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TITLE: Targeting Novel Neurotrophin Effectors for Treating Post-Traumatic Epilepsy

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Traumatic brain injury (TBI) affects about 800,000 children each year and has been linked to various life-long disorders including epilepsy, depression, and intellectual impairment. Despite the prevalence of pediatric TBI, most TBI studies focus on adult animal models. Yet the developing brain exhibits a completely distinctive injury response so it can be difficult to extrapolate the results from adult models to pediatric TBI. Thus, there is an urgent need for research into the effects of pediatric TBI and potential remediation. Neurotrophins are secreted proteins that are important for early brain development and are known to be neuroprotective after injury. However their short biological half-life and poor blood-brain barrier permeability have made it difficult to use neurotrophins in clinical settings. One promising strategy is to target cellular effector proteins of neurotrophins that can be manipulated pharmacologically. Recently, we identified two novel effector proteins within the neurotrophic pathways named Par1 and HuD. These two proteins remain unexplored in TBI research. We found that the levels of these two proteins decrease significantly after pediatric TBI. We also found that both Par1 and HuD are important for brain development and disruption of either protein leads to poor cognitive functions and increased seizure susceptibility, which are symptoms often observed after pediatric TBI. Thus, in this proposal, we will test the molecular and cellular mechanisms by which Par1 and HuD are involved in the pediatric brain injury response, using multifaceted approaches including laser capture and quantitative real time PCR, 3D serial reconstruction, live two-photon in vivo imaging, biochemical and behavioral analyses. We will also use genetic and pharmacological approaches to stimulate the Par1-HuD pathway to determine whether we can promote regeneration and improve behavioral outcomes after pediatric TBI.

15. SUBJECT TERMS

None Listed

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1. Introduction

Traumatic brain injury (TBI) affects about 800,000 children each year and has been linked to various life-long disorders including epilepsy, depression, and intellectual impairment. Despite the prevalence of pediatric TBI, most TBI studies focus on adult animal models. Yet the developing brain exhibits a completely distinctive injury response so it can be difficult to extrapolate the results from adult models to pediatric TBI. Thus, there is an urgent need for research into the effects of pediatric TBI and potential remediation.

Neurotrophins are secreted proteins that are important for early brain development and are known to be neuroprotective after injury. However their short biological half-life and poor blood-brain barrier permeability have made it difficult to use neurotrophins in clinical settings. One promising strategy is to target cellular effector proteins of neurotrophins that can be manipulated pharmacologically. Recently, we identified two novel effector proteins within the neurotrophic pathways named Par1 and HuD. These two proteins remain unexplored in TBI research. We found that the levels of these two proteins decrease significantly after pediatric TBI. We also found that both Par1 and HuD are important for brain development and disruption of either protein leads to poor cognitive functions and increased seizure susceptibility, which are symptoms often observed after pediatric TBI. Thus, in this proposal, we will test the molecular and cellular mechanisms by which Par1 and HuD are involved in the pediatric brain injury response, using multifaceted approaches including laser capture and quantitative real time PCR, 3D serial reconstruction, live two-photon in vivo imaging, biochemical and behavioral analyses. We will also use genetic and pharmacological approaches to stimulate the Par1-HuD pathway to determine whether we can promote regeneration and improve behavioral outcomes after pediatric TBI.

2. Keywords

Pediatric TBI, Par1, MARK, HuD, neurotrophin

3. Accomplishments

What were the major goals of the project?

Specific Aim 1: To determine Par1 and HuD-dependent molecular, cellular and circuit changes after pediatric TBI.	**Timeline**	**Site 1** (PI)	**Site 2** (co-PI)	**% Completed**
Major Task 1: To analyze Par1 and HuD-dependent expression of key forebrain development molecules	Months			
Subtask 1: Assess molecular markers at P22, P42, and P90 post-TBI and sham surgeries of WT, Par1b KO, and HuD KO mice, using immunohistochemistry and confocal imaging. Participating teams: Dr. Zhang will oversee the immunohistochemistry and confocal imaging, Dr. Crockett will oversee the TBI surgeries.	1-3	Dr. Zhang Dr. Crockett		50%
Subtask 2: Assess molecular markers at P22, P42, and P90 post-TBI and sham surgeries of WT, Par1b KO, and HuD KO mice, using microdissection by laser capture (LCM; MMI) of distinct layers and assessing levels of gene and protein expression by using quantitative real-time PCR (qRT-PCR) and Western blotting Participating teams: Dr. Rasin and Dr. Crockett	2-7		Dr. Rasin Dr. Crockett	50%

