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**TITLE:** microRNA Replacement Therapy for ALS Treatment

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E-Mail:

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**14. ABSTRACT**

The overall goal of this grant is to determine whether ALS affects the expression and/or function of a critical microRNA-218 (miR-218) using mouse genetics. We have generated a novel mouse line that detects miR-218 activity (tg-miR-218-rep) and used it to establish that we can induce deletion of miR-218 in adult motor neurons using a novel intercross of the following animals: miR-218-1 -/- ; miR-218-2 CreER/fl; tg-miR-218-rep +/-. This inducible system for eliminating miR-218 will help to establish for the first time whether both alleles of miR-218 are required in mature motor neurons. We have determined that our reporter detects levels of miR-218 in SOD1(G93A) and PFN1(C71G) mice at end-stage, as well as in advanced disease TDP43(Q331K) and aging, asymptomatic BAC500 mice. The sensitivity of our reporter gene suggests that motor neurons maintain detectable levels of miR-218 at late stages of disease. We have hypothesized that elevated miR-218 may be protective against some ALS-causing mutations. To test this hypothesis, we have generated a new self-complementing retroAAV virus carrying miR-218 that will be injected into ALS mouse models. Finally, we have tested and identified a miR-218 mimic molecule using cell culture screens. This modified oligonucleotide can be used as an alternative approach to increase miR-218 in ALS.

**15. SUBJECT TERMS**

ALS, microRNA, miR-218, motor neuron, neurodegeneration, PFN1(C71G), reporter, retroAAV, SOD1(G93A), TDP43(Q331K).

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## 1. INTRODUCTION:

Motor neuron death drives ALS progression either due to both cell-autonomous and non-cell-autonomous effects. Mutations in more than 40 genes have been linked to the development of ALS. Of these genes, C9orf72, TDP43, FUS/TLS and SOD1 account for most of the known familial cases. C9orf72, TDP43 and FUS/TLS are genes that interact with RNAs in control their processing and transport. In particular, TDP43 and FUS/TLS interact with the nucleases involved in microRNA generation from longer precursor RNAs. We previously characterized miR-218 as the most highly enriched microRNA in motor neurons, observing neurodegenerative-like defects in mice embryos lacking miR-218. MiR-218 controls the expression of ~300 genes, many of them involved in cell homeostasis. In this grant we hypothesize that reduced levels of functional miR-218 contribute to the progression of ALS. Consequently, we predict that genetically-raising miR218 levels will slow ALS progression, and if our hypothesis is correct we logically predict that genetically-reducing the levels of miR218 will accelerate the disease. Our proposal takes advantage of mouse genetics to determine the natural history of miR-218 and its regulatory network in adult mice in the context of ALS. Our studies will also examine whether the genes regulated by miR-218 themselves have therapeutic potential.

## 2. KEYWORDS:

ALS, microRNA, miR-218, motor neuron, neurodegeneration, PFN1(C71G), reporter, retroAAV, SOD1(G93A), TDP43(Q331K).

## 3. ACCOMPLISHMENTS:

The major goals of our project are to:

1. Create a detailed survey of miR-218 function in the spinal cord (~70% completed)
2. Perform miR-218-associated candidate therapeutic approach for ALS (~50% completed)
3. miR-218 mimics as ALS therapeutic agents (0% completed, ~30% completed under alternative)

**What was accomplished under these goals?**

Progress on each task is described below the statement of work for each set of experiments.

| Experiment | Timeline (Months) | Site Personnel (#mice) |
|---|---|---|
| **Aim 1** Detailed survey of miR-218 function in the spinal cord. | | |
| Task 1: IACUC approval currently in place (protocol 11-00020) | 0 | Dr. Sam Pfaff |
| Milestone: ACURO Approval | 3 | |

