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14. ABSTRACT
Our proposal will focus on special enzymes, called proteases that digest proteins and change the cellular environment surrounding the motor neurons in the spinal cord and the brain. Many of pathological events in the spinal cord and the brain can be traced to the local pro-inflammatory processes that trigger several downstream mechanisms including the activation of proteases, enzymes that cleave proteins in and around the cells of the brain. We propose a strategy to develop transmembrane proteins that can be delivered to the spinal cord and the brain, specifically designed to release therapeutic proteins at the sites affected by the disease, after being cleaved by proteases that are activated during ALS. Our team has developed a way to change these proteases so that instead of being harmful they can now release therapeutic proteins. Once inserted into the cell membrane of spinal cord and brain cells, these sensors can be activated by the injury, to release therapeutics in the spinal cord and the brain to avert loss of motor neurons and prevent the progression of ALS.

15. SUBJECT TERMS
amyotrophic lateral sclerosis, ALS, FTD, FTLD, Neuroinflammation, Gene Therapy, Adeno Associated Virus, AAV, brain, spinal cord

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1. INTRODUCTION:

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease of idiopathic etiology. Clinically, it is characterized by a rapid and progressive loss of motor neurons in the brainstem, spinal cord (SC), and motor cortex resulting in muscle atrophy, weight loss, and progressive paralysis leading to respiratory failure and death within 3 years of onset (Braak et al., 2013). Overwhelming majority of the ALS cases are sporadic (sALS), and only 10% are familial (fALS). Most of the fALS cases are due to genetic mutations in the Cu/Zn superoxide dismutase 1 (SOD1), repeat nucleotide expansions in the gene encoding C9ORF72, or 43kDa TAR-DNA binding protein (TDP-43). The neuropathological hallmarks of sALS are the ubiquitin-positive cytosolic aggregations of phosphorylated TDP-43 in postmortem brain and spinal cord motoneurons (Neumann et al., 2006). There is an increasing body of clinical and experimental evidence supporting a multi-factorial etiology of ALS including excitotoxicity, inflammation, oxidative stress, and proteasomal dysfunction, that critically contribute to the pathogenesis of ALS (Vucic et al., 2014). Pathological processes in ALS trigger chronic activation of local metalloproteinases (Lukaszewicz-Zajac et al., 2014). Metalloproteinases are essential for normal brain functioning during development and in adulthood by inducing cleavage of extracellular transmembrane proteins on neurons, glial, and endothelial cells, and for releasing soluble mediators. This phenomenon known as protein "ectodomain shedding" (Peschon et al., 1998) affects various transmembrane proteins, such as receptors, adhesion molecules, growth factors, cytokines, and extracellular proteases. Sustained inflammatory responses with NLRP3 inflammasome activation, secondary to protein misfolding and intermediate filament accumulation have been reported in post-mortem tissue from transgenic mice and ALS patients (Johann et al., 2015). Thus, targeting the critical molecules involved with proinflammatory pathways is a logical approach for treatment of ALS. To date, there are no effective treatments that can substantially prolong life of ALS patients. Currently approved treatments are symptomatic and ineffective in preventing the process of neurodegeneration. This raises the need for novel, preventative and therapeutic strategies that will prevent the loss of motor neurons in ALS. Here we propose an innovative strategy for ALS, whereby patients will be treated with a new disease-triggered gene therapy delivery system of genetically engineered "protease activity sensors" (PAS) carrying anti-inflammatory and neuroprotective plasma membrane ectodomains that will be released in the extracellular space when local pathological processes reach a critical threshold during ALS.

2. KEYWORDS:

Amyotrophic Lateral Sclerosis, ALS, FTD, FTLD, Neuroinflammation, Gene Therapy, Adeno Associated Virus, AAV, Brain, Spinal cord

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: To evaluate the plasma membrane expression and membrane shedding of disease-triggered PAS *in vitro* in motor neurons and *in vivo* in ALS mice using engineered adeno-associated virus (AAV) self-complimentary (sc)AAV-PHP.eB virus driven by the CAG promoter (Arotcarena et al., 2021; Chatterjee et al., 2022).

Major Task 1: Obtain ACC and ACURO regulatory approval for use of animal subjects

Major Task 2: To evaluate the plasma membrane expression and membrane shedding of disease-triggered PAS in vitro in motor neurons and in vivo in ALS mice using AAV.

Specific Aim 2: To evaluate the therapeutic efficacy of PAS in two mouse models of ALS, TAR6/6 and TAR4/4 transgenic mice

Major Task 3: Evaluation of two therapeutic-PAS in ALS animal models of spinal cord and brain motor neuron degeneration

Specific Aim 3: To characterize the proteome of the transmembrane proteins that are cleaved by activated matrix metalloproteinases (MPs) in mouse models of ALS

Major Task 4: To evaluate the plasma membrane expression and membrane shedding, expression and activity of metalloproteinases in ALS models

What was accomplished under these goals?

Specific Aim 1: To evaluate the plasma membrane expression and membrane shedding of disease-triggered PAS *in vitro* in motor neurons and *in vivo* in ALS mice using adeno-associated virus (AAV).

Major Task 1: Obtain ACC and ACURO regulatory approval for use of animal subjects

First, to evaluate the plasma membrane expression and membrane shedding of disease-triggered PAS in motor neurons in vitro that are delivered using adeno-associated virus (AAV) we developed animal protocol "Gene and Protein Expression in Neurological Diseases", IACUC protocol number A22-0200 and received regulatory approvals from the Animal Care Committee (ACC) at the University of British Columbia (UBC), or Canadian equivalent to IACUC, and subsequently received approval by ACURO at DoD.

Second, to breed ALS mice TAR4/4 mice (Wils et al., 2010), used to evaluate *in vivo* our inducible gene therapy for ALS we developed a breeding animal protocol: "Breeding Protocol to study Neurodegeneration", IACUC protocol number A23-0084 and received regulatory approvals from the UBC ACC, as the Canadian equivalent to IACUC, and subsequently obtained approval by ACURO specialists at DoD.

