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TITLE: Developing Cell-Based and Mechanism-Focused Preclinical Platforms with Diseased Upper Motor Neurons

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14. ABSTRACT

This is a two-year DoD grant, allowing us to develop a semi-high throughput drug discovery and verification platform that incorporates the health of diseased upper motor neurons as an outcome measure, so that the cortical component of ALS can also be incorporated into drug discovery efforts in ALS. Since, no compound in clinical trials has ever been tested for their efficacy on improving the health of diseased upper motor neurons, this proposal fills an important gap in the field. On our first year, we developed mixed cortical cultures from four different disease models, each of which recapitulate a different aspect of the disease, such as the misfolded SOD1 toxicity, TDP-43 pathology and absence of alsin and profilin function. We optimized the high throughput plate culturing, (ie number of cells, the confluency, time of culture and the mode of tile imaging) so that reproducible results can be obtained from each culture condition. The first year was important for optimization and we hope and plan to have more robust experimental output in the second year.

Upper Motor Neuron, Drug Discovery, High Throughput, Different Disease mechanisms

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Our goal is to develop a novel preclinical drug development framework which will allow incorporation of diseased upper motor neurons so that more effective and precise development of new treatments for ALS can be developed. We propose to use mixed cortical cultures in which upper motor neurons that are engineered to light up fluorescent green so that they can be identified among other cortical neurons and cells in culture and their response to treatment can be rapidly screened and potential therapeutic chemical compounds can be identified in a semi high throughput fashion.
Our goal is to develop a novel preclinical drug development framework which will allow incorporation of diseased upper motor neurons so that more effective and precise development of new treatments for ALS can be developed. We propose to use mixed cortical cultures in which upper motor neurons that are engineered to light up fluorescent green so that they can be identified among other cortical neurons and cells in culture and their response to treatment can be rapidly screened and potential therapeutic chemical compounds can be identified in a semi high throughput fashion.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Amyotrophic Lateral Sclerosis, Upper Motor Neurons, Disease Mechanisms; Drug Discovery

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Goals of the Project
Start Date – 05/01/2022

	Completed	Comments
Major Task 1 – Northwestern University institutional animal care and use committee (IACUC) and USAMRDC Animal Care and Use Review Office (ACURO) approval.		
Subtask 1 – Prepare animal protocols and submit for approval.	100%	
Subtask 2- Revise protocols in response to reviewers' questions and feedback	100%	
Subtask 3 – Update and resubmit annually.	100%	
Milestones Achieved: Approved protocols in place.		

Major Task 2 In reporter lines of UMNs that are diseased due to different underlying causes, the UMNs will be labeled with eGFP expression that allows their visualization and cellular assessment *in vitro*.		
Subtask 1 – Utilize the following mouse strains: A. UCHL1-eGFP (healthy control) B. hSOD1^{G93A}-UeGFP C. TDP-43^{A315T}-UeGFP D. AlsinKO-UeGFP E. PFN-UeGFP **F.** SPAST^{C448Y}-UeGFP (diseased) (minimum of 10 mice/genotype)	100%	We generated and have active colonies for all these mouse models of ALS
Subtask 2 - Isolate motor cortex cultures from mouse strains above and plate 5000 cells/well in 96-well black polystyrene plates in preparation for high throughput assays. Culture for 6-8 hours.	100%	We actually even improved the culture conditions since we submitted the grant.
Subtask 3 - Select and mark a 20X objective field for each well, where cell density is about 50 neurons/20X objective field. Perform live imaging of each well first in green fluorescent (label location of GFP+ UMNs in the view point) and then in brightfield. Continue taking photographs of exact locations for each well (10 second max exposure for fluorescent and 2 second max exposure to brightfield) for 5 hours. Collect imaging data for each well.	75%	We are still optimizing the 20X tile imaging and reproducible cellular assessment
Subtask 4 - Plates will be imaged on the ImageXpress High Content Imager and analyzed for Neurite Outgrowth using the MetaXpress Transfluor application module, which provides a robust, out of the box solution to quantitate neuronal morphology. Perform quantitative measures: Quantify changes in neurite length of GFP+ UMN in each well, measure average rate of axon outgrowth, average neurite length, number of branch points per neuron, and changes in the total number of eGFP+ neurons	90-100%	We mostly optimized the neuronal tracing in tiled images, we may still need to optimize brightness.
Subtask 5 - Repeat these experiments for dissociated motor cortex isolated from P3 pups of hSOD1^{G93A}-UeGFP, TDP-43^{A315T}-UeGFP, Alsin^{KO}-UeGFP, PFN-UeGFP, SPAST^{C448Y}-UeGFP (n=10 pups each; 2 plates, 40 wells for each pup; a total of 400 wells for each genotype). Use UCHL1-eGFP mice as healthy control.	50%	We are working on this step, and we set up cultures as the pups become availble

