous WT torsinA (not shown). However, this intervention did not rescue the clasping and gait phenotypes in DYT1 transgenic rats (not shown). On the other hand, downregulation of torsinA(ΔE) led to partial correction of a recently described molecular phenotype consisting on dysregulation of the eIF2α pathway (**Figure 8C, D** and not shown). There was no behavioral, molecular or histological evidence of toxicity with any of the vectors (not shown). These results indicate that downregulation of torsinA(ΔE) expression in the striatum of DYT1 rats after they develop motor dysfunction does not improve this phenotype, even after rescue of a molecular phenotype. The very important therapeutic implication of these experiments is that either the striatum is not the primary site of dysfunction in DYT1, or that the molecular intervention should be applied before the motor phenotype appears.

Multiple publications from different laboratories in the last few years suggest that motor dysfunction in DYT1 arises from dysfunction at a network level, and not only the striatum or cerebellum. For that reason, rather than repeating the same experiments just described in cerebellum rather than striatum, we reasoned that it would be more optimal to develop an approach in which we can modify expression of torsinA(ΔE) in all components of that network (olivo-cerebellar-thalamo-cortical-basal ganglia). Todd Golde previouysly showed that intracerebroventricular injections of AAV in neonatal mice lead to widespread brain transduction. As we have a rat model, we decided to evaluate this approach in neonatal rats. We designed a pilot experiment to test different concentrations of AAV.GFP for intracerebroventricular injections in rats at P1 (2, 3 or 4µl of 1E12 vg). Animals were euthanized 21 days later and the expression of the GFP reporter protein assessed by western blot and immunofluorescence, demonstrating that the lower dose (2µl) was sufficient for widespread and robust CNS transduction, including all components of this network (**Figure 9** and not shown). Moreover, although the number of animals was not sufficient to quantify silencing in all brain regions, western blot analysis to measure torsinA levels suggested efficacy of this approach (not

shown). Implementation and optimization of this new protocol started in month 30 and a properly powered therapeutic trial with an appropriate number animals and aged to 11 months was not timely feasible. However, this experimental data was used as preliminary evidence in a R21 grant proposal submitted to NIH/NINDS in October 2017, to be reviewed in March 2018.