Third, to initiate the work with the proposed TAR4/4 and TAR 6/6 (Wils et al., 2010; Scherz et al., 2018) experimental animal models of ALS and to evaluate membrane shedding of disease-triggered PAS *in vivo* in these models, AAV carrying an engineered IL-1ra as an anti-inflammatory plasma membrane ectodomain we developed a breeding animal protocol: "Gene Therapy for Neurological

Disorders", IACUC protocol number A22-0200 and received regulatory approvals from the UBC ACC, as a Canadian equivalent to IACUC, and subsequently by ACURO at DoD.

<u>Major Task 2:</u> To evaluate the plasma membrane expression and membrane shedding of disease-triggered PAS *in vitro* in cortical neurons and *in vivo* in ALS mice using AAV.

Subtask 1: We have developing PAS that carry **IL-1ra ectodomains** that was delivered *in vitro* and *in vivo* using **scAAV-PHP.eB** virus driven by CAG promoter (Arotcarena et al., 2021; Chatterjee et al., 2022). Reporter-tagged PAS will be analyzed using IHC, WB and ELISA.

We have genetically engineered "protease activity sensor" (PAS) as chimeric transmembrane protein that will be cleaved by activated metalloproteinase during the progression of ALS, thereby releasing their tagged-extracellular domains in the vicinity of affected motoneurons in ALS mouse models of ALS (Wils et al., 2010; Scherz et al., 2018).

For the present study we have selected AAV vectors that are well-established viral-based technology for gene delivery in the brain (Klein et al., 2008; Hester et al., 2009; Kotterman and Schaffer, 2014; Deverman et al., 2016; Challis et al., 2019; Konno and Hirai, 2020). AAV vectors generally lack pathogenicity, induce negligible immune response, and can establish long-term transgene expression in both, dividing and non-dividing brain cells. Initially, the evaluation of membrane shedding and the release of tagged ectodomains will be performed *in vitro* under various adverse conditions that are present in ALS such as: excitotoxicity (glutamate).

These membrane spanning PAS construct consists of 2 reporter elements, including the Flag tag (Einhauer and Jungbauer, 2001), and a hemagglutinin A (HA) tags (Zhao et al., 2013). A protease cleavage site, engineered to be sensitive to injury/inflammation activated membrane associated proteases of the ADAM and MMP families (Candelario-Jalil et al., 2009; Rosenberg, 2009) is located just extracellular to the membrane spanning domain of the construct. In the earlier reports we demonstrated that upon infection with AAV carrying our construct, HEK293 cells in culture expressed the engineered HA-tagged PAS proteins. The HA-tagged ectodomains from our engineered constructs were detected in the extracellular medium within minutes following stimulation with ionomycin.

Our most recent generation of constructs (**Figs. 1 and 2**) consists of a reporter peptide hemagglutinin (HA) fused to the extracellular domain of transmembrane protein chemokine fractalkine (FKN), which is constitutively expressed in neurons, glial, and endothelial cells (Erichsen et al., 2003). We have demonstrated that upon neuronal injury, the FKN-based constructs are cleaved by metalloproteinases ADAM10 and ADAM17 (Erichsen et al., 2003; Hundhausen et al., 2003; Mizuno et al., 2003; Wildenberg et al., 2008) within minutes of being exposed to various adverse neuronal conditions including excitotoxicity (**Fig. 2**).

Subtask 2: Testing the delivery AAV carrying the reporter-tagged PAS constructs and PAS shedding *in vitro* using HEK293 cells and/or neurons (Fig. 1).