**Aim 1, Task 1**. As described in the Year 1 report, our project received ACURO approval in January 2019. All of our aims involve work in mice and therefore animal crosses for the project began at this stage. We have obtained ALS mouse models animals for SOD1(G93A), TDP-43(Q331K) and PFN1(C71G). These animals

have been intercrossed with miR-218 knockout mutants and with a novel miR-218 mouse reporter line (tg-miR-218-rep; described below in task 4) to generate the genetic combinations needed for experimentation. Initial characterization of the off spring has begun. Overall, these genetic experiments are underway as described in the grant.

| | | |
|---|---|---|
| Task 2: LCM capture of motor neuron for SmartSeq2 and Taqman analysis, together with histology analysis in WT mice. | 0-4 | Dr. Giancarlo Costaguta<br>Shawn Driscoll<br>Dr. Sam Pfaff<br>(12 WT mice) |
| Task 3: LCM capture of motor neuron for SmartSeq2 and Taqman analysis, together with histology analysis in ALS mouse models and tamoxifen-inducible 218DKO mice. | 4-12 | Dr. Giancarlo Costaguta<br>Shawn Driscoll<br>Dr. Sam Pfaff<br>(12 mice of each SOD1(G93A), PFN1(C71G), Tam-218DKO) |

**Aim1, Tasks 2 and 3.** In our Year 1 report we explained our switch from SmartSeq/Taqman analysis to single-nuclei RNA sequencing. Our expectation was to perform next generation sequencing from nuclei isolated from mice cohorts bearing miR-218-1 -/- miR-218-2 CreER/fl tg-miR-218-rep +/- and a control cohort miR-218-1 -/- miR-218-2 +/fl tg-miR-218-rep +/- that have been injected with Tamoxifen to induce Cre-dependent expression and deletion of miR-218 from adult motor neurons. Histological analysis of mice 3 months after Tamoxifen addition to induce conditional miR-218 deletion did not detect nuclear GFP fluorescence, suggesting that miR-218 is still present in motor neurons (Fig 1). We have observed that conditional deletion of miR-218 in adults produces partial neuromuscular junction (NMJ) loss ~12-15 month after Tamoxifen injection. We are waiting for our mouse cohorts to reach 12 months after Tamoxifen injection to perform our sequencing assay and will be addressed under a no-cost extension period.