Major Task 2: To assess changes in neocortical axonal circuits				
Subtask 1: Perform P21 CHI on transgenic mice that have distinct fluorescently labeled subpopulations of neocortical projection neurons. Participating teams: Dr. Zhang and Dr. Crockett	5-6	Dr. Zhang Dr. Crockett		10%
Subtask 2: Confocal imaging and 3D serial reconstruction of axonal circuits	7-12		Dr. Rasin	10%
Major Task 3: To determine the effect of the Par1-HuD pathway on post-injury recovery of neuronal connectivity in mice subjected to early TBI				
Subtask 1: Analyze pre- and postsynaptic markers at various time points after injury Participating teams: Dr. Zhang will oversee the immunohistochemistry and confocal imaging, Dr. Crockett will oversee the TBI surgeries.	1-3	Dr. Zhang Dr. Crockett		50%
Subtask 2: Perform two photon imaging on mice after P21 TBI. Participating teams: Dr. Zhang and Dr. Crockett	2-12	Dr. Zhang		40%
Specific Aim 2: To determine if acutely inducing Par1 and HuD promotes regeneration and improve behavioral outcomes after pediatric TBI.				
Major Task 4: Effects of genetically inducing Par1 and HuD expression on post-injury recovery				
Subtask 1: Perform E13 in utero electroporation of inducible Par1 or HuD expression vectors Participating teams: Dr. Rasin	12-14		Dr. Rasin	60%
Subtask 2: Analyze circuitry changes using molecular markers and 3D serial reconstruction Participating teams: Dr. Zhang and Dr. Rasin	13-18	Dr. Zhang	Dr. Rasin	50%
Subtask 3: Perform behavioral tests on mice from each experimental condition. Participating teams: Dr. Zhang and Dr. Crockett	13-16	Dr. Zhang Dr. Crockett		0%
Major Task 5: Effects of pharmacologically inducing Par1 and HuD on post-injury recovery				
Subtask 1: Perform two-photon in vivo imaging on injured mice treated with metformin Participating teams: Dr. Zhang and Dr. Crockett	13-24	Dr. Zhang Dr. Crockett		20%
Subtask 2: Examine the effects of metformin on intracortical and subcortical connectivity in injured mice Participating teams: Drs. Rasin and Dr. Crockett	13-24		Dr. Rasin Dr. Crockett	10%
Subtask 3: Examine the effects of metformin on behavioral outcomes of injured mice Participating teams: Drs. Zhang and Dr. Crockett	13-24	Dr. Zhang Dr. Crockett		95%

Milestone #1: Prepare manuscript on Par1 and HuD dependent changes in synaptogenesis and global gene expression changes in mice undergone adolescent TBI. *Participating teams: Drs. Zhang, Rasin, Crockett.*	18-24	Dr. Zhang Dr. Crockett	Dr. Rasin	50%
Specific Aim 3: To determine the role of Par1 in neuroinflammation after pediatric TBI.				
Major Task 6: Role of Par1 in post-injury gliosis and inflammatory cytokine secretion				
Subtask 1: Examine molecular markers of inflammation using Western blot and immunohistochemistry.	25-28	Dr. Zhang		70%
Subtask 2: Examine cytokine profile in WT and Par1b KO mice of different experimental conditions.	31-34	Dr. Zhang		70%
Major Task 7: Examine whether stimulation of Par1 activity can reduce neuroinflammation after TBI				
Subtask 1: Examine molecular markers of inflammation using Western blot and immunohistochemistry after metformin treatment of injured mice	27-30	Dr. Zhang		70%
Subtask 2: Examine cytokine profile in injured mice after metformin treatment.	31-34	Dr. Zhang		30%
Milestone #2: Prepare manuscript on effects of Par1 on neuroinflammation. *Participating teams: Drs. Zhang, Rasin and Crockett.*	30-36	Dr. Zhang Dr. Crockett	Dr. Rasin	95%

What was accomplished under these goals?

1) Major activities
We have made progress in all Major Tasks listed above.