Subtask 6- Electroporate dissociated motor cortex preparations to visualize mitochondria and/or lysosome. Plate them as previously described to perform live imaging. Image 4-5 wells/genotype/condition to assay dynamic changes in organelle movement in both healthy and diseased UMNs.	20%	We performed initial experiments
Milestone(s) Achieved: Develop automated semi high-throughput quantitative outcome measure for the: a) average percentage of healthy and diseased UMNs numbers (survival); b) average axon length for the healthy and diseased UMNs; c) maximum axon length for the healthy and diseased UMNs; d) average branches of healthy and diseased UMNs; e) dynamic changes of organelles inside healthy and diseased UMNs.		
Major Task 3 - Diseased UMNs have reduced survival rates in culture and they display diseased phenotype. If their health is improved, this will be detected by robust quantitative measures**.**		
Subtask 1: Prepare dissociated motor cortex cultures from P3 GFP+ healthy mouse pups (n=10) in 96-well black polystyrene plates for high throughput screening. Plate 5000 cells/well in 96 well plates. Culture neurons for 6-8 hours prior to imaging.	100%	Yes, we completed culturing of healthy neurons
Subtask 2: Treat cultures for 3 days with: a) Riluzole (FDA-approved ALS Drug; 500nM; reported working concentration); b) Edaravone (FDA-approved ALS Drug; 1mM; reported working concentration); c) Amylyx AMX-0035 compound (1mM 4-Phenylbutyric acid + 100µM Tauroursodeoxycholic Acid Sodium Salt); d) NU-9 (compound our group discovered to improve the health of diseased UMNs; 400nM working concentration); and e) Control Vehicle.	30%	We are still in the process of treating them with the drugs and compounds
Subtask 3: Select and mark a 20X objective field for each well, where cell density is about 50 neurons/20X objective field. Perform live imaging of each well first in green fluorescent (label location of GFP+ UMNs in the viewpoint), and then in brightfield. Continue taking photographs of exact locations for each well (10 second max exposure for fluorescent and 2 second max exposure to brightfield) for 5 hours. Collect imaging data for each well.	25%	We are in the process of optimizing cellular assays with 20X imaging
Subtask 4: Perform quantitative measures: Quantify changes in neurite length of GFP+ UMN in each well, measure average rate of axon outgrowth, average neurite length, number of branch points per neuron, and changes in the total number of eGFP+ neurons within the 20X objective field.	50%	We made progress in this but realized that 10X tile gives better results for axon measurement
Subtask 5: Repeat experiments for treated and untreated dissociated motor cortex isolated from P3 mutant pups *(n=10 each)* creating 2 plates, 40 wells for each pup; a total of 400 wells for each genotype. A. hSOD1^{G93A}-UeGFP	25%	We are in the process of

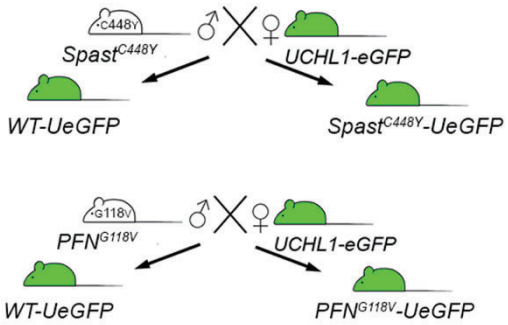
B. TDP-43^{A315T}-UeGFP C. Alsin^{KO-}UeGFP D. PFN-UeGFP E. SPAST^{C448Y}-UeGFP F. UCHL1-eGFP mice as healthy control		these experiments
*Subtask 6: Electroporate healthy and diseased UMN to visualize mitochondria or lysosome and r*epeat experiments for treated and untreated dissociated motor cortex isolated from P3 mutant pups *(n=10 each)* creating 2 plates, 10 wells for each pup; a total of 20 wells for each genotype. A. hSOD1^{G93A}-UeGFP B. TDP-43^{A315T}-UeGFP C. Alsin^{KO-}UeGFP D. PFN-UeGFP E. SPAST^{C448Y}-UeGFP F. UCHL1-eGFP mice as healthy control	20%	We are in the process of these experiments
Milestone(s) Achieved: Develop and validate capabilities of automated semi high-throughput cell-based assays to quantitate UMN survival and health and response to treatment for: a) survival of healthy and diseased UMNs numbers; b) average axon length for the healthy and diseased UMNs; c) maximum axon length for the healthy and diseased UMNs; d) average branches of healthy and diseased UMNs with and without drug treatment; and e) quantitative measurement for the dynamic movement of organelles		
Aim 3. Define the baseline characteristics of astrogliosis and microgliosis in the motor cortex of mice with mSOD1 toxicity, TDP-43 pathology, lack of alsin function, profilin mutations, and Spastin in culture with respect to healthy UMNs.		
Major Task 4 - Investigate the impact of compound treatment on neuro-immune modulation and UMN health.		
Subtask 1: Prepare dissociated motor cortex cultures from 10 pups each from the following mouse strains: A. UCHL1-eGFP (healthy control) B. hSOD1^{G93A}-UeGFP C. TDP-43^{A315T}-UeGFP D. AlsinKO-UeGFP E. PFN-UeGFP F. SPAST^{C448Y}-UeGFP (diseased) mice 40 pups will be required per strain to obtain the desired genotypes (240 mice total)	25%	We are in the process of these experiments
Subtask 2: At the end of culture period, fix samples and use GFAP- and IBA1-antibody staining to assess the average number of astrocytes (GFAP+) and microglia (IBA1+) in vitro.	25%	We are in the process of these experiments