FIGURES

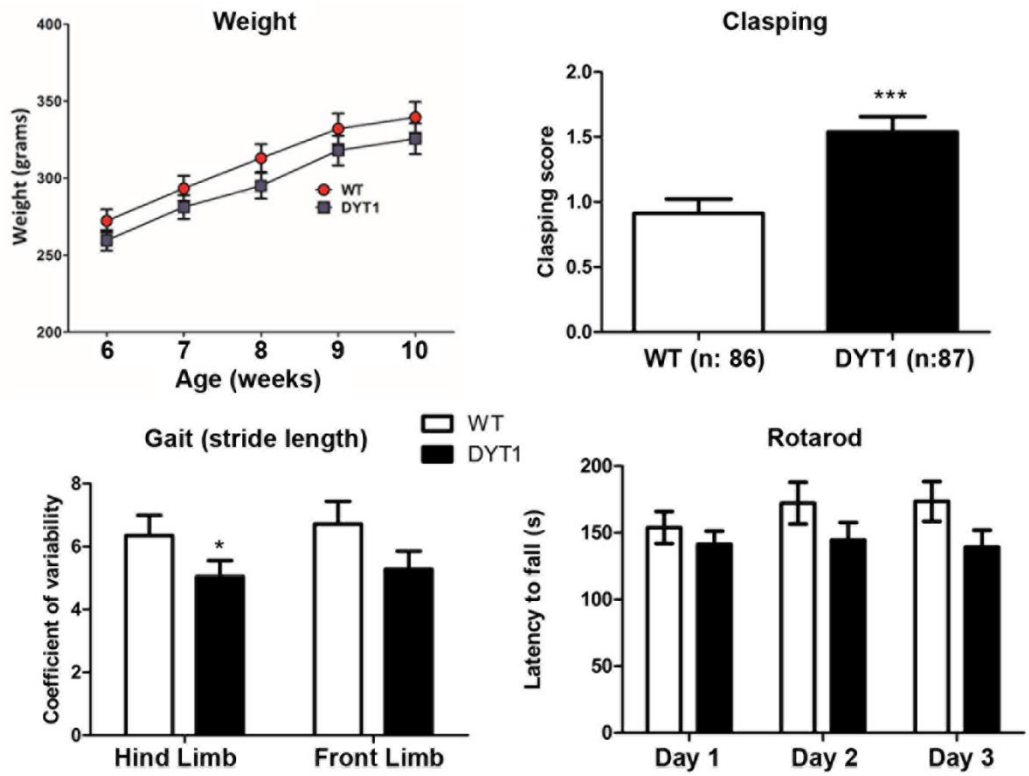

Figure 1. Baseline behavioral analysis. DYT1 transgenic (n: 87) and control WT littermates (n: 86) underwent behavioral evaluation at 3-4 weeks of age before implantation of an Alzet pump to receive ASO or control injections