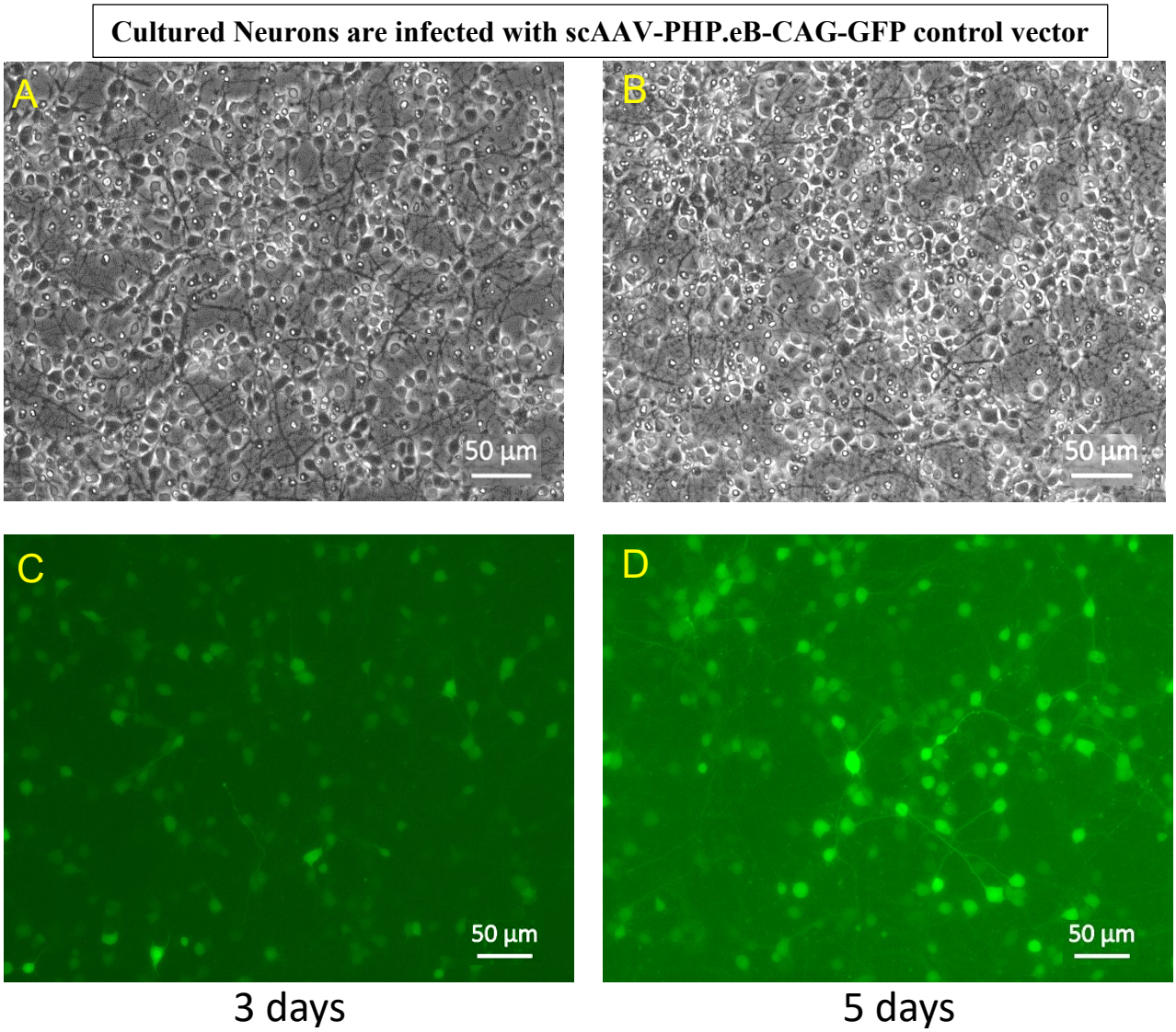

Figure 1. We used engineered adeno-associated virus (AAV) self-complimentary (sc) AAV-PHP.eB virus driven by CAG promoter to transduce primary cortical culture (at $5x10^4$ MOI). The presence of GFP in the cytoplasm was confirmed with GFP fluorescence microscopy, 3 (**A, C**) and 5 (**B, D**) days after infection. **A-B** bright field images of infected neurons. **C-D** presence of GFP-tagged constructs in the cytoplasm was confirmed with GFP fluorescence microscopy (scale bar 50µm; green=GFP).

Subtask 3: Evaluating delivery, expression of reporter tagged-PAS ***in vivo*** in mice (Fig. 3).

Milestone(s) Achieved*: We have successfully demonstrated expression and release (membrane shedding and releasing ectodomains in the extracellular space) of scAAV-PHP.eB virus-delivered PAS-IL-1ra-HA construct in vitro* (Fig. 2A and 2B) and *in vivo* (Fig. 7C).

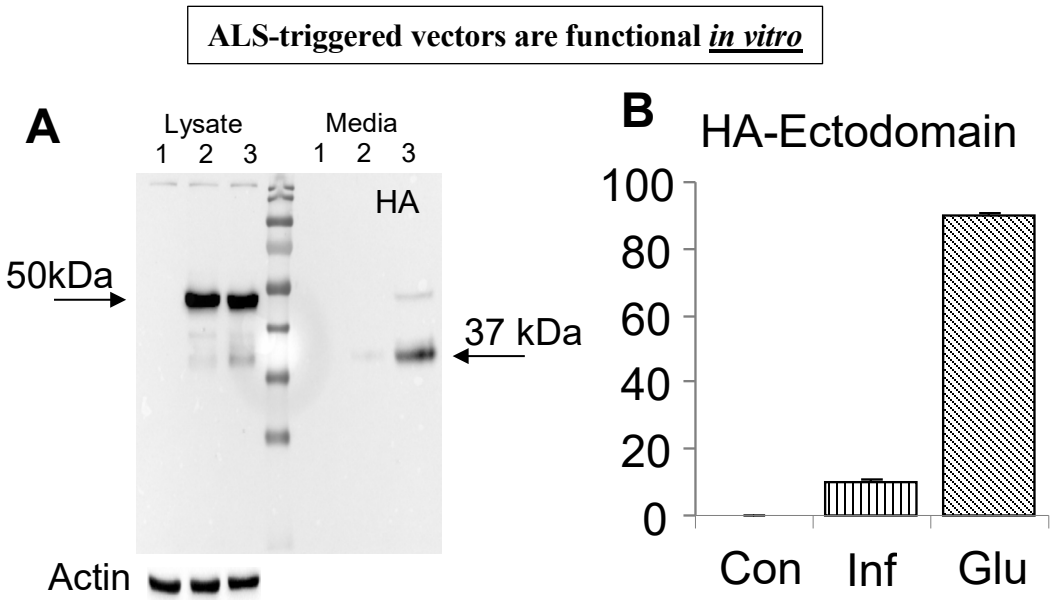

Figure 2. ALS-triggered vectors are functional *in vitro*. Glutamate activated proteases cleave our engineered transmembrane protein at the protease cleavage site, leading to the release of the HA-tagged IL-1ra therapeutic ectodomain (37 kDa). **(A)** A representative Western Blot (WB) from primary cultured cortical neurons. The whole lysate and the media after 100μM glutamate (Glu) treatment obtained from AAV vector-infected neurons (Inf) demonstrates membrane shedding and release into the medium of the HA-tagged extracellular domain of the glutamate-triggered vectors. **(B)** Summary WB data from 3 repeats in **A**.

Specific Aim 2: To evaluate the therapeutic efficacy of PAS in two mouse models of ALS, TAR4/4 and TAR6/6 transgenic mice

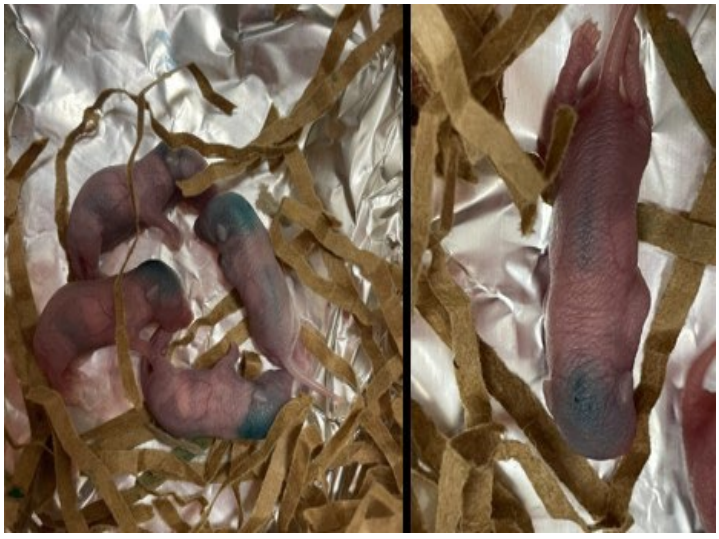

Fig. 3 Methylene blue (MB) injection into cisterna magna (ICM). To approximately outline the area in the CNS that is covered by ICM of AAV, newborn C57/Bl6 pups (P0) were injected with 1μL of methylene blue as a tracer (10mg/mL), into cisterna magna. Within minutes, the brain, medulla and the spinal cord were stained with MB. CSF is produced by the choroid plexus flows from the ventricles to the subarachnoid space via the cisterna magna, that is the most favorable and the least invasive route for direct AAV delivery into CNS.

