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TITLE: microRNA Replacement Therapy for ALS Treatment

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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. In preliminary experiments we have found that the reporter encoded by tg-miR-218-rep detects miR-218 in SOD1(G93A) mice (n=2), whereas miR-218 activity is reduced in ALS caused by Fig4 mutation (n=1). This result needs to be confirmed, but suggests that different forms of ALS may impact miR-218 function differently. We are now testing whether miR-218 function is perturbed in TDP43(Q331K) and PFN1(C71G) mouse models. We have hypothesized that elevated miR-218 may be protective against some ALS-causing mutations. To test this 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. ACCOMLIPSHMENTS

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

The major goals of our project are to:

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

3b. 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. 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 Shawn Driscoll Dr. Sam Pfaff (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 Shawn Driscoll Dr. Sam Pfaff (12 mice of each SOD1(G93A), PFN1(C71G), Tam-218DKO)

Aim1, Tasks 2 and 3. Motor neurons affected by ALS represent a small percentage of spinal cells, therefore it is necessary to purify these neurons in order to accurately profile their gene expression. Previous studies adapted laser capture microscopy (LCM) for isolating motor neurons and we have likewise previously used this method. Therefore we proposed to use LCM to profile motor neuron gene expression in our grant application [Nicherwitz et al., Nat Commun. 2016 Jul 8;7:12139. doi: 10.1038/ncomms12139]. However, there are technical drawbacks with laser capture including contamination from non-motor neurons, low throughput in cell numbers, and poor RNA quality which limits the depth of gene coverage. Since the time between grant submission and funding the field of gene profiling has made enormous technical advances. It is now much more common to use nuclear purification in combination with next generation sequencing (NGS) to profile gene expression [Sathyamurthy et al., Cell Rep. 2018 Feb 20;22(8):2216-2225. doi: 10.1016/j.celrep.2018.02.003]. We have published several papers using next generation sequencing (NGS) of neurons. Because NGS is an improvement over our proposed method and this technology is well established in the lab we have adopted this as a minor change to our experimental plan. Importantly, this does not change the goal of this aim to profile gene expression in motor neurons affected by ALS. The laboratory has performed several NGS experiments on wild type mice and miR-218 knockout mice at birth. These data sets have allowed us to identify target RNAs regulated by miR-218 during embryogenesis.

Our next step is to determine whether miR-218 regulates gene expression in adult motor neurons, as proposed in the grant. We have generated 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. The half-life of microRNAs in non-dividing cells has been estimated to lie on average between 5 to 10 days [Gantier et al., Nucleic Acids Res. 2011 Jul; 39(13): 5692–5703. doi: 10.1093/nar/gkr148]. We expect to process these animals by February 2020, allowing the decrease of residual miR-218 to sub-optimal levels in the conditional miR-218 delete strains.

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 Dr. Sam Pfaff (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. 1). 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, we have crossed this reporter into several mouse models of ALS (see task 1 above).

We have tested pre-symptomatic (p70), disease bearing (p100-p120) and close to end-stage (p130) SOD1(G93A) mice and still detected miR-218 activity in motor neurons. This is not an unexpected result, since the SOD1(G93A) mutations may affect motor neurons through different (non-microRNA related) pathways from genes such as TDP43 and FUS. Crosses to the other models of ALS will be analyzed during the coming year.

Deletions in the FIG4 gene (ALS11) cause ALS [Chow et al., Am J Hum Genet. 2009 Jan;84(1):85-8. doi: 10.1016/j.ajhg.2008.12.010]. The pale tremor mouse (*plt*, JAX # 017800) carries a retrotransposon insertion in FIG4, and homozygous mice for *plt* show clear signs of neurodegeneration during their first post-natal week and usually die within their first two weeks. We generated FIG4 *plt/plt* tg-miR-218-rep +/- mice and preliminary experiments indicate that miR-218 function may be affected at p8, suggesting that miR-218 levels may decrease dramatically before cell death in this form of ALS (Fig. 2).