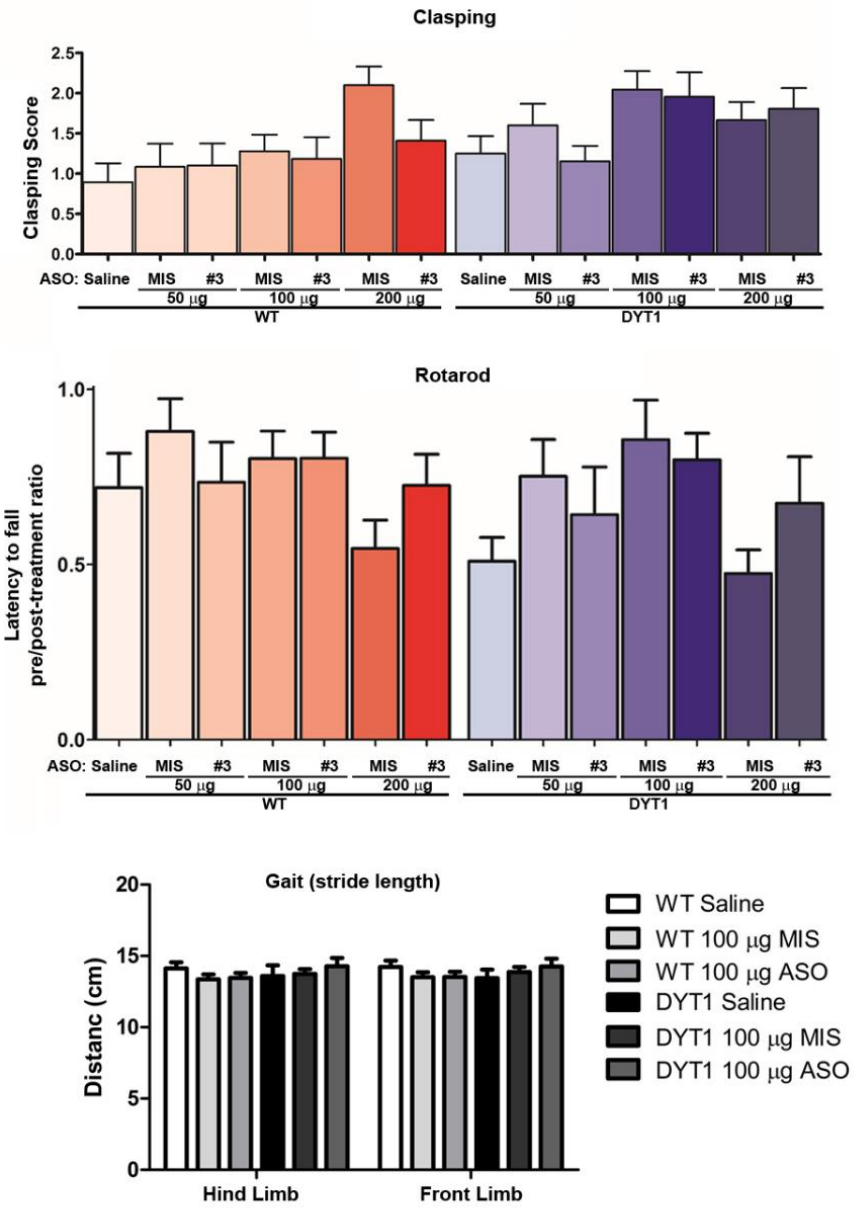

Figure 2. Post-ASO infusion behavioral analysis. DYT1 transgenic and control WT littermates received either saline or increasing concentrations of ASO#3 (active) or a missense ASO (control) and underwent behavioral evaluation during the 2 weeks after the infusion was completed. There was a trend towards worse clasping scores in DYT1 transgenic animals receiving any ASO (active or control) over those receiving saline.

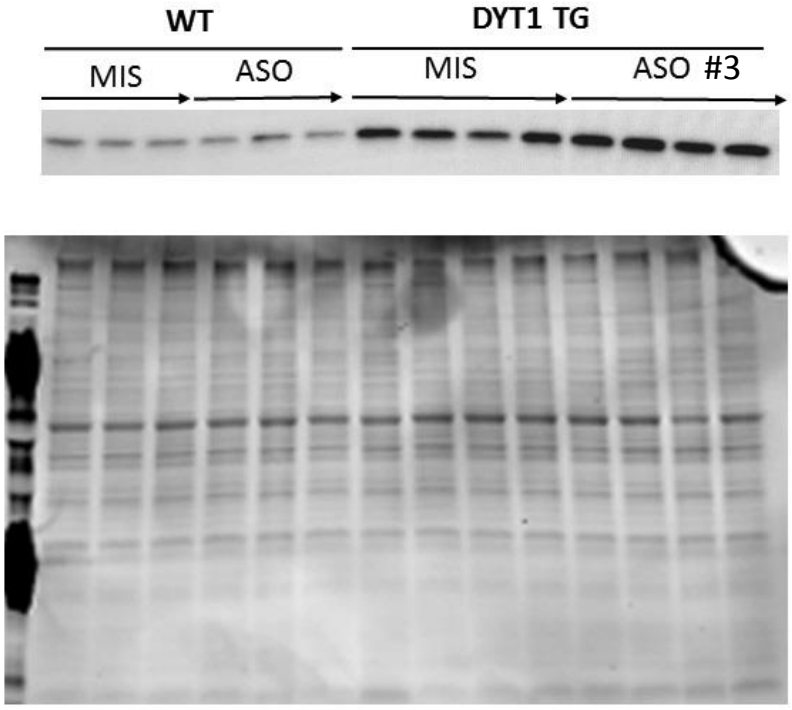

Figure 3. Western blot analysis of human torsinA(ΔE) expression in rats receiving ASO-control (MIS) or "active" (#3). Tissue lysates were obtained from brain tissue of rats treated with ASO after euthanasia. Upper panel shows levels of torsinA (endogenous and transgene), and bottom panel total protein content as a loading control. These experiments showed increased torsinA expression in DYT1 transgenic rats over WT as expected. However, it did not show any evidence of silencing of the human transgene in DYT1 trangenic rats by the active ASO#3 over control missense. Shown is a representative blot of prefrontal cortex tissue. Similar results were obtained in other tissues such as striatum and cerebellum.