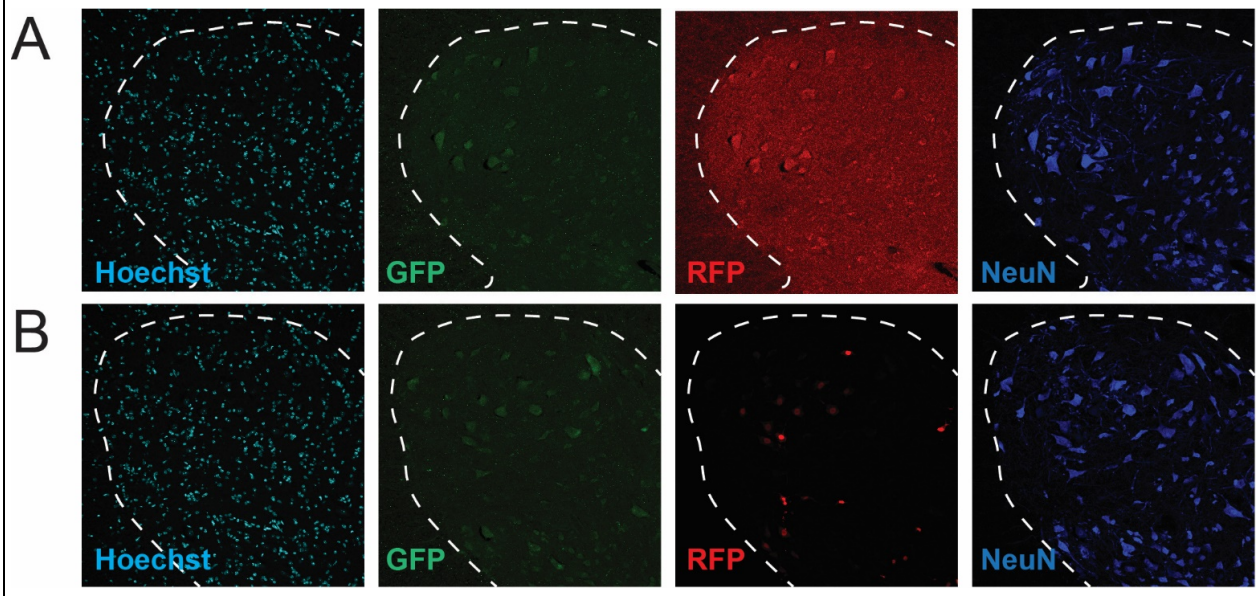


**Fig. 1. MiR-218 expression 3 months after conditional deletion**. Adult mice (A) WT and (B) miR-218-1 $^{-/-}$ miR-218-2 $^{fl/CreER}$ tg-miR-218-Rep $^{+/-}$ were injected with tamoxifen to induce conditional deletion of miR-218. Representative ventral sector of lumbar spinal cord shows expression of nuclear mRFP from the reporter and absence of nuclear GFP signal, suggesting the presence of miR-218.

| | | |
|---|---|---|
| Task 4: Histological analysis of motor neurons from mice bearing a miR-218 reporter in WT and ALS mouse models. | 6-12 | Dr. Giancarlo Costaguta<br>Dr. Sam Pfaff<br>(12 mice of each WT, SOD1(G93A), PFN1(C71G), tam-218DKO, all crossed to tg-miR-218rep) |

**Aim 1, Task 4.** We have created a functional reporter for miR218 activity in mice by making a novel transgenic mouse line (tg-miR-218-rep) that produces a red/green ratiometric signal based on miR218 activity in vivo. We have shown in control mouse experiments that this reporter is an extremely sensitive detector of miR218. As expected, the reporter detects the repressive-activity of miR-218 in embryonic motor neurons expressing high levels of the microRNA. To assay the sensitivity of the synthetic reporter for miR-218 activity we generated a panel of mouse crosses that combine the reporter (tg-miR-218-rep) with different deletions of miR-218-1 and miR-218-2 (Fig. 2). These genetic experiments show that the miR-218 sensor encoded by tg-miR-218-rep is sensitive to levels of miR-218 that are even below the functional level needed for proper motor neuron function. Thus, as part of our Year 1 report we crossed tg-miR-218-rep into several mouse models of ALS (see task 1 above).

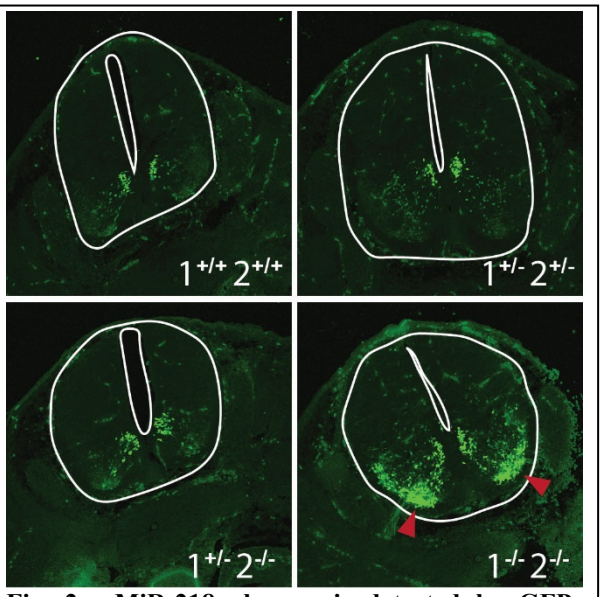


**Fig. 2. MiR-218 absence is detected by GFP expression.** Mouse strains with different allele deletions of miR-218 and bearing tg-miR-218-rep. Note that complete deletion of miR-218 results in appearance of GFP fluorescence in the ventral area of the developing spinal cord (red arrowheads) at e12.5.