Currently, we have ALS mouse models [TDP-43(Q331K), PFN1(C71G)] combined with tg-miR-218-rep aging to reach mid and end-stage to maximize the possible detection of miR-218 activity.

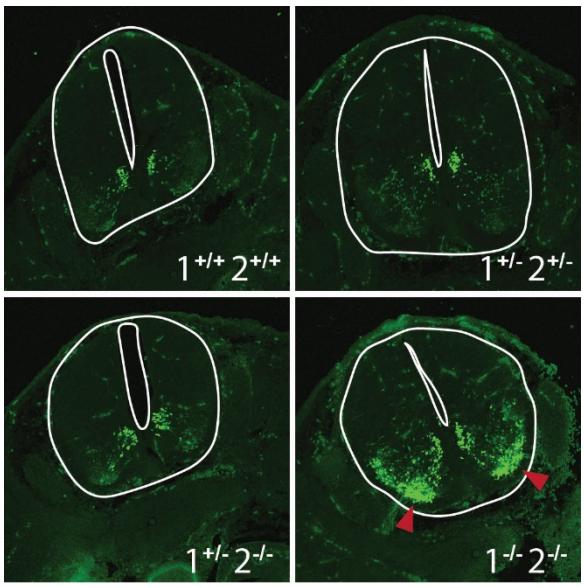

Fig. 1. MiR-218 absence is detected by GFP expression. Mouse strains with different allele deletions of miR-218 and bearing tg-miR-218-rep. Note that complete deletion of miR-218 results in appearance of GFP fluorescence in the ventral area of the developing spinal cord (red arrowheads) at e12.5.

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

Aim 1, Milestone. As we approach the first year of our ACURO authorization (~January 2020) we are actively pursuing the characterization of tg-miR-218-rep in SOD1(G93A) mice and are close to start analysis of TDP-43(Q331K) and PFN1(C71G) mice near disease end stage. 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 the function of miR-218 in embryos). We have established breeding colonies for all of the mouse lines described in the proposal. We have performed control experiment to establish the sensitivity of the in vivo miR-218 sensor (tg-miR-218-rep), and we have established a new motor neuron specific Tamoxifen-inducible Cre line and tested its efficacy in control experiments. These experiments will allow us to the first time define the function of miR-218 in adult motor neurons and determine whether the functional-levels of this microRNA change during the course of different types of ALS.

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 Dr. Giancarlo Costaguta Dr. Sam Pfaff 6 WT mice

Aim 2, Task 1. Soon after the start of the project, our lab acquired self-complementing retroAAV, which can efficiently infect cells in the spinal cord after a single intramuscular injection into one hindlimb of newborn mice (p0-p1) (Fig. 3). We initially proposed to use intrathecal injections of scAAV9, however this is more complex and is not as reliable at targeting motor neurons. Therefore a minor improvement on the original grant is to use the retroAAV capsid in combination with intramuscular injection to deliver genes rather than intrathecal injection of AAV with capsid 9. Importantly, both scAAV9 and retroAAV are self-complementing viruses, meaning that using retroAAV greatly simplifies the delivery method without compromising the experimental design.

We have cloned the retroAAV virus for overexpressing miR-218 constitutively or conditionally in cre-expressing cells (Fig 4). The viruses are engineered so infectivity in neurons can be monitored by mCardinal fluorescence.

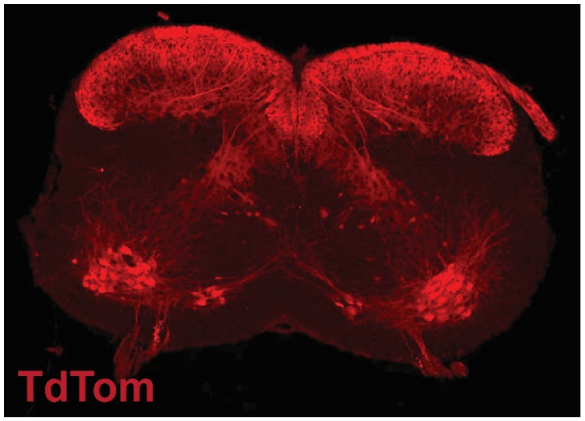

Fig. 3. Retro-AAV-Cre activates reporter in the spinal cord. Ai14 reporter strain bearing TdTom whose expression depends on the presence of the Cre recombinase. Ai14 mice were infected with a retroAAV virus bearing the Cre recombinase as described in the text. Motor neurons in the ventral horn as well as cells in the dorsal horn demonstrate viral infection. Experiment by N. Moore, G. Senturk and M. Hayashi.