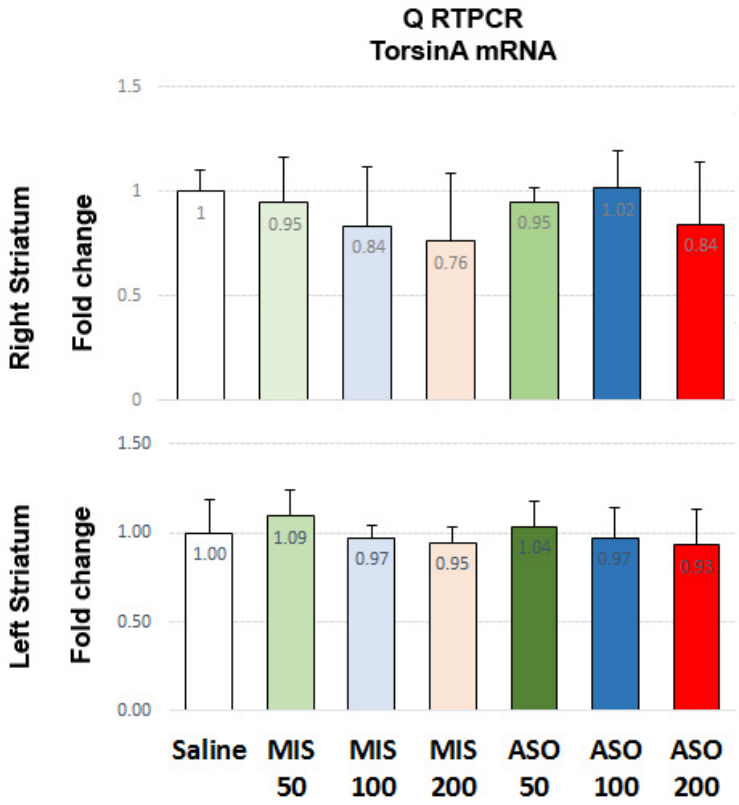

Figure 4. Q-RT-PCR analysis of human torsinA(ΔE) expression in DYT1 transgenic rats receiving ASO-control (MIS) or "active" (ASO) at increasing doses. Brain tissue was obtained after euthanasia as in figure 3 and processed for RT-PCR to measure levels of the transgene. We found no evidence of silencing by the active ASO over control missense. Shown is representative data of striatal tissue. Similar results were obtained in other tissues such as prefrontal cortex and cerebellum.