Subtask 3: Quantify average number of cells per 1000 cells in healthy and diseased motor cortex cultures of: a) GFAP+ astrocytes: b) IBA+ microglia; c) MCP1-releasing (RFP+) cells; d) CCR2-expressing (GFP+) cells	25%	We are in the process of these experiments
Major Task 5: Use MCP1 (RFP+) and CCR2 (GFP+) reporter cells to investigate neuroimmune cells *in vitro*.		
Subtask 1: Prepare dissociated motor cortex cultures from (10 pups each): (a) MCP1-CCR2 (control); (b) MCP1-CCR2-SOD1^{G93A} (diseased); and (c) MCP1-CC2-TDP43^{A315T} (diseased) mice	10%	We are in the process of these experiments
Subtask 2: Quantify average number of cells per 1000 cells in healthy and diseased motor cortex cultures of: a) GFAP+ astrocytes: b) IBA+ microglia; c) MCP1-releasing (RFP+) cells; d) CCR2-expressing (GFP+) cells	10%	We are in the process of these experiments
Subtask3: Treat cultures for 3 days with: a) Riluzole (FDA-approved ALS Drug; 500nM; reported working concentration); b) Edaravone (FDA-approved ALS Drug; 1mM; reported working concentration); c) Amylyx AMX-0035 compound (1mM 4-Phenylbutyric acid + 100µM Tauroursodeoxycholic Acid Sodium Salt); d) NU-9 (compound our group discovered to improve the health of diseased UMNs; 400nM working concentration); and e) Control Vehicle	10%	We are in the process of these experiments
Subtask 4: Quantify average number of cells per 1000 cells in healthy and diseased motor cortex cultures, with and without treatment, of: a) GFAP+ astrocytes: b) IBA+ microglia; c) MCP1-releasing (RFP+) cells; d) CCR2-expressing (GFP+) cells	10%	We are in the process of these experiments
Milestones Achieved: Automated semi high-throughput assay to quantitate the average number of GFAP+ astrocytes, IBA+ microglia, MCP1- releasing (RFP+), and CCR2- expressing (GFP+), per 1000 cells in healthy and diseased dissociated motor cortex cultures, and their response to treatment with ALS therapeutics.		

What was accomplished under these goals?

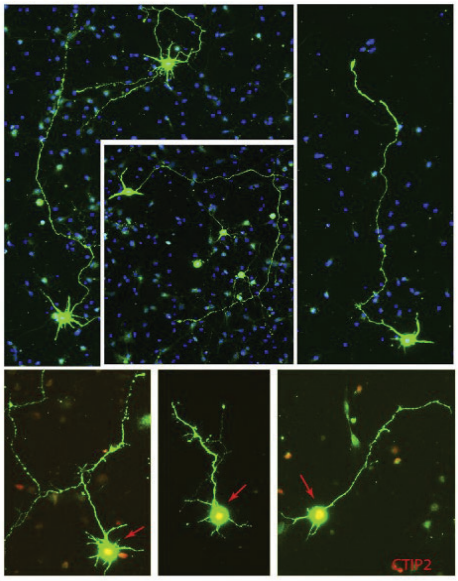
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

We made important progress on the project and completed almost all tasks that were planned to be completed in the first year. For example, in specific Aim 1, Major task 1 is completed, (we have an active animal protocol including all mice used in the study); Major Task 2, subtask1 is completed, (we utilized all the mouse strains included in the study, Fig. 1).



In addition to the hSOD1^{G93A} and the TDP-43^{A315T} mouse models, we also crossed the UCHL1-eGFP mice with the SpastC^{448Y} and the PFN^{G118V} mouse models of HSP and ALS, respectively. We now have very active breeding cages, producing about one litter every 10 days.

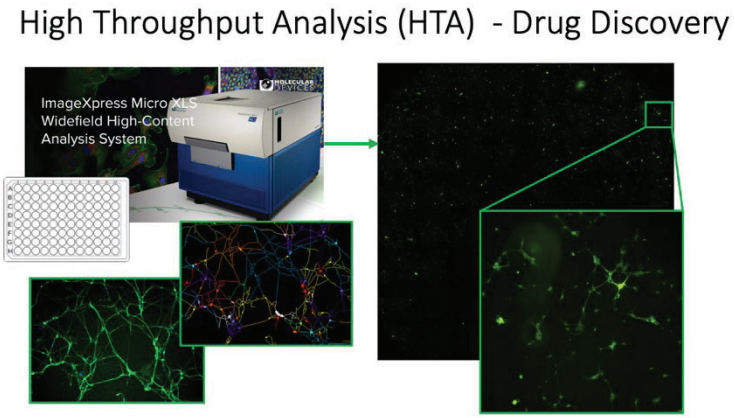
Fig. 1: Reporter lines of Spastin and Profilin mice are generated so that very active breeding cages are generating litters every 10 days.



Subtask 2 is completed (we isolated motor cortex cultures from these mouse strains and optimized the neuron numbers to be plated and the culture conditions);

We previously isolated the motor cortex and established mixed motor cortex cultures in which the upper motor neurons (UMNs) are eGFP labeled and are fluorescent. They can be distinguished among other cells and neurons in the culture. We also confirmed their UMN identity with molecular marker expression, such as CTIP2 (Fig. 2).

Fig. 2: When dissociated motor cortex cultures are prepared from diseased mice crossed with the UCHL1-eGFP mice (experimental) or the UCHL1-eGFP mice (healthy control), the UMNs are distinguished among other cells or neurons. They also express UMN marker CTIP2 in culture.