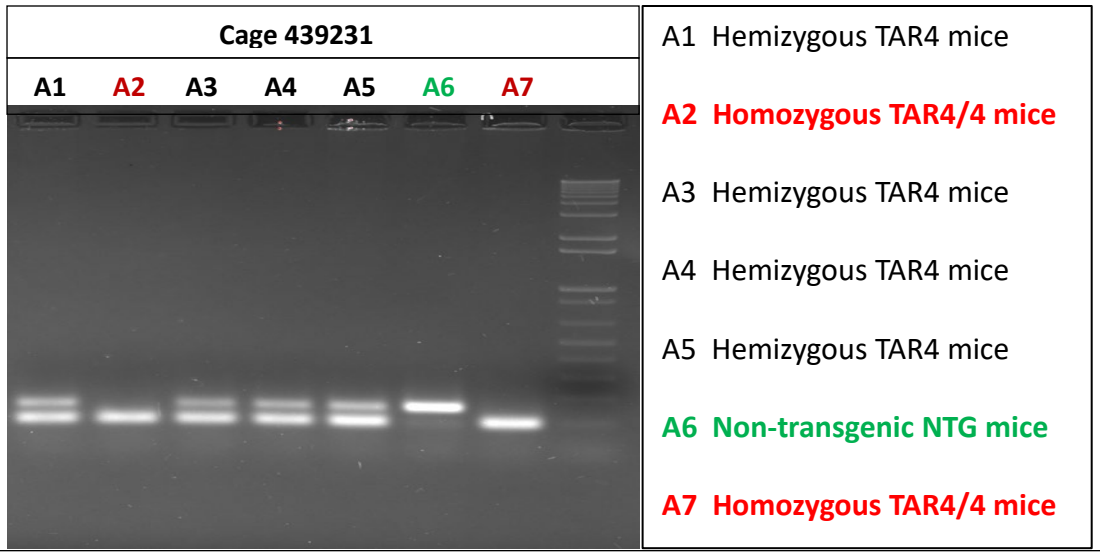

Figure 4. Genotyping of ALS TAR4 (Hemi), TAR4/4 (Homo), NTG mice. We have genotyped all NTG, hemi TAR4 and homo TAR4/4 mice at 14 days of age based on the "touchdown" cycling protocol and the primers designed by the Jackson Laboratory (USA). This assay can distinguish hemizygous (TAR4) from homozygous (TAR4/4) and NTG mice. The assay is designed around the transgene insertion site known to be on the mouse Chr 6. **Left:** A representative Blot of the PCR products for one litter (the 160 bp product is from mouse wtTDP43, and the 100 bp product is amplified from human wtTDP43). **Right:** Assigned genotypes for 7 mice from a single litter.

Phenotype of homozygous Tar4/4 mice

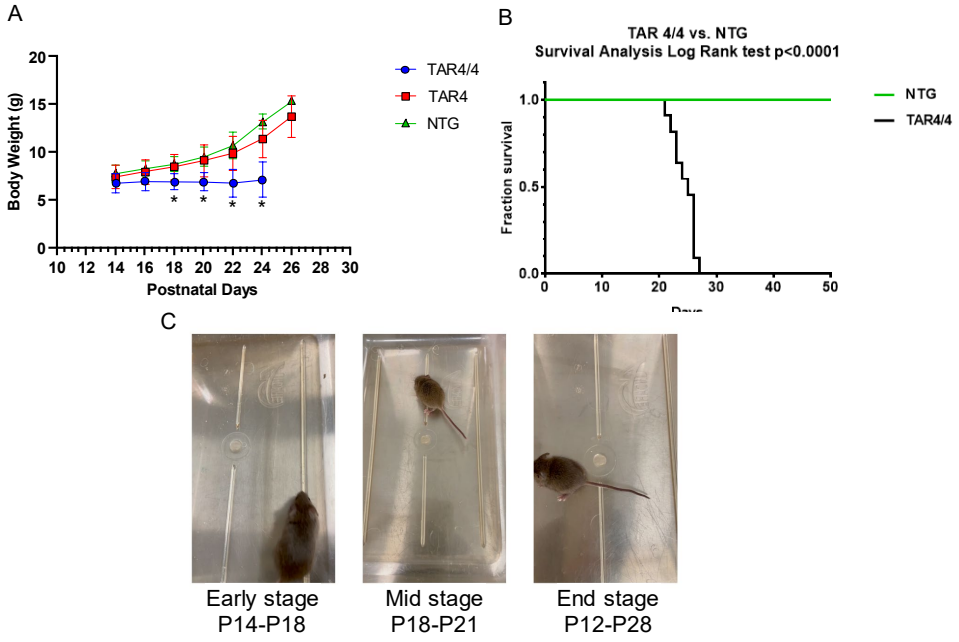

Figure 5. Phenotype of human TDP43 overexpressing homozygous Tar4/4 mice. These mice exhibit progressive cortical atrophy, muscle denervation, motoneuron (MN) loss and muscle atrophy: (A) retarded growth and weight loss after 14 days of age, (B) followed by an extremely rapid disease progression with complete paralysis and median survival of 25 days. (C) impaired mobility develop after 14 days with abnormal walking with axial tremulous movement and falling sideways after 22 days. The movies presented in C could not fit the size limit of the report.