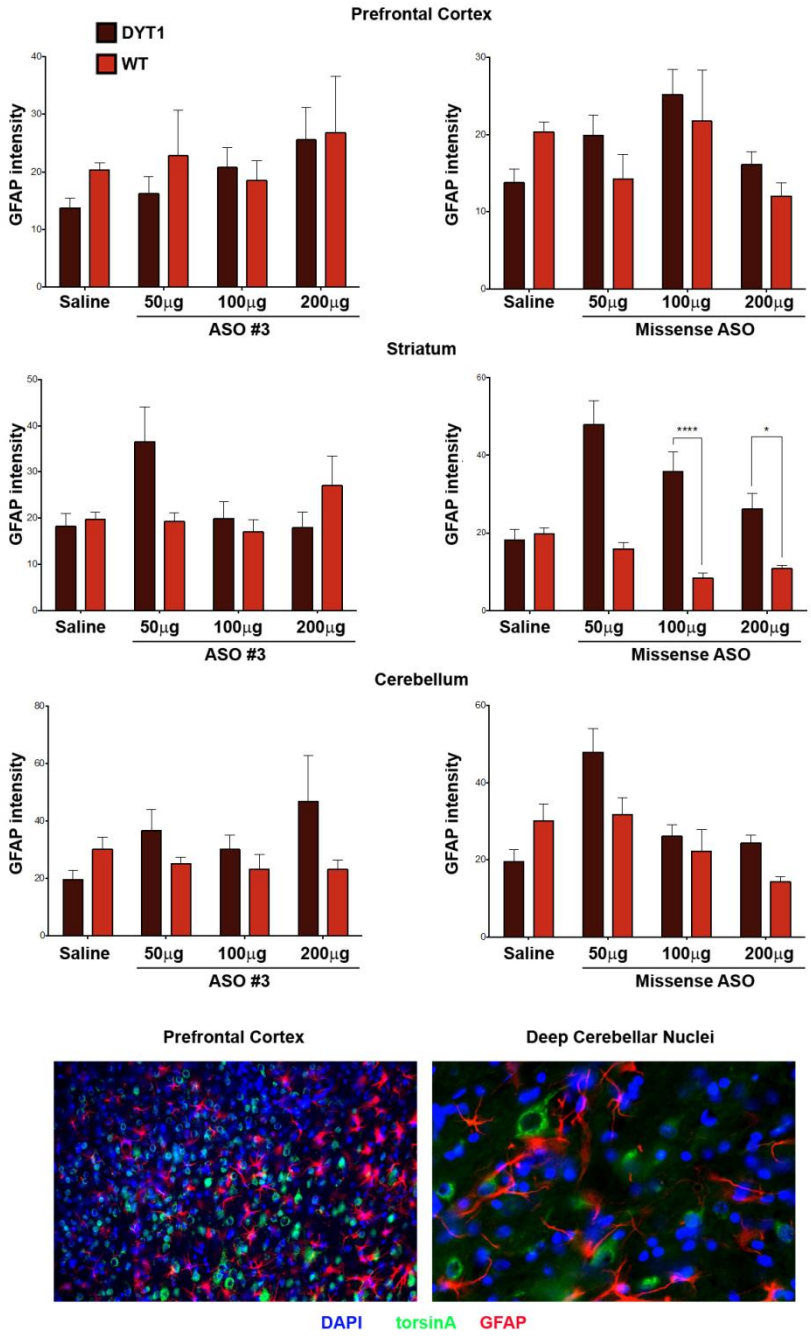

Figure 5. Histological analysis of brain tissue in rats after treatment with ASO. Immunofluorescence microscopy analysis of activated GFAP (marker of astroglial reaction) in different brain regions (quantified 3 equivalent slides per animal from each region selected). We found a trend and at times a significant increase in GFAP optical density (systematically measured by a blinded reader) caused by either ASO (active #3 or control) and more pronounced in DYT1 transgenic rats, indicating a susceptibility towards toxicity driven by this genotype. Below are shown representative images (20X in cortex and a digitally magnified detail of deep cerebellar nuclei) demostrating the quality of the staining for torsinA and GFAP). We found no differences in torsinA expression in any brain region (quantification not shown)

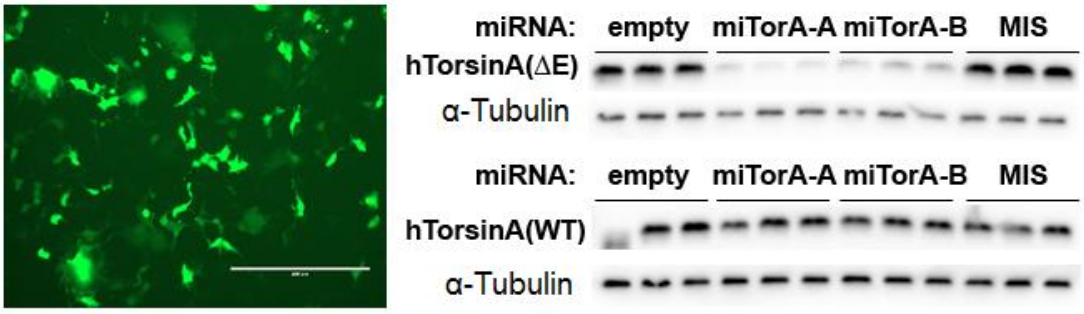

Figure 6. Generation of AAV1 expressing miRNAs targeting human torsinA(ΔE). AAV1 were generated to express only GFP as a reporter of transduction (empty), or GFP and miRNAs targeting human torsinA(ΔE) (miTorA-A and miTorA-B) or a missense miRNA (MIS). HEK293 cells were co-transfected with human torsinA(WT) or torsinA(ΔE) cDNA and these vectors. Immunofluorecence analysis demonstrated efficient cell transfection as illustrated by GFP expression. Transgene expression was assessed 48 hrs later via western blotting of whole cell lysates, showing efficient silencing of human torsinA(ΔE) by the two miRNAs targeting this transcript, but no silencing of the human torsinA(WT) cDNA (demonstrating allele-specificity)