2) Specific Objectives
   a. Continue analysis on key forebrain development molecules in Par1b KO mice.
   b. Continue analysis on the role of Par1 in post injury gliosis and determine the role of metformin in post injury gliosis
   c. Construct HuD isoform-specific expression vectors, perform in utero electroporation with HuD isoform-specific expression vectors and analyze expression of HuD isoforms in the neocortex.

3) Significant results
In our last reporting period, we had found out that loss of Par1b affects excitatory synapse formation. We have now further characterized the synaptic phenotypes including dendritic spine formation, inhibitory synapse formation, and mEPSCs.

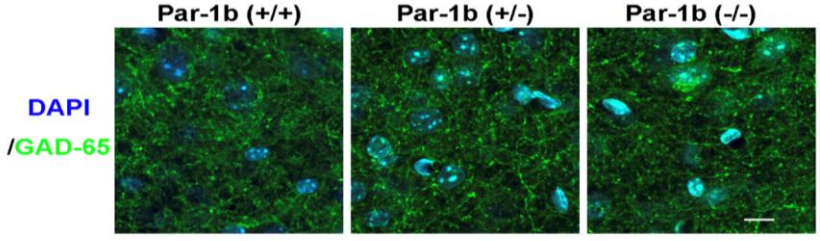


Figure 1. Loss of Par1b does not significantly impact inhibitory synapse formation.

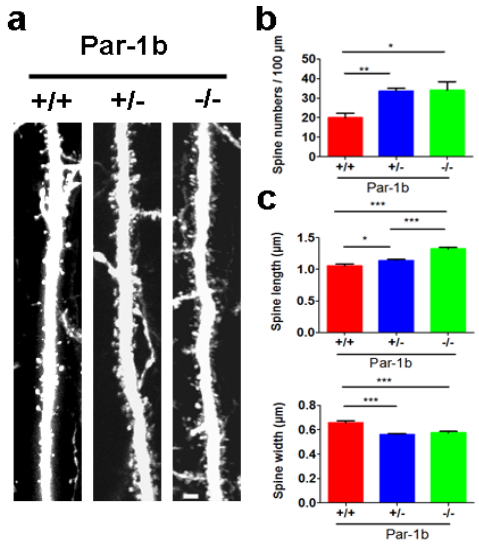


Figure 2. **Par1b KO mice show abnormal spine morphogenesis**. Par1b KO mice were crossed with Thy1-YFP mice to label Layer V pyramidal neurons. a. Spine images from the cortex of three week old KO and littermates were collected (scale bar: 2 µm) and quantified. As compared with littermate controls, Par1b (-/-) mice have an overall increase in the density of protrusions (b) and have longer and thinner dendritic protrusions (c). Similar effects were observed in the hippocampus (data not shown). *p<0.05, **p<0.01, ***p<0.001. Data are mean±SEM. n=1200-1300 spines from 15 neurons per group.

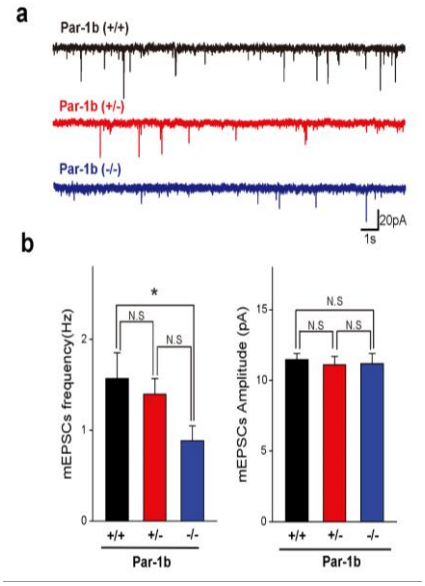
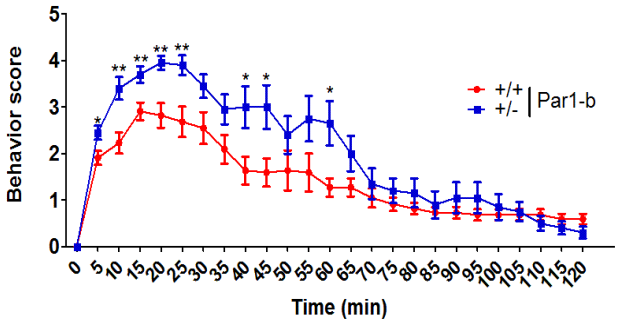


Figure 3(←). Electrophysiological analysis of synaptic functions of Par1b. A. Representative mEPSCs trace from Par1b KO mice and littermate controls. B. Average frequency and amplitude of mEPSCs from acute hippocampal slices of Par1b KO mice and littermate controls. **p<0.01. Triangle, open and close circles represent the averages of each KO mouse and their respective littermate. n=20 (control) and 16 (KO) cells.