As described In the Year 1 report, we did detect miR-218 expression in SOD1(G93A) mice at all stages, including, disease end stage. Using our reporter line we have now observed miR-218 expression on PFN1(C71G) at disease mid- and end-stage, and on TDP-43(Q331K) at advanced disease stage (Fig. 3), suggesting that levels of miR-218 as detected by our reporter, are present in motor neurons at advanced stages on different ALS disease models. Finally, 1 year old BAC500 female mice that don't show disease manifestation show presence of miR-218 in motor neurons (Fig.3).

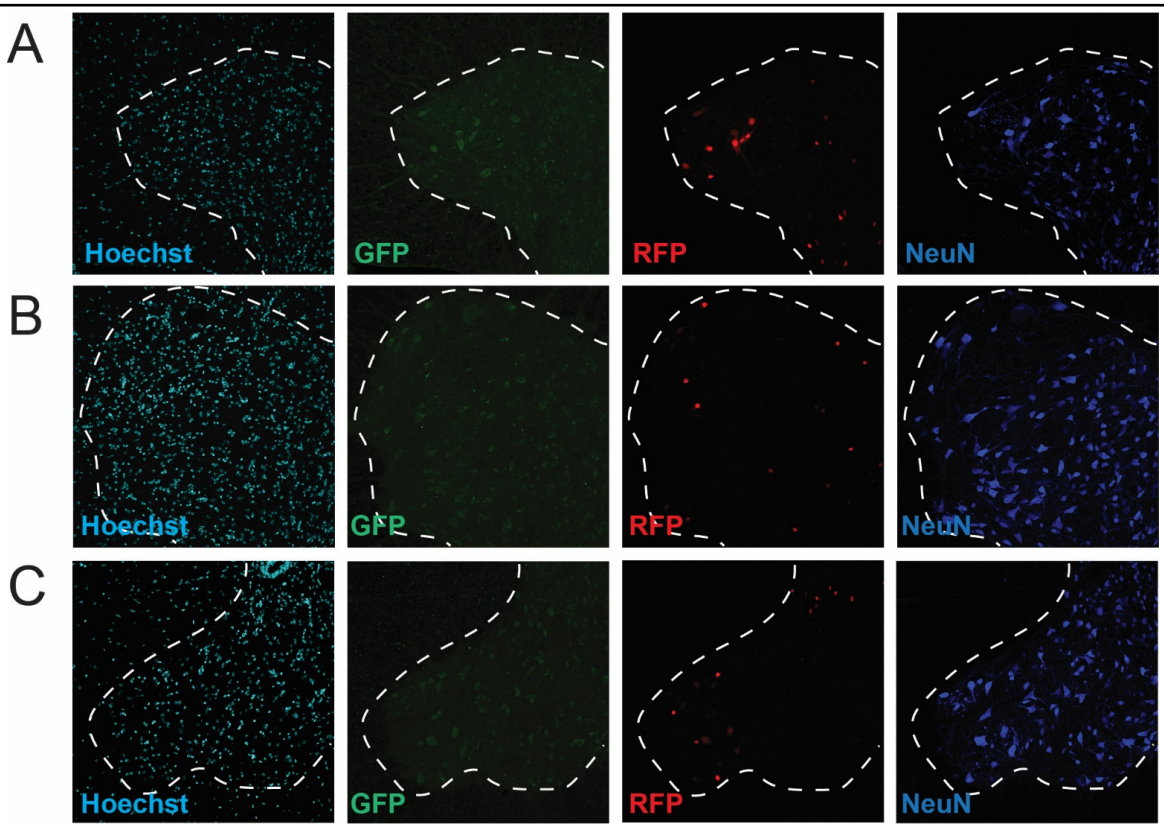


**Fig. 3. MiR-218 expression is detected in advance and end-stage ALS mouse models**. (A) 13 m.o. Thy1.2-PFN1(C71G) $^{+/-}$ Prnp-PFN1(C71G) $^{+/-}$ tg-miR-218-Rep $^{+/-}$ at disease end-stageand; (B) 17 m.o. Prnp-TARDBP(Q331K) $^{+/-}$ tg-miR-218-Rep $^{+/-}$ at advanced disease stage; and (C) 13 m.o. BAC500 $^{+/-}$ tg-miR-218-Rep +/- asymptomatic. Representative ventral sector of lumbar spinal cord shows expression of nuclear mRFP from the reporter and absence of nuclear GFP signal, suggesting the presence of miR-218.