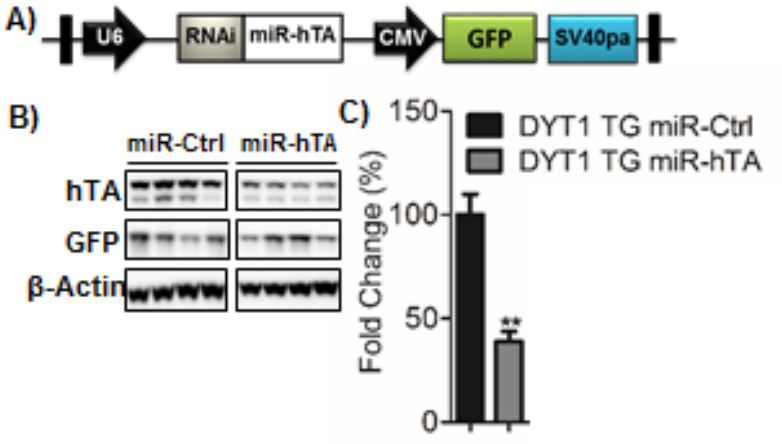

Figure 7. Supressing striatal expression of human torsinA(ΔE) *in vivo*. DYT1 transgenic rats received unilateral striatal injections of AAV1 expressing control missense miRNA (miR-Ctrl) or miRNA targeting human torsinA(ΔE) (miR-hTA) and GFP as a reporter of transduction (A). (B) Animals were euthanized 3 weeks later and whole striatal lysates used for western blotting with antibodies to detect torsinA (to measure silencing), GFP (reporter of transduction) and b-actin (loading control). (C) Quantification of normalized western blot signal showed that levels of torsinA(ΔE) are significantly reduced by the miRNA targeting the transgene by about 50%.

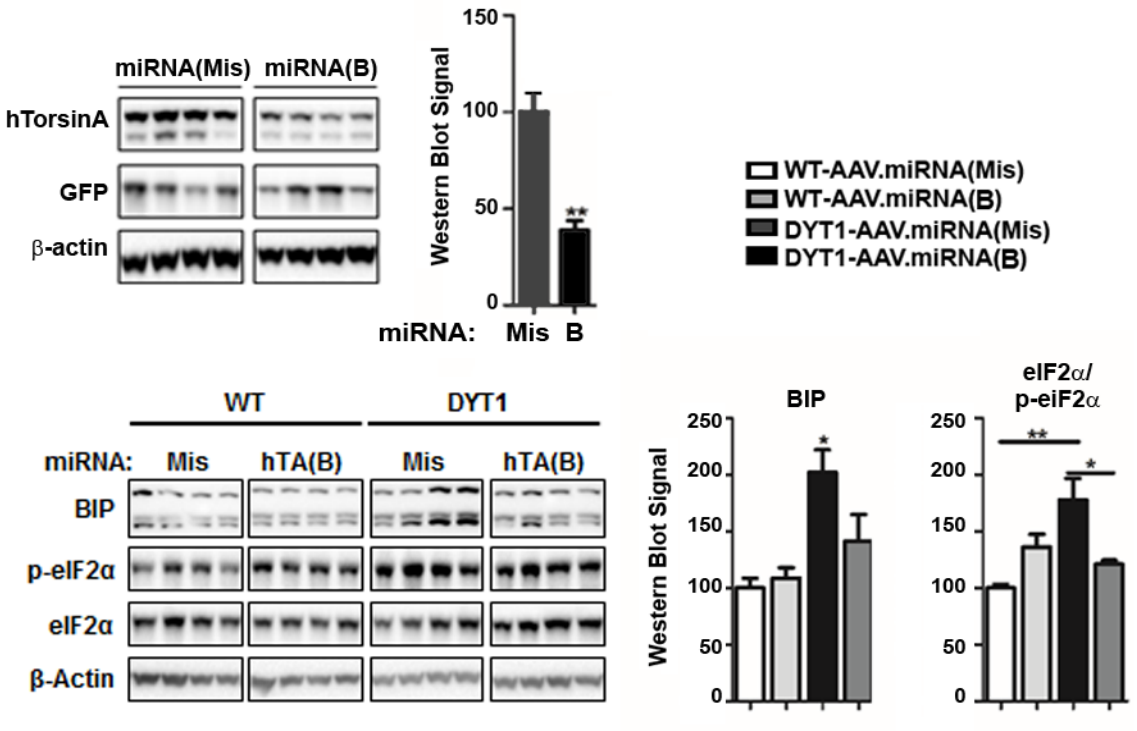

Figure 8. Downregulation of human torsinA(ΔE) in striatum of 12-month-old rats. (A) Western blot analysis of striatal lysates from DYT1 rats 2 months after injection with AAV1.miRNA (missense (Mis) or targeting the transgene (B)). Representative images from 4 animals/ group are shown, all from the same blot and same exposure. Intermediate lanes have been excised for clarity. Injections in WT rats not shown as they do not express human torsinA (B) Quantification of experiments as in A (n:8/group). (C) Western blot analysis and (D) quantification of striatal lysates from DYT1 and WT rats injected as in (A) showing rescue of levels of BIP (upstream component of the ER eIF2α pathway) and the abnormal eIF2α/p-eIF2α ratio in DYT1 animals.