In addition, we started culturing neurons on high throughput glass bottom dark 96-well plates with a neuronal density of 20,000 live cell/well and culturing for 3 days in vitro (DIV). We initially tried different concentration of cells and different culture times and found 20,000 cells/well gave the optimum confluency for future cellular assessments in vitro (Fig. 3).

Fig. 3: Using the High Throughput platform, we began to perform mixed cortical cultures on the 96-wells. UMNs can be identified and visualized so that they are imaged with different magnifications for detailed cellular assessments.

We completed 90% of Subtask 3 (imaging 20X objective field to capture the images of UMNs, astrocyte and microglia. We have not yet fully optimized the imaging and quantification of astrocytes.) We completed about 90% of Subtask 4 (imaging plates on the ImageXpress High Content Imager + analysis of neurite outgrowth) as we continue to image the plates, We completed 75% of Subtask 4, as we continue to increase our n numbers for each genotype.

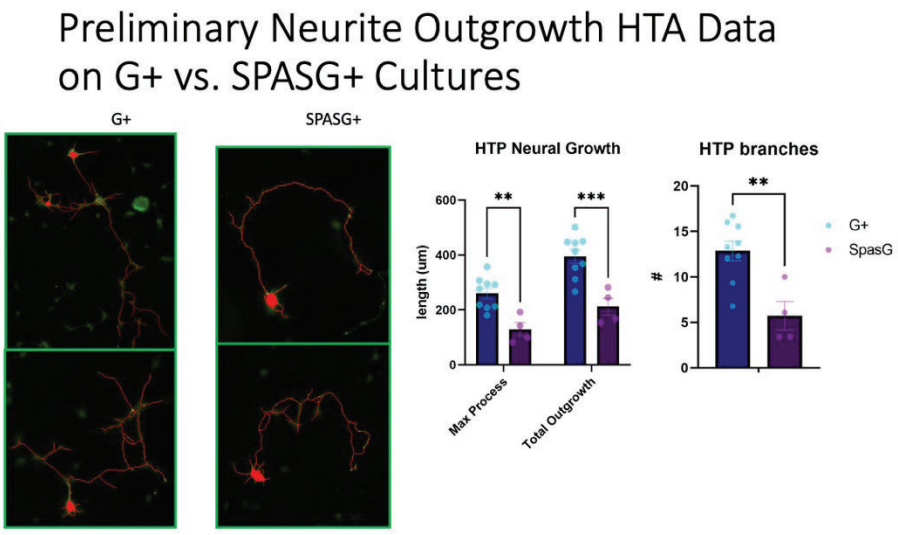
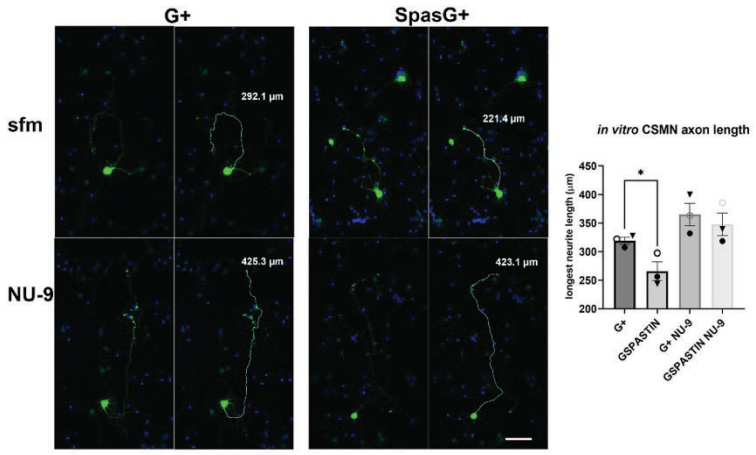


Fig. 4: The High Throughput algorithm allows tracing of neurons so that the total neurite length and the extent of branching can be quantitatively assessed in an unbiased fashion. Our preliminary results suggests that the UMNs that are diseased with mutation in the *Spastin* gene have shorter neurite length and have less branching and arborization.

We had to write different algorithms and set different thresholds to be able to trace neurites in the most effective way with no or limited error. Even though we continue to improve it, our current settings and algorithms can distinguish neurites and can measure them effectively. Using these parameters, we completed at least 3 experiments for the spastin model and found that the total neurite length is shorter in the diseased UMN and they also have much less branching and arborization pattern. (Fig. 4). These are ongoing experiments, but our preliminary results point us in the right direction.



In addition, we began our experiments with different compound treatments, and utilized the same tracing approach to obtain cellular readings. Our ongoing preliminary results with NU-9 treatment suggest an improvement in the axonal length of UMNs diseased with spastin after 3DIV with NU-9. (Fig. 5)

Fig. 5: Preliminary results of the ongoing studies suggest that NU-9 treatment helps enhance the axon outgrowth of UMNs diseased due to Spastin mutation in the SpastC^{448Y}-UeGFP mice.

We are making important progress in Specific Aim 2: We began to treat some Spastin, SOD1 and TDP-43 cultures with riluzole and NU-9 and we began to image them for quantitative analyses and our goal is to complete these studies by the end of the second year (Fig. 5).