Figure 4 (↓). Par1b (+/-) mice exhibit enhanced seizure susceptibility. Seizure was induced by pilocarpine injection and scored on a scale of 1 to 6 as compared to wild type mice. * p< 0.05,
**p<0.01.



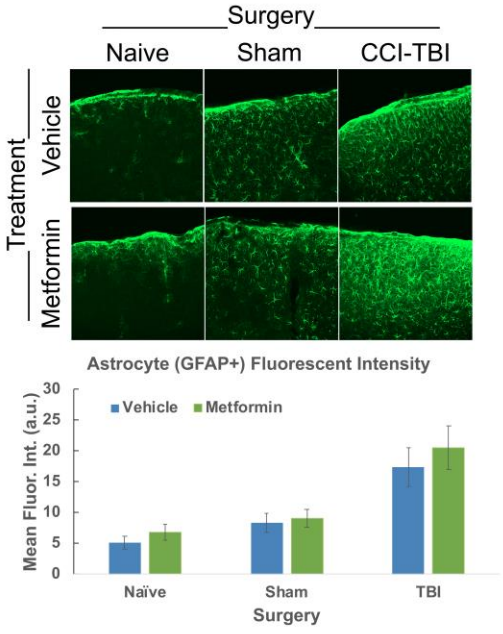


Figure 5. Astrocyte activation is not significantly altered following Metformin treatment. Confocal images were acquired of the ipsi-lateral cortex of CCI-TBI, sham-operated and naïve control mouse tissue and the fluorescent intensity was manually quantified of GFAP+ cells using ImageJ. No significant difference in fluorescent intensity of GFAP+ labeled astrocytes was found between vehicle or metformin treated Naïve, sham-operated control or CCI-TBI mice. Data represent the mean ± SEM. A 2-way ANOVA was performed. N (animals/images) = Vehicle: Naive=8/24, Sham=12/32, TBI=10/31; Metformin: Naive=10/27, Sham=12/37, TBI=11/36, *p<0.05, **p<0.01 by type III test of fixed effects using a linear model analyses.

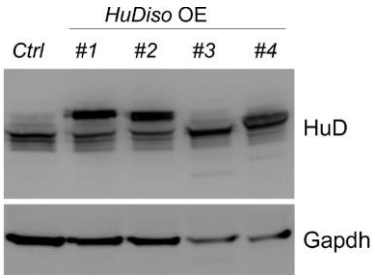


Figure 6. Validation of HuD isoform expression constructs. Western blot analysis of N2a cells transfected with each isoform and control.

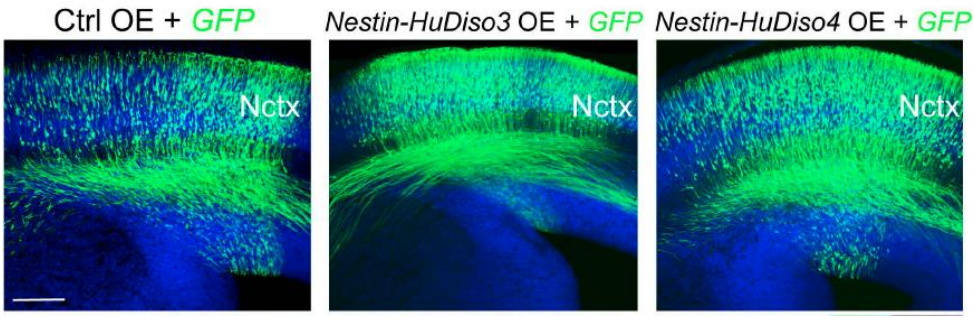


Figure 7. Representative confocal images of in utero electroporated neocortices electroporated with *GFP* and either Control (Ctrl), *Nestin-HuDiso3*, or *Nestin-HuDiso4* OE plasmids. GFP is expressed by electroporated cells and their axons. DAPI is shown in blue.