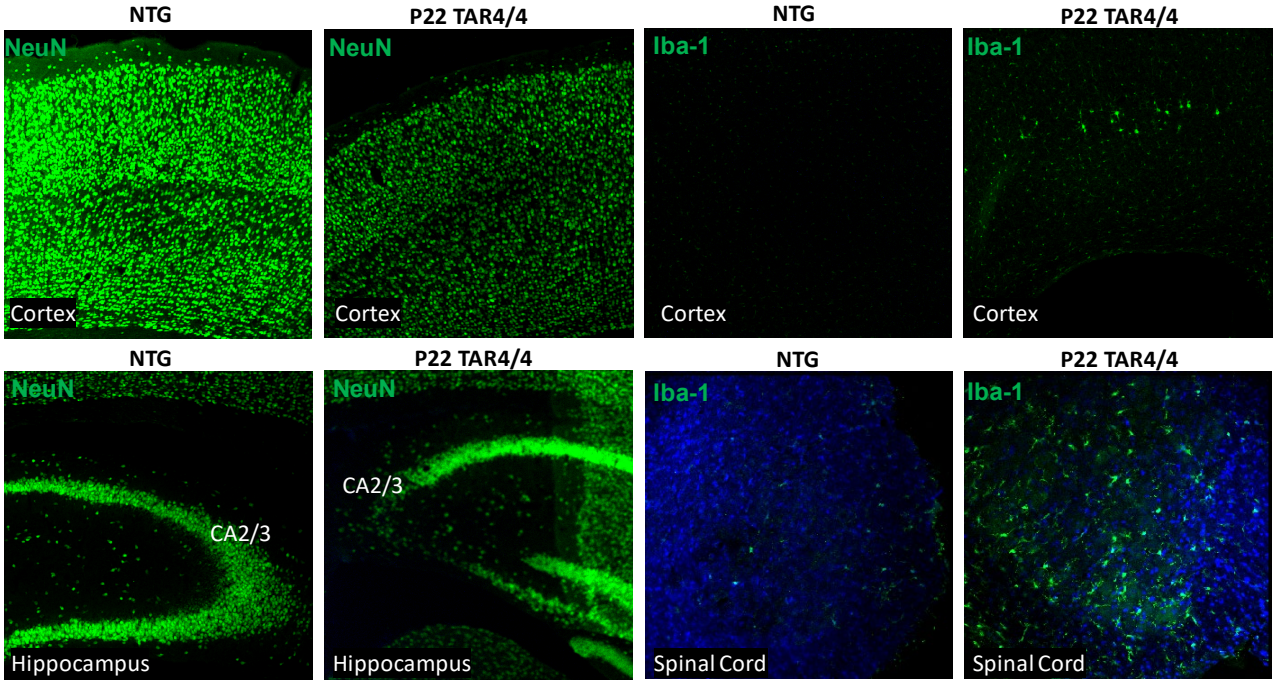

Figure 6. Cortical and hippocampal neurodegeneration, and cortical and spinal cord microgliosis in Tar4/4 mice. (A) There was loss of neurons (cortex and hippocampus) and microgliosis in the cortex and ventral horns of the lumbar spinal cord by P22.

Animal models of ALS

We selected **TAR4/4** transgenic mice from Kumar-Singh Lab (Wils et al., 2010) that overexpress wild-type human TARDBP (wt-hTDP43) under the control of a neuronal murine Thy-1 promoter, which drives pan-neuronal expression around postnatal day seven (P7). These mice are used as animal model for familial (and to some extent) sporadic ALS/FTD, for developing and testing new therapies for ALS. Our choice of these mice was based on their strong and stable phenotype caused by abnormal TDP-43 accumulation. **TAR4** mice, hemizygous for wt-hTDP43 are viable, fertile, and grossly normal. Hemizygous TAR4 mice**,** JAX - B6;SJL-Tg(Thy1-TARDBP)4Singh/J, will be used as carriers to produce the homozygous **TAR4/4** transgenic mice for our project. At birth, the mice homozygous **TAR4/4** are morphologically indistinguishable from their hemizygous littermates. However, at 14 days **TAR4/4** mice display profound motor dysfunction, a hindlimb clasping reflex when lifted by the tails, characterized by retraction of hind legs toward the trunk rather than extending them. Early on, they also display a shortened stride, a wide stance, and frequent stumbling, resulting in a complete inability to walk around P21 and death in the fourth week of life (Wils et al., 2010).

Homozygous **TAR6/6** mice have 3.8 fold wt-hTDP-43 higher protein expression levels compared to endogenous murine TDP-43, develops abnormal hind limb reflex at 2 months, shows up to 6-fold reduced motor performance at 4 months exhibiting an ALS/FTLD like phenotype with a moderate progression rate. Histological analyses reveals neuronal loss and neuroinflammation in the motor cortex as well as in the spinal cord in 6 months old TAR6/6 mice. At birth, the **TAR6/6** mice are morphologically indistinguishable from their hemizygous littermates. At 2 months, the mice develop

abnormal hindlimb reflex, early motor deficits in the clasping- and wire suspension test (Scherz et al., 2018) and 6-fold reduction in motor activity at 4 months (Wils et al., 2010), decreased anxiety in the elevated plus maze, with an average survival time of 6.5 months (Wils et al., 2010).

These transgenic models exhibit progressive cortical atrophy, muscle denervation, motoneuron (MN) loss and muscle atrophy. These mice also develop reactive microgliosis and astrocytosis in the spinal cord resulting in release of pro-inflammatory cytokines, chemokines, prostaglandins and reactive oxygen species (Wils et al., 2010; Scherz et al., 2018). Recently, we have also suggested a role for TDP-43 in regulating inflammation by competting with NF-κB for the nuclear transporter importin α3 (Zhu et al., 2015).

<u>Major Task 3:</u> Evaluation of two therapeutic-PAS in ALS animal models of spinal cord and brain motor neuron degeneration

Subtask 1: We have used **scAAV-PHP.eB virus** driven by the CAG promoter (Arotcarena et al., 2021; Chatterjee et al., 2022), and our engineered protease activity sensors (PAS) to carry **IL-1ra** as **therapeutic ectodomain** that was delivered *in vivo* (Fig. 7A and 7B).