In summary, we made important progress during the first year of our award. First of all, we performed a massive expension of our colony by mating UCHL1-eGFP mice with hSOD1^{G93A}, TDP-43^{A315T}, Alsin^{KO}, SpastC^{448Y} and PFN^{G118V} mice. All these disease models have been previously shown to have prominent UMN loss. This mating strategy resulted in the generation of hSOD1^{G93A}-UeGFP, TDP-43^{A315T}-UeGFP, Alsin^{KO}-UeGFP, SpastC^{448Y}-UeGFP, and PFN^{G118V}-UeGFP mice, all of which are UMN disease models in which UMNs are genetically labeled with eGFP expression that is stable and that enables identification of UMNs in culture, when motor cortex is dissected out prior to culturing. We performed extensive breeding in the first year to generate breeding cages and active and constant supply of pups and litters. We currently have one litter born every 10 days and this is perfect for the experimental plans and procedures.

We also established and optimized mixed motor cortex cultures from these disease models that represent different underlying causes of the disease. For example, we optimized the total number of cells that needs to be plated, the time of culturing period and the optimum coating of the glass bottom. We also had to optimize the data acquisition algorithm such that cellular tracings are optimum and cells are properly and reliably traced. We tried individual imaging then forming tiles and performing individual imaging using tiles. We tried different magnification of images, such that size of data file will be optimum without losing resolution.

We also began compound treatment studies, especially after we realized a difference between healthy and diseased UMNs, which would allow assessments of compound-mediated improvements. As outlined in the “Timeline” section of our application, we completed the tasks that were proposed to be completed within the first year. As we continue our studies, we thank you for your support.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

This project generated an immense learning experience for a large group of scientists.
Dr. Mukesh Gautam is now a Research Assistant Professor at the Department of Neurology and he used his expertise to develop his own research program investigating the survival requirements of diseased upper motor neurons within the context of hereditary spastic paraplegia.
Dr. Christopher Quintanilla, the postdoctoral fellow in the Ozdinler Lab became a postdoctoral fellow on an NIH-T32 traning grant thanks to the learning experience this DoD grant has given to him.
Omar Kashow, a postbac student in the lab has presented some early parts of this project at the NEALS meeting and received Best Poster award. He is now going to apply for PhD programs.
Rosalind Wang, an undergraduate student took some parts of this project as her Honors Thesis for graduation and she wrote a Thesis based on the initial results of the High Throughput experiments. She is now accepted to the PhD program at the University of Washington Medical School.
In addition, our project has also been a learning experience at the Core facility as we begin to improve the use and application of this approach so that other investigators can also benefit from them.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report yet. We are still in the optimization and data collection phase. We hope and plan to publish next year and beyond.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Next year will be the time to collect data and to publish. The first year was mainly the year of optimization and now we will enter the phase of production. We will be able to perform reliable and reproducible quantitative outcome measures that reveal the cellular response of diseased upper motor neurons to treatment.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of

knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We believe that we are making an important impact in the field, which became even more evident when we attended the ALS Drug Discovery summit in Boston (May 16-18, 2023). The drug companies appreciate the fact that they have not incorporated the upper motor neuron biology or pathology in their pre-clinical drug discovery efforts and they are curious whether their compound of interest would also help the diseased upper motor neurons. Many companies asked us how our investigations are going on and they showed interest working with us after we optimize and finalize our current DoD project.
We are very happy that we will be including the upper motor neuron health as a readout in preclinical assays, especially for upper motor neuron diseases.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

In ALS both upper and lower motor neurons degenerate, but there are some diseases in which primary neuron loss is observed in the upper motor neurons. Hereditary spastic paraplegia (HSP) and primary lateral sclerosis (PLS) are examples to such upper motor neuron diseases. Currently there are no drug discovery efforts for them and there are no potential treatments. Therefore, our investigations carry an immense importance for the HSP and PLS patients. We have been approached by the Spastic Paraplegia foundation and have been invited to submit grant proposal. Likewise, CureSPG4 foundation began to show interest in our investigations and we hope to make an important contribution to other upper motor neuron diseases.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

We have not yet made an impact on technology transfer, but our goal is to be active in this field upon completion of our proposal.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

I think we are making a significant impact beyond science and technology. For years, it was believed that ALS progresses as a one-way street going from the periphery to the brain and that neurodegeneration in the brain, in the motor cortex, was a byproduct of an ongoing degeneration and that the brain component of ALS did not really matter when it comes to building therapies or developing solutions. Thanks to our efforts and efforts or many other labs around the globe, this idea or that way of thinking has been changing. Now people have begun to appreciate the importance of the brain and this will be a game-changer in our ability to discover and identify novel treatment strategies for ALS.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

We did not have any change in the experimental design or the statement of work. We also did not run into major problems.

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We did not have major problems during the first year.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

We did not have any change that led to a significant impact on expenditure.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

None; as we do not utilize any specimens from human patients for this study.

Significant changes in use or care of vertebrate animals

We did not have significant changes in the use of vertebrate animals.

Significant changes in use of biohazards and/or select agents

We did not have significant changes in the use of biohazards or select agents.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  We have not yet published our findings.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

We have not yet published our results.

We presented some of our early investigations at two conferences.