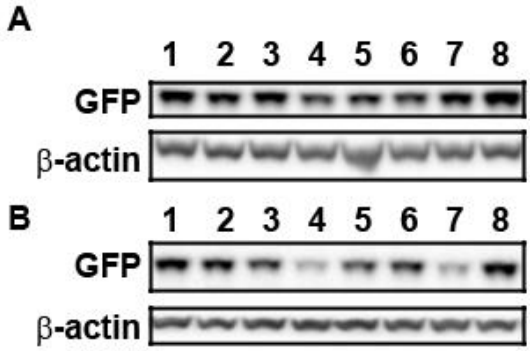

Figure 9. Widespread neuronal transduction with AAV1.GFP. Eight P1 rats received intracerebroventricular injections of AAV.GFP at P1. At age 3 weeks, brains were dissected and lysates obtained for western blot analysis of GFP expression. Shown are (A) whole cerebellar and (B) whole striatal lysates. Numbers indicate different animals.

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

During the completion of this work, Genevieve Beauvais (postdoctoral scholar) and Jaime Watson (Technician) have learned from the principal investigator protocols involving behavioral testing in rodents, design and generation of AAV vectors, injection of AAV into rodent brain and molecular/histological analyses. This has resulted in high proficiency for both researchers on this techniques.

How were the results disseminated to communities of interest?

Part of the data generated in this grant was presented by Dr. Gonzalez-Alegre (PI) to dystonia researchers during a workshop co-organized by Duke University and the Dystonia Medical Research Foundation. The workshop was on "Dystonia Cellular Mechanisms: Emerging Molecular Pathways" and took place in Durham, NC on October 13, 2017. Dr. Gonzalez-Alegre's talk was entitled: "eIF2a pathway and dystonia: pathogenic, compensatory or coincidental?" In addition there are 2 upcoming publications supported by this work. The first one is current *under review* in Neuroscience and is entitled "Exploring the interaction between eIF2α dysregulation, acute endoplasmic reticulum stress and DYT1 dystonia in the mammalian brain". The second one is *in preparation* and will be tentatively entitled "Downregulation of torsinA(ΔE) expression in the striatum of DYT1 transgenic rats rescues molecular phenotypes but does not reverse motor dysfunction" Finally, we anticipate presenting this data in additional dystonia-oriented scientific meetings over the next 2 years.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Overall, most of the work was completed as initially planned, with the two significant data-driven deviations from the initial protocol described above. First of all, the lack of silencing of ASO#3 *in vivo* suggests that this sequence is not therapeutically helpful for DYT1 dystonia. However, we were able to conclusively determine that suppressing expression of torsinA(ΔE) in the striatum once motor dysfunction has developed is not a viable therapeutic strategy in DYT1. This will be a major contribution of this grant to the field of dystonia research and experimental therapeutics. In addition, it adding very valuable information on the biological link between torsinA(ΔE) expression, eIF2α dysregulation and motor dysfunction in DYT1. Our initial report of a link between dystonia and eIF2α dysregulation was confirmed by a follow up report by the laboratory of Nicole Calakos (Duke) (Rittiner et al, 2016 *Neuron*). Our finding that rescue of normal eIF2α pathway expression does not necessarily imply phenotypical reversal will

be of great interest to the dystonia community as this has been a very significant unanswered question.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS.