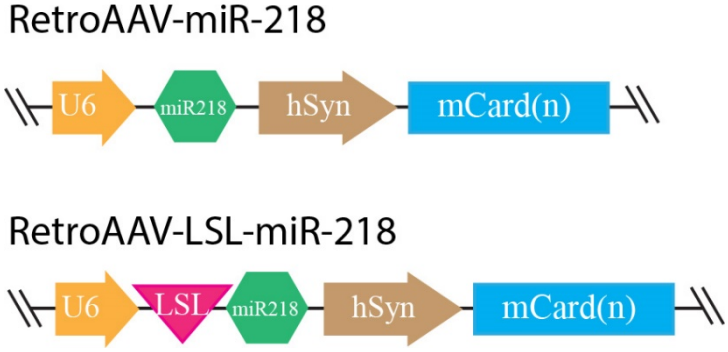

Fig. 4. Retro-AAV viruses for miR-218 expression. We have built retroAAV viral backbones that express miR-218 under the control of the human U6 promoter, based on strategy by Garwick-Coppens et al. [BMC Biotechnol. 2011 Nov 16;11:107. doi: 10.1186/1472-6750-11-107]. The top version drives constitutive expression of miR-218 (RetroAAV-miR-218) while the bottom version drives Cre-dependent conditional expression of miR-218 (RetroAAV-LSL-miR-218). Neurons infected by either virus will be monitored by expression of the far-red fluorescent protein mCardinal.

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

Aim 2, Task 2. The switch from scAAV9 to retroAAV does not change the strategy from our original proposal when using the constitutive retroAAV. We designed a conditional miR-218 retroAAV to target the expression of miR-218 to only motor neurons if we find that constitutive expression of the microRNA in

non-motor neurons is toxic For targeted expression of miR-218 in motor neurons of ALS models, we have already introduced ChAT:CRE into the SOD1(G93A) mouse and we are introducing ChAT:CRE in the PFN1(C71G) mouse.

Task 3: Delivery of scAAV9 bearing Relay218 genes to ALS mouse models with behavioral and histological analysis.	9-21	Miriam Gullo Dr. Giancarlo Costaguta Dr. Sam Pfaff (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 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 underway

Aim 3 MiR-218 mimics as ALS therapeutic agents.		
Task 1: Intrathecal delivery conditions for [miR-218].	3-6	Miriam Gullo Dr. Giancarlo Costaguta Dr. Sam Pfaff (20 Tam-218DKO mice)
Task 2: LD50 determination for [miR-218].	6-9	Miriam Gullo Dr. Giancarlo Costaguta Dr. Sam Pfaff (30 WT mice)
Task 3: Delivery of [miR-218] to mouse models of ALS.	9-24	Miriam Gullo Dr. Giancarlo Costaguta Dr. Sam Pfaff (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]). 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 have 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.

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

Aim 3, Milestone. We have identified a miR-218 mimic molecule that targets the expected gene network in cultured motor neurons. The efficacy and toxicity of this molecule 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.

3c. 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.

3d. How were 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.

3e. What do you plan to do during the next reporting period to accomplish the goals?

During the current reporting period we have set the groundwork to reach the proposed milestones. Many of our experiments involve the production of mouse strains with 2-4 gene modifications, a task that requires 2-3 generations (6-8 months). Most of the relevant mouse strains have been developed or are close to completion, allowing us to focus on the main experiments to complete our milestones by the second reporting period.

4. IMPACT

4a. 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.

4b. 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.

4c. 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.

4d. What was the impact on society beyond science and technology?