1) Ahrens A., Genc B., Jara J., Sanchez S., Lagrimas A., Gozutok O., Kocak N., Zhu Y.L., Ozdinler H. Upper Motor Neurons are a Target for Gene Therapy and UCHL1 is Necessary and Sufficient to Improve Cellular Integrity of Diseased Upper Motor Neurons, Muscle & Nerve. 66:S3-S4, 2022. The 21st Northeast ALS Consortium (NEALS) meeting in Clearwater, Florida Nov. 01-03, 2022

2) Christopher Alexander Quintanilla,, Omar Iyad Kashow,,Baris Genc, Rosalind Wang,, Sara Fernandez Dunne, Hande Ozdinler. Developing A Semi-High Throughput Platform to Advance Drug Discovery Efforts for Upper Motor Neuron Diseases. The 2nd Annual ALS Drug Discovery Summit, May 16-18, 2023, Boston, MA.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

None yet.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

None yet.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

None yet.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

None yet.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: Hande Ozdinler, PhD
Project Role: PI
Researcher Identifier:
Nearest person month worked: 2
Contribution to Project: Overseeing all of the experiments and major tasks of the proposal.

Name: Chi-Hao Luan, PhD
Project Role: Co-Investigator
Researcher Identifier:
Nearest person month worked: 1
Contribution to Project: Design of high-throughput assays and working closely with Dr. Ozdinler on the establishment of UMN cultures as a drug screening platform within HTA.

Name: Mukesh Gautam, PhD
Project Role: Research Scientist
Researcher Identifier:
Nearest person month worked: 2
Contribution to Project: Setting up cell cultures, and performing imaging experiments, and data analyses.

Name: Baris Genc, PhD
Project Role: Postdoctoral Fellow
Researcher Identifier:
Nearest person month worked: 9
Contribution to Project: Working with the members of the high throughput core and setting up cultures for semi high throughput assays, imaging, quantitation, and data collection.

Name: Christopher Quintanilla, PhD
Project Role: Postdoctoral Fellow
Nearest person month worked: 2
Contribution to Project: Generates the transgenic mice used in this proposal and sets up cultures and performes imaging and statistical data analyses

Name: Rosalind Wang
Project Role: Undergrad Student
Nearest person month worked: 1
Contribution to Project: Collects high throughput data, writes code to analyze data with different parameters. Helps with statistical analyses.

Name: Omar Kashow
Project Role: Postbaccalaureate student
Nearest person month worked: 2
Contribution to Project: Fixes cultures and performs immune, help with imaging and data collection.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

No change in active support on the present project. All changes in support for key personnel are indicated as follows. (# indicates change)

CHANGES IN ACTIVE SUPPORT

OZDINLER, P. HANDE, PhD

ACTIVE (# indicates change)
#New Award (This Award)
CDMRP/USAMRDC – W81XH-21-ALSRP-TIA **05/01/2022 – 04/30/2024** 1.20 CM
Ozdinler (PI)
Developing Cell-Based and Mechanism-Focused Preclinical Platforms with Diseased Upper Motor Neurons
We will develop a semi high-throughput drug discovery/verification platform that utilizes diseased UMNs and obtains data directly from their cellular responses to treatment allow the establishment of cell-based and mechanism-focused drug discovery. These studies will help identify the most effective treatment strategy for a distinct underlying cause or pathology.
• Specific Aim 1:. Develop a semi high-throughput platform to define the baseline characteristics of UMNs diseased due to mSOD1 toxicity, TDP-43 pathology, lack of alsin function, profilin mutations, and Spastin mutations in vitro with respect to healthy UMNs.
• Specific Aim 2:. Investigate the extent of diseased UMN's response to compound treatment in vitro.
• Specific Aim 3: Investigate the impact of compound treatment on neuro-immune modulation and UMN health.
Sponsor Contact
Dr. Sarah Dougherty
I

Overlap: This is the award being reported on.

#New Award
CDMRP/USAMRDC – W81XWH-21-ALSRP-CDA 09/01/2022 – 08/31/2024 0.84 CM
Ozdinler (PI)
Identification and Utilization of Upper Motor Neuron Biomarkers for ALS
This multi-institutional team will apply its expertise and resources towards the goal of identifying UMN biomarkers in ALS. These studies will be used to determine whether specific patterns of protein in blood can be used to diagnose ALS, develop more precise and sensitive clinical trials, and provide a reliable measure of the impact of treatment on the cortical component of ALS more quickly and accurately. The development of biomarkers for UMN degeneration has the potential to substantially improve the diagnosis, treatment and prognosis of individuals with ALS.

- Specific Aim 1: Determine the protein profile of serum and plasma samples isolated from ALS patients with prominent UMN loss, ALS patients with minimal cortical involvement, and age and sex-matched healthy controls. Determine whether identified "key proteins" are specific to ALS patients with prominent UMN loss, or also observed in HSP and PLS patients.
- Specific Aim 2: Determine whether serum/plasma protein profiles can be used to reliably and accurately assess disease progression and response to treatment. We will quantitate changes in proteins and their isoforms over time in alignment with clinical markers of UMN involvement and in response to disease progression and/or therapeutic intervention.

Sponsor Contact
Dr. Sarah Dougherty

Overlap: None

Stealth BioTherapeutics, Inc. 08/01/19 - 07/31/23 1.20 CM
Ozdinler (PI) (Total Costs, **decreased**)
SRA: Preclinical Investigation of SBT Discovery Compounds in Upper Motor Neuron Cell Based Model of ALS
The aim of this grant is to investigate whether compounds at reduced protein aggregation will be beneficial for neurodegenerative diseases.
• Specific Aim 1: We will investigate putative neuroprotective effects of two discovery stage mitochondrial therapies in a primary cell culture model of ALS.