ICM Injection of AAV-PHP.eb-CAG-IL1ra-HA in Neonatal mice

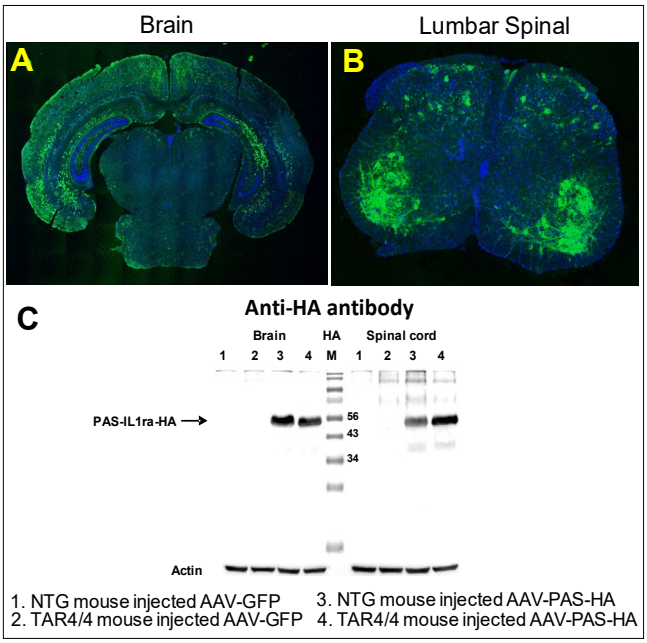

Figure 7. *In vivo* delivery of scAAV9.PHP.eB-CAG-IL-1ra-HA in mice brain and spinal cord. We used self-complementary (sc)AAV9.PHP.eB virus (1μL 10^{13} vg/mice) to successfully deliver CAG-PAS-IL-1ra-HA therapeutic DNA construct in mouse brain and spinal cord via injection in cisterna magna (ICM). Photo-micrographs demonstrate the presence of therapeutic PAS-IL-1ra-HA 7 days after ICM injection in mouse brain (A) and in ventral horns of spinal cord (B); (C) representative Western Blot (WB) using brain and spinal cord whole cell lysates, obtained from TAR4/4 and NTG mice injected with either control scAAV9.PHP.eB-CAG-GFP (lane 1, 2) or therapeutic scAAV9.PHP.eB-CAG-PAS-IL-1ra-HA virus (lane 3, 4). The membrane was probed with anti-HA antibody; IHC: blue (DAPI), green (anti-HA-Ab).

ICM Injection of AAV-PHP.eb-CAG-IL1ra-HA alleviate weight loss and prolongs survival

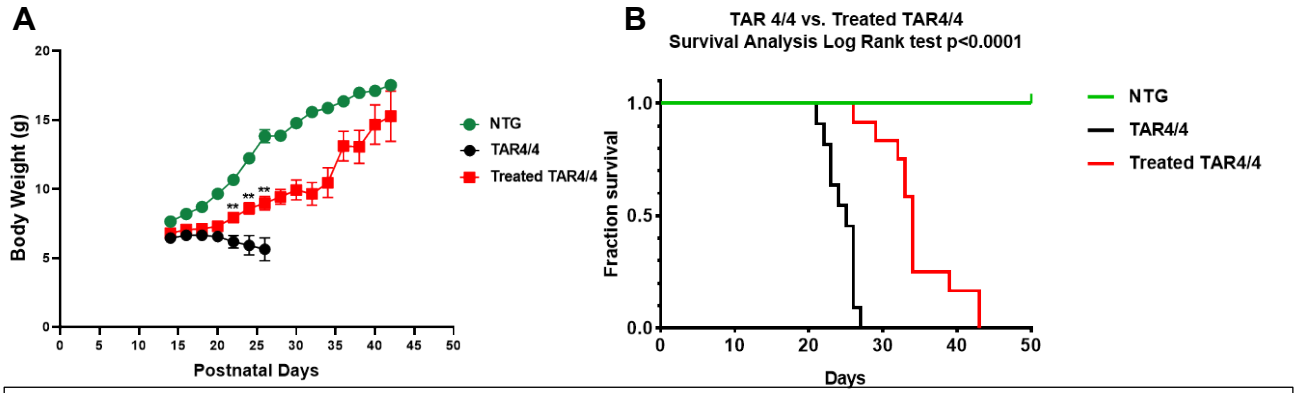

Figure 8. Therapeutic effects of AAV delivered PAS-IL-1ra in TAR4/4 ALS mouse model. Using the self-complementary (sc)AAV9.PHP.eB virus (1µL 10^{13} vg/mice) we delivered therapeutic PAS-IL-1ra construct via cisterna magna to the brain and the ventral roots of spinal cord. (A) treatment with **scAAV9.PHP.eB-CAG-IL-1ra-HA** virus (AAV-PAS-IL-1ra) carrying the inducible therapeutic PAS-IL-1ra (red) significantly alleviated ($p<0.01$) the loss of weight in TAR4/4 mice (black), (B) Therapeutic PAS-IL-1ra significantly increased the median survival time of TAR4/4 mice (red) vs treated TAR4/4 mice (black), from 25 to 34 days (40 %, $p<0.0001$).

Subtask 3: We have evaluated the efficacy of PAS carrying **therapeutic IL-1ra ectodomain** in **TAR4/4** mouse model of ALS (Fig. 8 and 9). Briefly, PAS-IL-1ra significantly alleviated ($P<0.01$) the loss of weight in TAR4/4 mice (Fig. 8A) ($p<0.01$), significantly increased the median survival time in treated TAR4/4 mice (Fig. 8B), from 25 to 34 days (40 %, $p<0.0001$), significantly improved the Hind limb reflex in treated TAR4/4 mice (Fig. 9A), significantly reduced the hind limb impairment (Fig. 9B), and reduced tremor in treated TAR4/4 mice (Fig. 9C) ($p<0.001$).