| | | |
|---|---|---|
| Milestone: Expression and miR-218 activity map in WT and ALS mouse models. | 12 | |

**Aim 1, Milestone.** We have detected miR-218 in motor neurons at end-stage of SOD1(G93A) and PFN1(C71G) mice, advanced disease stage for TDP-43(Q331K), and aging BAC500 mice. The presence of miR-218 at advance disease stage of the TDP-43(Q331K) and end stage of SOD1(G93A) and PFN1(C71G) models suggests that detectable levels of miR-218 may be present near or at the time of motor neuron death. A single allele of miR-218-1 can partially suppress expression of GFP in our reporter (Fig. 2). Unpublished data from our laboratory places miR-218-1 +/+ as contributing ~20% of total miR-218 in embryonic motor neurons (Amin and Pfaff, manuscript in preparation). These data suggest that motor neurons at terminal disease stage in our ALS models contain miR-218 at levels of at least 20% as observed in healthy motor neurons. We are currently in the process of using single nuclei purification and NGS to profile motor neurons in adult animals to define the genetic networks regulated by miR-218 (previous studies have only characterized miR-218 gene netowork in embryos). These experiments will allow us for the first time define the function of miR-218 in adult motor neurons.

| | | |
|---|---|---|
| **Aim 2** MiR-218-associated candidate therapeutic approach for ALS. | | |
| Task 1: Conditions for quantitative scAAV9 delivery to motor neurons in WT mice. | 0-4 | Miriam Gullo<br>Dr. Giancarlo Costaguta<br>Dr. Sam Pfaff<br>6 WT mice |

**Aim 2, Task 1.** In our Year 1 report we explained the switch from using scAAV9 virus to retroAAV, and had cloned the retroAAV virus for overexpressing miR-218 constitutively or conditionally in cre-expressing cells.

| | | |
|---|---|---|
| Task 2: Delivery of scAAV9 bearing miR-218 or mock to ALS mouse models with behavioral and histological analysis. | 4-12 | Miriam Gullo<br>Dr. Giancarlo Costaguta Dr.<br>Sam Pfaff |
| | | (48 mice of each SOD1(G93A), PFN1(C71G)<br>and 60 FUS(R521C) mice) |

**Aim 2, Task 2.** We have generated breeding mouse pairs SOD1(G93A)$^{+/-}$ x ChAT:Cre$^{+/+}$ for targeted expression of miR-218 in motor neurons using our conditional miR-210 virus. Salk Institute closures and work restrictions in response to the Covid-19 pandemic delayed our viral injections, and they will be completed under the no-cost extension period.

| | | |
|---|---|---|
| Task 3: Delivery of scAAV9 bearing Relay218 genes to ALS mouse models with behavioral and histological analysis. | 9-21 | Miriam Gullo<br>Dr. Giancarlo Costaguta Dr.<br>Sam Pfaff<br>(48 mice of each SOD1(G93A) and PFN1(C71G)) |

**Aim 2, Task 3.** We are in the process of cloning retroAAV viruses that will express the transgenes ANXA2 and PRPH. These are genes that become downregulated in motor neurons lacking miR-218, and we predict they may have therapeutic function

| | | |
|---|---|---|
| Milestone: Disease modification by miR-218 overexpression. | 21 | |

**Aim 2, Milestone.** We have identified a highly efficient transgenic AAV system that can be delivered by simple intramuscular injection. scAAV-retro clones have been generated for miR-218 and are underway for other target genes. The ALS mouse models needed for targeting motor neuron expression with the conditional vectors are ready to start the viral injections.