Sponsor Contact
Name:Dennis Keefe

Overlap: None

Spastic Paraplegia Foundation, Inc. 07/16/20 - **#09/29/2023** 0.60 CM
Ozdinler (PI) **(NCE)** (Total Costs)
Directed Gene Delivery to Upper Motor Neurons

The aim of this grant is to investigate the approaches for directed gene delivery to the upper motor neurons.

- Specific Aim 1: We will investigate AAV-mediated gene delivery approached to the motor cortex such that selective transduction of upper motor neurons can be achieved with a one-time motor cortex injection. Studies will be made on the motor cortex in a mouse model and the macaque monkey with the goal of translational efforts being developed in the near future. We will collaborate with Dr. Hatsopoulous at University of Chicago, an expert on cortical connectivity in non-human primates.

  Sponsor Contact
  Name: Mark Weber

Overlap: None

NIH/NIA - R01 AG061708 08/01/19 - 04/30/24 4.80 CM
Ozdinler (PI) (Total Costs)

Novel Protein Aggregation Inhibitors and Upper Motor Neuron Stabilizers for ALS and other Neurodegenerative Diseases

Upon successful completion, our proposal will result in the development of a novel drug discovery platform and will identify new drug candidates for amyotrophic lateral sclerosis (ALS) and, more broadly, for other age-related neurodegenerative diseases in which protein aggregation is the underlying cause, such as Alzheimer's disease, Parkinson's disease, and ALS/FTD.

- Specific Aim 1: To synthesize new analogues of pyrazolone and cyclohexane-1,3-dione compounds that inhibit protein aggregation.
- Specific Aim 2: To identify the target of action of our pyrazolone class of compounds, including edaravone, and our cyclohexanediones.
- Specific Aim 3: Investigate the impact of selected compounds on their ability to improve the health and stability of diseased UMNs *in vitro*.
- Specific Aim 4: To investigate whether compounds of interest improve overall motor neuron function and motor neuron health *in vivo*.

  Sponsor Contact
  Name: Coryse St.Hillaire-Clarke

Overlap: None

#New Award

Spastic Paraplegia Foundation, Inc. **12/22/2022 – 12/21/2024** 0.60 CM
Ozdinler (PI) (Total Costs)

Investigation of NU-9 and its impact on upper motor neurons diseased by spastin mutations in HSP

This proposed research is an investigation of NU-9 and its impact on disease models, which recapitulate HSP disease, an upper motor neuron disease. All experiments will be performed in the Ozdinler Lab, and all experiments are tissue culture experiments to reveal the underlying mechanism of action.

- Specific Aim 1: To investigate the optimum concentration of NU-9 to improve the health of upper motor neurons diseased due to mutations in the spastin gene (SPG4)
- Specific Aim 2: To investigate the cellular mechanisms by which NU-9 improves the health of diseased CSMN with Spastin mutations

Sponsor Contact
Name: Mark Weber

Overlap: None

#New Award

NIH/NINDS – R21 NS125465 **09/01/2022 - 08/31/2024** 1.20 CM
Ozdinler (PI) Total Costs

Profiles of Common and Unique aspects of Upper Motor Neuron degeneration in HSP and ALS

Using pure populations of upper motor neurons that are diseased due to HSP and ALS pathologies, the goal of the project is to investigate the dynamic changes in their gene expression profile and metabolomic alterations during presymptomatic and symptomatic stages of neurodegeneration. Our results will reveal the common and unique aspects of upper motor neuron vulnerability and progressive degeneration in ALS and HSP, and will lay the foundation for future detailed therapeutic strategies for these upper motor neuron diseases.

- Specific Aim 1: Identify the dynamic changes in gene expression profiles of UMN that become diseased due to HSP and ALS.
- Specific Aim 2: Identify the dynamic changes in protein profiles of UMN that become diseased due to HSP and ALS.

Sponsor Contact
Name: Karrah Benson

Overlap: None

#New Award

Revalesio Corporation - RC AGMT 10/19/22 10/19/2022 - 10/18/2024 0.24 CM
Mukesh Gautam, PI Total Costs

Preclinical Investigation of RNS60 Discovery Compounds in TDP-43^{A315T} mouse model of ALS

The goal of the study is to investigate putative neuroprotective effects of RNS60 mitochondrial therapy in an ALS mouse model of TDP-43 pathology.

- Specific Aim1: Phase I: In vivo treatment of prpTDP-43^{A315T}-UeGFP mouse with a predetermined dose of RNS60 to investigate its effect on mitochondria and UMN health

- Specific Aim 2: Phase II: Assessment of mitochondrial function in prpTDP-43A315T-UeGFP upper motor neuron in vivo at optimized doses of RNS60

Sponsor Contact:
Name: Supurna Ghosh
Overlap: None

LUAN, CHI-HAO, PhD

ACTIVE (# indicates change)
#New Award (This Award)
CDMRP/USAMRDC – W81XH-21-ALSRP-TIA **05/01/2022 – 04/30/2024** 0.6 CM
Ozdinler (PI)
Developing Cell-Based and Mechanism-Focused Preclinical Platforms with Diseased Upper Motor Neurons
We will develop a semi high-throughput drug discovery/verification platform that utilizes diseased UMNs and obtains data directly from their cellular responses to treatment allow the establishment of cell-based and mechanism-focused drug discovery. These studies will help identify the most effective treatment strategy for a distinct underlying cause or pathology.