ICM Injection of AAV-PHP.eb-CAG-IL1ra-HA improves mobility in ALS mice

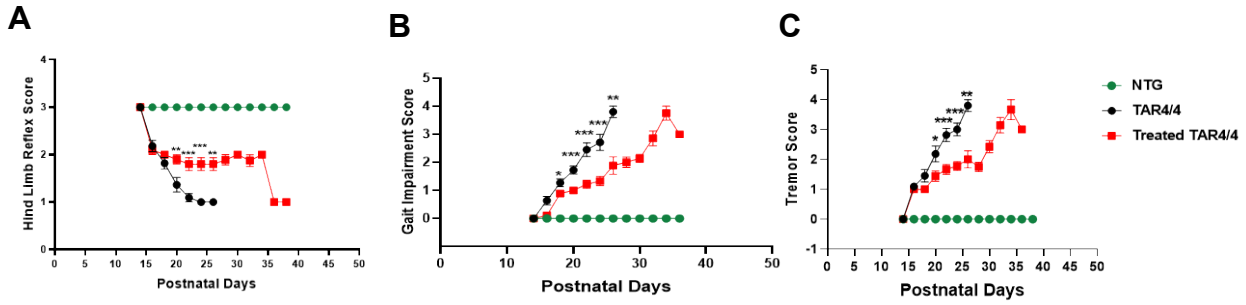

Figure 9. Therapeutic effects of AAV delivered PAS-IL-1ra in TAR4/4 ALS mouse model. Using the self-complementary (sc)AAV9.PHP.eB virus (1µL 10^{13} vg/mice) we delivered therapeutic PAS-IL-1ra construct via cisterna magna to the brain and the ventral roots of spinal cord. (A) treatment with **scAAV9.PHP.eB-CAG-IL-1ra-HA** virus (AAV-PAS-IL-1ra) carrying the inducible therapeutic PAS-IL-1ra (red) significantly improved ($p<0.001$) the Hind limb reflex in TAR4/4 mice (black), (B) Therapeutic PAS-IL-1ra significantly reduced the hind limb impairment ($p<0.001$) and (C) reduced tremor in treated TAR4/4 mice (red) ($p<0.001$).

***Milestone(s) Achieved**: We have demonstrated significant therapeutic effects of AAV-PAS therapeutic constructs in TAR4/4 transgenic mouse model of ALS* (Fig. 8 and Fig. 9)*. Publication in peer reviewed journals are considered.*

Major achievements in the First Year of the project

1. In the first year we developed two viral vectors that are used to deliver genes to the brain and spinal cord of experimental mouse models of ALS. The therapeutic scAAV-PHP.eB-CA**G-PAS-IL-1ra-**HA-Flag and the control scAAV-PHP.eB-CAG-GFP viruses are designed to carry the protease "inducible" (PAS) interleukin 1 receptor antagonist (IL-1ra), and GFP respectively. We have democtrated their expression in neuronal cells *in vitro* (Fig. 1 and Fig. 2) and *in vivo* in the brain and spinal cord using (Fig. 6) using immunofluorescence (Fig. 1) and IHC (Fig. 2 and Fig. 6 top panel).

2. We have successfully established a colony of TAR4 breeders (Wils et al., 2010). By breeding TAR4 we can yield approximately 50% TAR4, 25% non-transgenic, and 25% homozygous TAR4/4 mice. As was addressed above, only TAR4/4 develop the severe signs of ALS within the first month.

3. We have successfully evaluated intra cisterna magna (ICM) injections using nontoxic dyes to trace its distribution in the sensomotory regions of the brain, cervical and lumbar segments of the spinal cord (Fig. 3).

4. We have successfully adapted the Jackson Lab genotyping protocol that have allowed us to identify the hemizygous TAR4, non-transgenic NTG, and the homozygous TAR4/4 mice (Fig. 4).

5. We have characterize the phenotype of homozygous TAR4/4 mice in our facility (Fig. 5). We confirmed that homozygous TAR4/4 mice are morphologically indistinguishable from their hemizygous littermates. At 14 days, TAR4/4 mice display profound motor dysfunction, a hindlimb clasping reflex when lifted by the tails, characterized by: retraction of hind legs toward the trunk rather than the normal extension, a shortened stride, a wide stance, and frequent stumbling, resulting in a complete inability to walk around P21 and death in the fourth week of life (Wils et al., 2010).

6. We have shown that TAR4/4 mouse develop cortical and hippocampal neurodegeneration, and microgliosis in the cortex and ventral horns of the lumbar spinal cord by postnatal day 22 (Fig. 6).

7. We successfully delivered the CAG-PAS-IL-1ra-HA therapeutic DNA construct in mouse brain and spinal cord after intra cisterna magna injection of scAAV9.PHP.eB-CAG-IL-ra-HA virus (Fig. 7). The presence of therapeutic PAS-IL-1ra-HA protein was demonstrated with immunofluorescence staining and IHC of brain and spinal cord (Fig 7A and 7B), and with Western Blot of brain cell lysates (Fig. 7C).

8. ICM treatment with the self-complementary **scAAV9.PHP.eB-CAG-IL-1ra-HA** virus carrying the inducible therapeutic PAS-IL-1ra transmembrane construct significantly **alleviated the loss of weight**, significantly **increased the median survival** time in treated TAR4/4 mice from 25 to 34

days (40 %,) (Fig. 8), significantly **improved the Hind limb reflex**, significantly **reduced the hind limb impairment** and **reduced tremor** in treated TAR4/4 mice (Fig. 9).

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

The Centre for Brain Health (CBH) is the host institution that combines clinical and basic research under the same roof. Through regular meetings such as the weekly and monthly staff meetings, the proposed research is disseminated to the clinicians who in turn will disseminate results to patients, clinical colleagues and industrial contacts. Complementary feedback allows the scientists to be kept abreast of the latest results of relevant clinical trials by the clinicians who attend these meetings. This information exchange provides both, clinicians and scientists, with a new synthesis of ideas and strategies which will feed into the development and planning of future experiments towards our end goal of developing a novel strategy to complement and improve treatments for ALS. Further knowledge translation occurs, as our investigators are active speakers both nationally and internationally and results will be presented at international meetings. The researchers at the Centre for Brain Health Publications regularly publish in high impact factor journals that are an important measure of research output and productivity. CBH hosts a variety of journal clubs and seminars offering an excellent opportunity for researchers and students alike to meet each other and learn more about what their colleagues are studying. The journal clubs provide opportunities for students, postdocs, and faculty to discuss recent research. Graduate students and postdocs are encouraged to present topics relevant to their own studies, and everyone is welcome to attend. A multidisciplinary perspective is promoted, with discussions related to genetics, imaging, animal models, biochemistry/molecular biology, as well as clinical research.