| | | |
|---|---|---|
| **Aim 3** MiR-218 mimics as ALS therapeutic agents. | | |

| | | |
|---|---|---|
| Task 1: Intrathecal delivery conditions for [miR-218]. | 3-6 | Miriam Gullo<br>Dr. Giancarlo Costaguta Dr.<br>Sam Pfaff<br>(20 Tam-218DKO mice) |
| Task 2: LD50 determination for [miR-218]. | 6-9 | Miriam Gullo<br>Dr. Giancarlo Costaguta Dr.<br>Sam Pfaff<br>(30 WT mice) |
| Task 3: Delivery of [miR-218] to mouse models of ALS. | 9-24 | Miriam Gullo<br>Dr. Giancarlo Costaguta Dr.<br>Sam Pfaff<br>(120 mice of each WT and SOD1(G93A), 144 PFN1(C71G) mice) |

**Aim 3, Tasks 1 through 3.** In addition to using a viral delivery system for miR-218, we speculated that it may be possible to use a stable oligonucleotide that mimics the activity of miR-218 (indicated as [miR-218]). As reported for Year 1 we performed a series of pilot experiments with cultured motor neurons to establish whether oligonucleotides could mimic the activity of miR-218, which required gene profiling to confirm. We identified a modified oligonucleotide that can be taken up by culture cells and mimics miR-218. At present there are concerns that off target effects could complicate the interpretation of our experiments, and we plan to focus our efforts on establishing the proof-of-concept studies with the retro-AAV system targeted to motor neurons. If this proves successful we will have the control system needed to test the efficacy of the [miR-218] mimic we identified. The delay on our retro-AAV injections negatively influenced this Aim. Will make every effort to follow through during the no-cost extension period.

| | | |
|---|---|---|
| Milestone: Disease modification by intrathecal delivery of [miR- 218]. | 24 | |

**Aim 3, Milestone.** The efficacy and toxicity of the miR-218 mimic chracterized on year 1 will be compared to the retro-AAV system that is capable of selectively targeting only motor neurons for expression of miR-218 in mice with Cre-expressing motor neurons.

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

The support provided by this grant has been of great value in the professional development of Dr. Costaguta, allowing him to attend and present our work in meetings relevant for ALS and related motor neuron diseases:

i) The 23rd International SMA Researcher Meeting. June 2019. Poster presentation.

ii) Gordon Research Conference - Amyotrophic Lateral Sclerosis (ALS) and Related Motor Neuron Diseases. Mechanisms of Motor Neuron Degeneration and Therapeutic Intervention. July 2019. Poster presentation.

iii) SBP's 40th Annual Symposium - Regulation of Neural Function in Health and Disease. November 2019. Poster presentation.

**How were the results disseminated to communities of interest?**

We have presented posters with our results in conferences with participation of the ALS as well as SMA and neurodegenerative diseases scientific communities.

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

The Covid-19 pandemic caused interruptions and delays in our work. As part of a no-cost extension request, we expect to complete our experiments to identify the adult miR-218 regulatory network and assess whether viral delivery of miR-218 improves disease outcomes for ALS mouse models.

## *4.* IMPACT:

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

We are investigating how miR-218, a molecule with the ability to regulate the expression of about 300 genes in motor neurons, changes through life and in ALS in order to gain insights on the pathways that maintain proper motor neuron function. We hypothesize that elevated expression of miR-218 in motor neurons with insufficient levels of this microRNA could slow-down progression or delay onset of ALS. This approach would represent an entirely new direction for targeting ALS disease.

**What was the impact on other disciplines?**

The tools generated in this study have helped to inform neuroscientists about how gene networks are regulated, and provided mouse genetic tools for studying the spinal cord.

**What was the impact on technology transfer?**

If miR-218 holds therapeutic potential for ALS, it would be an attractive molecule for industry to adopt in their development for new ALS interventions.

**What was the impact on society beyond science and technology?**

The laboratory has hosted students and lay visitors. We have collaborated with an artist to develop art work based on our scientific images.