• Specific Aim 1:. Develop a semi high-throughput platform to define the baseline characteristics of UMNs diseased due to mSOD1 toxicity, TDP-43 pathology, lack of alsin function, profilin mutations, and Spastin mutations in vitro with respect to healthy UMNs.
• Specific Aim 2:. Investigate the extent of diseased UMN's response to compound treatment in vitro.
• Specific Aim 3: Investigate the impact of compound treatment on neuro-immune modulation and UMN health.

Sponsor Contact
Dr. Sarah Dougherty

Overlap: This is the award being reported on.

R01 AI146073 Seifert (PI) 08/01/20 - 07/31/24 **#1.2 CM**
NIH/NIAID Total Costs
Targeting the functions of the gonococcal Type IV pilus

This project will determine how the Neisseria gonorrhoeae (Gc) pilus functions to modulate available iron within the bacterial cell. The work will also identify and validate chemicals that interfere with pilus expression and may become novel types of antimicrobials.
Role: Co-Investigator

Specific Aims

1) Define the mechanisms behind pilus-mediated protection from PMN oxidative and non-oxidative killing.
2) Identify compounds that inhibit Mpg activity.

Sponsor Contact:
Name: Connolly, Kristie Lee

Overlap: None

A-015 Leis/Luan (PIs) 09/01/2020 – 08/31/2023 **#0.12** CM
Chicago Biomedical Consortium
Prazole Analogs to Block Budding of Viruses
We identified small molecules, called viral budding inhibitors (VBIs), that quantitatively block budding of HIV-1 and HSV-1/2. Since the same VBIs block both viruses, they have potential for broad-spectrum applications including the possibility of blocking budding of SARS-CoV-2.
Role: Co-PI

Specific Aims

1) Continue the medicinal chemistry campaign to improve potency and drug-like properties of VBIs. Test drug-like properties using standard panels, off-target effects, and cytotoxicity.
2) Run counter-screens to validate drug action and initiate drug metabolism, and pharmacokinetic (DMPK) studies to determine small-molecule behavior.
3) Expand the HIV antiviral cell-based assays to include known mutant HIV, HIV isolated from patients, and other cell types.
4) Concurrent with our efforts against HIV, we will test our potent prazole compounds to inhibit SARSCoV-2 and improve the antiviral activity of Remdesivir.
5) Initiate HTS program to identify novel chemical scaffolds for developing back-up series of compounds.

Sponsor Contact:
Name: Kimberly Corn

Overlap: None

5 P30 CA060553-25 Platanias (PI) 8/1/2019 – 7/31/2023 **#2.88** CM
NIH/NCI $581,056 (to the HTAL Shared Resource)

Robert H. Lurie Comprehensive Cancer Center: HTAL Shared Resource

The Robert H. Lurie Comprehensive Cancer Center of Northwestern University is an NCI-designated, university based, matrix cancer center conducting a broad range of multidisciplinary clinical, laboratory and population science research. The High Throughput Analysis laboratory (HTAL) enables LCC researchers to develop, perform, and analyze large-scale experiments that elucidate the fundamental biology of cancer and launch the discovery of novel therapeutic agents

Role: Scientific Director of High Throughput Analysis Laboratory

Specific Aims

1) HTA staff will collaborate with LCC researchers to adapt bench-scale studies into assays that are tractable for such large-scale experiments and to use these assays to run screens. HTA is configured to develop and run assays ranging from fundamental biochemistry to cell-based studies involving high throughput confocal microscopy (known as high content screening or “HCS”).
2) The facility maintains a suite of instruments for large-scale experiments that include advanced liquid handling platforms for nanoliter to microliter volumes, a diverse set of high-end photometric systems, and two different platforms for high-content screening (one of which is a new confocal instrument). The facility’s staff acquires and analyzes data from all instruments as a service, assists with experimental design, and trains researchers to use its analytical and acquisition software independently. Importantly, HTA is a fully-equipped conventional laboratory for cell and molecular biology with significant bench space and a newly renovated in-house tissue culture facility. Users can run complex experiments entirely in the facility. T
3) HTA actively maintains a productive collaboration with ChemCore, speeding the cycle of lead optimization and ensuring that compound library selection maximizes the potential for medicinal chemistry with screening hits. Moreover, HTA works with the Developmental Therapeutics Core to develop new assays that use advanced tissue culture approaches.

Sponsor Contact:
Name: Krzysztof Ptak

Overlap: None

#New Award

R01 MH130838 Penzes/Luan (MPI)
1.0 CM Years 02-03. 07/19/2022 – 06/30/2026 2.75 CM Year 01,
NIH/NIMH

Targeting Postsynaptic GTPase Regulators

This project will employ high-throughput primary screens and biological hit validation assays to discover novel small-molecule inhibitors of the synaptic RaAC-guanosine nucleotide exchange factor (GEF) kalirin. Such molecules could be developed into probes to study GEF function, synaptic plasticity, and the neurobiological bases of mental illness.
Role: PI and Core Scientific Director

Specific Aims
1) Hit discovery by HTS to identify small molecules binding to kalirin's DHPH domain and inhibiting its GEF activity.
2) Hit validation in cellular and neuronal assays.
3) Characterization of the mechanism of action of hit compounds.

Sponsor Contact:
Name: Yong Yao

Overlap: None

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS:** Nothing to Report

   COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

   N/A

   QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

   N/A

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

   N/A