Changes in approach and reasons for change

There are two changes in approach from the initial plan based on results obtained, as described in Annual Progress Reports and noted above:

1) The findings observed upon completion of tasks 1 and 2 led us to conclude that ASO#3 does not reduce expression of torsinA(ΔE) transgenic in rats *in vivo*. Furthermore, we found evidence of toxicity that could be even more prominent in transgenic rats over wild type littermates. As a consequence, it was not scientifically reasonable to complete the proposed Task 3. Task #3 was proposed as a therapeutic trial of ASO#3 in aged rats using the dose identified as efficacious in Task#2. As we found no efficacy with any dose, and detected potential toxicity (as potentially predicted in the Alternative Outcomes and Strategies section of the initial application), we decided to move on to Tasks #4 and #5. This was approved after submission of the Progress Report for Year 2.

2) Cerebellar injections: since the beginning of this project, there have been additional data reported from different laboratories indicating that, rather than targeting the striatum and cerebellum in isolation, both might need to be targeted simultaneously. In light of that data, we felt it made more sense to develop a protocol to target both simultaneously. In fact, this is what we initially planned to achieve with ASOs, although their lack of efficacy and toxicity precluded us from completing that experiment. Based on mouse data, we hypothesized that intracerebroventricular injections of AAV into P0-P1 rat brain, would achieve widespread neuronal transduction. We completed these pilot experiments and optimized the protocol. Although this required more time and the experiment could not be completed, this data has been used as preliminary data in a submission of an R21 grant proposal to NIH/NINDS in October 2017 (study section review pending).

Actual or anticipated problems or delays and actions or plans to resolve them.

As explained in the previous subheading.

Changes that had a significant impact on expenditures.

None.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

None

6. PRODUCTS.

Publications, conference papers, and presentations.

- Dr. Gonzalez-Alegre (PI) presented part of this data to dystonia researchers during a workshop co-organized by Duke University and the Dystonia Medical Research Foundation. The workshop was on “Dystonia Cellular Mechanisms: Emerging Molecular Pathways” and took place in Durham, NC on October 13, 2017. Dr. Gonzalez-Alegre’s talk was entitled: “eIF2a pathway and dystonia: pathogenic, compensatory or coincidental?”

- There are 2 upcoming publications supported by this work:

1) The first one is current *under review* in Neuroscience and is entitled “Exploring the interaction between eIF2α dysregulation, acute endoplasmic reticulum stress and DYT1 dystonia in the mammalian brain”.

2) The second one is *in preparation* and will be entitled “Downregulation of torsinA(ΔE) expression in the striatum of DYT1 transgenic rats rescues molecular phenotypes but does not reverse motor dysfunction”

Website(s) or other Internet site(s)

None.

Technologies or techniques

None.

Other Products

None.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS.

What individuals have worked on the project?

Name: Pedro Gonzalez-Alegre
Project Role: PI
Nearest person month worked: 3
Contribution to Project: No change.

Name: Beverly Davidson
Project Role: Co-Investigator
Nearest person month worked: 1
Contribution to Project: No change.

Name: Genevieve Beauvais
Project Role: Postdoctoral Associate
Nearest person month worked: 12
Contribution to Project: No change.

Name: Jaime Watson
Project Role: Research Technician II
Nearest person month worked: 12
Contribution to Project: Ms. Watson assists Dr. Beauvais in colony maintenance, animal genotyping, surgeries, behavioral, histological and molecular assessments.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Gonzalez-Alegre has the following changes to report:

New Grants

Penn Health Tech Pilot Grant. Flavia Vitale (PI)
1/1/18-12/31/18 0.6 cal mos
MINT: A multipoint injection system for intraparenchymal drug delivery
Role: Investigator

Terminated:

Collaborative Center for X-Linked Dystonia Parkinsonism (CCXDP); Gadue (PI)
3/1/16-2/28/17 0.6 cal mos
Response of DYT3 iPSC to ER and Metabolic Stressors

Dr. Davidson has the following changes to report:

New Grants

NONE

Terminated

Allele-selective knockdown of mHTT using ASOs
Wave Life Sciences, Davidson (PI)
9/1/16-3/31/17 0 Cal Mos

5R01NS045667
NIH/NICHD
Cell Signaling and Neurodegenration, Orr (PI)
5/1/14-4/30/17 0.6 Cal Mos

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS.

None

9. APPENDICES.

None.