How were the results disseminated to communities of interest?

Nothing to Report.

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

During the next 12 months we plan to achieve the following grant objectives:

1. **Develop and evaluate** *in vitro* and *in vivo* new gene therapy, the disease **triggered release of the vascular endothelial growth factor** (**VEGF**) (Storkebaum et al., 2005; Keifer et al., 2014), to be extracellularly released at the sites of tissue damage in ALS, by the "protease activity sensors" (**PAS),** as their therapeutic ectodomains. These sites are center of the pathological events driven by ALS that results in neurodegeneration (loss of neurons) and neuroinflammation (microgliosis and astrogliosis). The transgenes expression will be driven by the CAG promoter to provide long-term and robust expression of therapeutic genes in the CNS.
2. **Introduce a new animal model of ALS**, the TAR6/6 mouse (Wils et al., 2010; Scherz et al., 2018). TAR6/6 model develops a later disease phenotype onset compared to TAR4/4 and an average survival time of 6.5 months, that validates its suitability for testing new therapies for ALS, a devastating, and currently incurable disease.

3. **Evaluate the expression and distribution** of the scAAV9.PHP.eB virus carrying the CAG-**VEGF**-HA therapeutic transgene using an *in vitro* model of cortical neurons.
4. Evaluate the expression and distribution of the scAAV9.PHP.eB virus carrying the CAG-**VEGF**-HA therapeutic transgene after intra cisterna magna (ICM) delivery.
5. **Evaluate the therapeutic potential** of disease-induced release of CAG-**VEGF**-HA therapeutic delivered by an scAAV9.PHP.eB-CAG-**VEGF**-HA viral vector in the TAR4/4 mouse model of ALS.
6. **We will analyze the proteome of transmembrane proteins** that are cleaved by matrix metalloproteinases (MMPs), and a disintegrin and metalloproteinases (ADAMs) activated in ALS. We will use quantitative shotgun proteomics and tandem mass spectrometry (MS/MS) to identify the transmembrane proteins that are specifically cleaved in ALS, and utilize their molecular structure to design ALS-specific PAS carrying therapeutic extracellular domains in mouse models of ALS. To accomplish this goal, we will use SC and brain samples from: mouse model of ALS (Wils et al., 2010; Scherz et al., 2018), at different stages of disease progression.

4. IMPACT:

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

Nothing to Report

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

Nothing to Report

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

We have anticipated that 3 months would be sufficient to complete that Major Task 1. Instead of the planned, and allocated 3 months, in the SOW statement, it took almost 5 months to obtain all approvals. The complexity of our models (ALS animals with complex morbidities and high mortality) required more time form the UBC Animal Care Committee (IACUC) veterinarians and additional questions to be addressed. Although the Covid-19 pandemic is behind us, the dynamic of animal protocol approval may have taken a hit. These protocols were send to ACURO in May and June 2023, instead of the usual two months review ending in April, so the protocols were send during June 2023 for the DoD ACURO approval. The summer vacation period also took a toll, and the protocols were approved in the middle of July, a two month delay. To maximize the time we started with the *in vitro* work as soon as the in vitro protocol was approved. When in the second half of July the breeding and treatment protocols were approved we start ordering animals. Instead of 3 months this process playout in 5 months. Starting immediately with the *in vitro* soon after the approval, and purchasing the *in vitro* work and molecular lab supply we have mitigated somewhat of the delays in the process.

This delay would not involve any change in the approved objectives or scope of the project, and would not require any additional funding.

Changes that had a significant impact on expenditures

Nothing to Report

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

Nothing to Report

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

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

  Journal publications.

  Nothing to Report

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

  Nothing to Report

  Other publications, conference papers and presentations.

  Nothing to Report

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

  Nothing to Report

- **Technologies or techniques**

  Nothing to Report

- **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?

PDs/PIs on the project

Name:	**Dr. Max Cynader**
Project Role:	PD/PI
Researcher Identifier (eRA ID):	MAXCYNADER
Nearest person month worked:	3
Contribution to Project:	Dr. Cynader is reviewing and coordinating the preparation and the filing of all required regulatory documents; he is overseeing the *in vitro* and *in vivo* experiments used for testing the PAS constructs in animal models of ALS, lead and provide major direction to timely execute the goals and objectives of the project.

Name:	**Dr. Ljubomir Kojic**
Project Role:	Co-PD/PI
Researcher Identifier (eRA ID):	LJUBOMIRKOJIC
Nearest person month worked:	6
Contribution to Project:	Dr. Kojic is directly involved in the designing of the protease activity sensors (PAS) constructs developed for the treatment of ALS in cellular and animal models; he will prepare the animal protocol and other regulatory documents, and submitting them and communicating with the IACUC and DoD regulatory agencies, assuring their final approval. He is involved in the preparing, monitoring and proper execution of the experimental design. He is directly involved in completing and filing the yearly and final reports.

Name:	**Dr. Yanhua Wen**
Project Role:	Research Assistant
Researcher Identifier (eRA ID):	
Nearest person month worked:	12
Contribution to Project:	Dr. Wen is involved in the development and testing of the AAV vectors and PAS constructs

	in vitro and *in vivo* experimental models, preforming the experimental protocols, preparing and analyzing data for the yearly and final reports.
Name:	**Dr. Jingyan Zhu**
Project Role:	Research Associate
Researcher Identifier (eRA ID):	
Nearest person month worked:	12
Contribution to Project:	Dr. Zhu is involved in the development and testing of the AAV vectors and PAS constructs *in vivo*, performing the immunohistochemistry and western blotting of brain and spinal cord from animal models and preparing and analyzing data for the yearly and final reports.

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

Nothing to Report

What other organizations were involved as partners?

No other partner organizations were involved.

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS:

Nothing to Report

QUAD CHARTS:

9. APPENDICES:

10. REFERENCES:

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