## 5. CHANGES/PROBLEMS:

**Changes in approach and reasons for change**

Nothing to report.

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

Due to the Covid-19 pandemic the Salk Institute was closed for approximately 2 months and has been partially operational during the last 4 months. This has delayed our ability to access animals for ALS experiments and has drastically reduced the function of critical core facilities at the Salk. We have requested a 12 month no-cost extension to complete the proposed work.

**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**

No significant changes. All procedures on mice are currently approved as in our IACUC protocol 11-00020.

**Significant changes in use or care of human subjects**

No human subjects

**Significant changes in use or care of vertebrate animals**

None

**Significant changes in use of biohazards and/or select agents**

Nothing to report.

**6. PRODUCTS:**

- **Publications, conference papers, and presentations**

**Journal publications.**

None

**Books or other non-periodical, one-time publications.**

None

**Other publications, conference papers and presentations**.

SBP's 40th Annual Symposium - Regulation of Neural Function in Health and Disease. November 2019. Poster presentation.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

All technologies developed will be shared according to the scientific community guidelines.

i) **Mouse reporter lines for miR-218**. Allows for the detection of low levels of miR-218 by the observation of GFP expression in motor neurons.
ii) **Mouse line with conditional expression of miR-218 reporter**. The reporter is activated by the presence of Cre recombinase, allowing the analysis of miR-218 levels in different cell types and tissues. Currently under characterization.
iii) **retroAAV-miR-218**. It delivers ectopic expression of miR-218. Currently under characterization.
iv) **retroAAV-LSL-miR-218**. It delivers conditional expression of miR-218 to cells or tissues expressing the Cre recombinase. Currently under characterization.

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

### What individuals have worked on the project?

| | |
|---|---|
| Name: | Samuel L. Pfaff |
| Project Role: | Principal Investigator |
| Researcher Identifier | |
| Nearest person month worked: | 1.8 |
| Contribution to project | Plan and interpret experiments, ensure that regulatory and reporting requirements are met, prepare manuscripts and coordinate the sharing of reagents and communication of results. |
| Funding Support: | HHMI |

| | |
|---|---|
| Name: | Giancarlo Costaguta |
| Project Role: | Senior Research Assistant |
| Researcher Identifier | |
| Nearest person month worked: | 12 |
| Contribution to project | Performs experiments in Aims 1-3. He is an experience molecular-geneticist with a background in the cellular pathways associated with neurological disease. |
| Funding Support: | DOD |

| | |
|---|---|
| Name: | Shawn Driscoll |
| Project Role: | Bioinformatics Specialist |
| Researcher Identifier | |
| Nearest person month worked: | 1.2 |
| Contribution to project | Performs statistical analysis and bioinformatics characterization of the gene expression data. |
| Funding Support: | HHMI |

| | |
|---|---|
| Name: | Miriam Gullo |
| Project Role: | Research Technician |
| Researcher Identifier | |
| Nearest person month worked: | 12 |
| Contribution to project | Assist with all mouse experiments. She is a highly experienced technician who helps to breed, genotype, and process tissue from the mice used in Aims 1-3. |
| Funding Support: | DOD |

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

Since the last reporting period, the HHMI and Target ALS grants ended. Additionally, a new grant was secured:

1 U19 NS112959-01 (Goulding, PI) 09/15/2019-06/30/2024
NIH/NINDS
Spinal Circuits for the Control of Dextrous Movement
The overall goal of this project is to define the functional organization of motor circuits in the cervical spinal cord that control forelimb movements. Project 3, Cell Phenotyping: Intrinsic physiology and genetic characteristics (S. Pfaff, Project Lead), will characterize cellular and molecular properties that define pre-motor IN subtypes and ask how they map across motor pools and joints, to assess whether diversity increases with movement dexterity.

**What other organizations were involved as partners?**

None

## 8. SPECIAL REPORTING REQUIREMENTS

**Not Applicable**

## 9. APPENDICES

**None**