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

5. CHANGES/PROBLEMS

5a. Changes in approach and reasons for change.

i) **Aim 1: Technical change from LCM/SmartSeq to single nuclei and NGS.** Between our proposal's approval and its start date the field of single cell sequencing had progressed greatly, prompting a reconsideration of our strategy. LCM involves manual collection of 10-20 motor neurons from spinal cords, a process that is very time consuming and that relies on very small samples to make quantitative assessments. It is very challenging to purify motor neurons from adult spinal tissue in meaningful amounts, and that was the main reason we chose LCM for our proposal. However, breakthroughs in single cell sequencing have allowed the use of purified nuclei as a representative sample of the whole cell, and it has already been successfully applied to the adult spinal cord [Sathyamurthy et al., Cell Rep. 2018 Feb 20;22(8):2216-2225. doi: 10.1016/j.celrep.2018.02.003]. We realized that purification of nuclei from our mouse reporter strain (tg-miR-218-rep) followed by FACS in the context of miR-218 loss or ALS will yield enrichment of motor neurons, allowing for the generation of more quantitative data.

ii) **Aim 2: Technical change from scAAV9 to retroAAV.** Between our proposal's approval and its start date our lab had initiated work with retroAAV for gene delivery to the spinal cord. scAAV9 requires intrathecal injections to efficiently deliver its cargo to motor neurons, while for retroAAV it suffices with a single intramuscular injection in one hindlimb of p0-p1 mice. It became clear to us that by eliminating the difficulties of intrathecal injections of scAAV9 for simple intramuscular retroAAV injections, we could greatly improve the success rate of our infections. Both scAAV9 and retroAAV are self-complementing viruses, so a positive result with retroAAV should be easily translatable into viruses relevant for human gene therapy.

5b. Actual or anticipated problems or delays and actions or plans to resolve them.

i) **Autofluorescence in the green channel of the adult spinal cord.** It greatly affects the positive detection of GFP. To ameliorate or bypass this problem we are currently testing treatments that decrease autofluorescence (i.e. incubation of fixed tissue with glycerol) or using anti-GFP antibodies in channels that show less autofluorescence.

ii) **Delays on Aim1 sequencing data.** As expressed on 5ai, we switched from LCM to single RNA sequencing. The mouse strains that allow us to genetically label and FACs-purify nuclei added a slight delay to the sequencing experiment, but the crosses for these experiments are underway and we expect to reach our goal of profiling adult motor neuron gene expression in a variety of genetic backgrounds by the end of the reporting period.

5c. Changes that had a significant impact on expenditures.

None

5d. 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 on IACUC protocol 11-00020.

5e. Significant changes in use or care of human subjects.

No human subjects

5f. Significant changes in use or care of vertebrate animals.

None

5f. Significant changes in use or care of biohazards and/or select agents.

Nothing to report.

6. PRODUCTS

6a. Publications, conference papers, and presentations.

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.

6b. Website(s) or other internet site(s).

Nothing to report.

6c. 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.

6d. Inventions, patent applications, and/or licenses.

Nothing to report.

6e. Other products.

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7a. 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

7b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

W81XWH1810120 (Pfaff, PI)09/01/2018-08/31/2020
Department of Defense
MicroRNA Replacement Therapy for ALS Treatment
The major goal of this project is to determine whether miR-218 is a therapeutically viable target for ALS patients in animal models.

Howard Hughes Medical Institute (Pfaff, PI) 09/01/2018-08/31/2019
Motor Neuron Development and Connection
The major goal is to understand how spinal circuits control movement

Research Grant (Pfaff, PI) 05/01/2017-04/30/2019
Target ALS Foundation, Inc.
Relevance of miR-0218 in ALS genetics, therapeutics and diagnostics
The major goal of this project is to investigate whether miR-218 can be used to modulate ALS and serve as a quantitative biomarker.

7c. What other organizations were involved as partners?
None.

8. SPECIAL REPORTING REQUIREMENTS

8a. Collaborative awards.

Nothing to report.

8b. QUAD charts

N/A

9. APPENDICES

None.