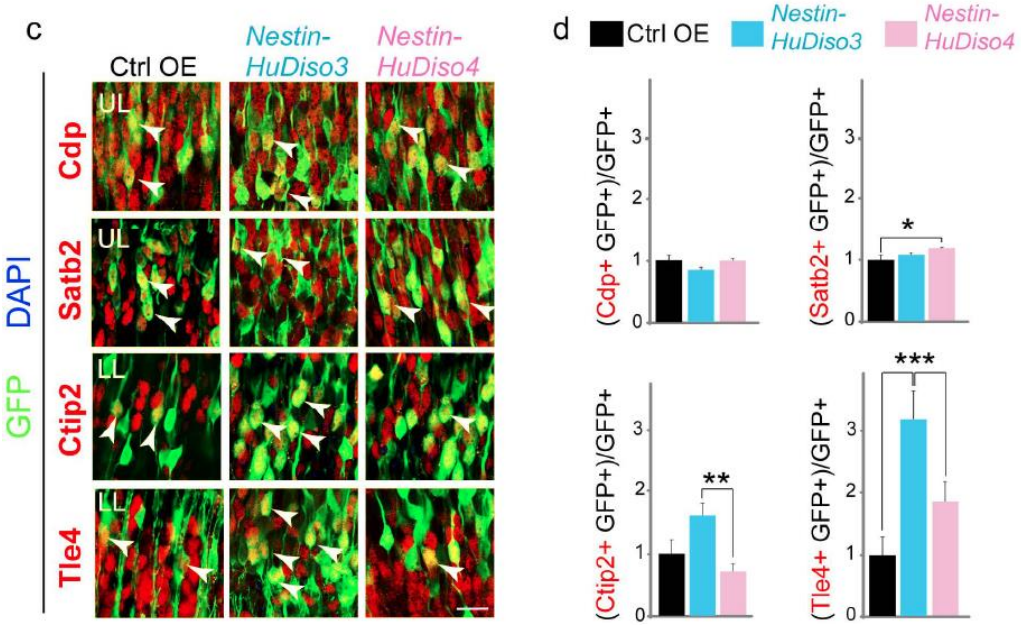


Figure 8. C. Representative confocal images of E17 IUE neocortices electroporated with GFP and either Control (Ctrl) (n = 3), *Nestin-HuDiso3* (n = 4), or *Nestin-HuDiso4* (n = 4) OE plasmids immunostained for GFP (green), and cortical transcription factor identity markers Cdp, Satb2, Ctip2, or Tle4 (red). DAPI is shown in blue. D. Quantification of colocalization between cortical identity markers and GFP normalized to the level of colocalization in the controls. Data represent mean and SEM. Statistical analysis is one-way ANOVA with a post-hoc Tukey test for multiple comparisons (*p<0.05, **p< 0.01). Comparisons not demarcated are not statistically significant.

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

Nothing to report (grant not intended for training and professional development).

How were the results disseminated to communities of interest?

In the current reporting period we have engaged in community outreach activities to enhance public understanding and increase interest in careers in science. First, the PI, Dr. Huaye Zhang, and one of the co-PIs, Dr. Mladen-Roko Rasin, are involved in the annual Central New Jersey Brain Bee, which has ~80

high school student participants in addition to their siblings, parents and teachers. Dr. Zhang gave a powerpoint presentation at the Brain Bee to discuss the latest neuroscience research and careers in science. Second, the PI and co-PIs usually organize a booth called “Gray Matters” at the annual Rutgers Day, which normally attracts nearly 100K visitors to all Rutgers campuses. We discuss with the public about how the brain works through fun games. In addition, we discuss the importance of both preventing TBI and research on TBI therapies. This year, the PI has partnered with Dana Foundation to make the event part of the Brain Awareness Week events. Unfortunately, the event had to be cancelled at the end because of the coronavirus pandemic. However we hope and plan to resume the event next Spring.

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

In the next reporting period, we plan to continue the CHI experiments on P21 mice and analyze molecular markers of forebrain development at different time points after injury, as proposed in Aim 1 of the grant. We will continue our in utero electroporation studies to determine the effects of specific HuD isoforms on post injury recovery, as proposed in Aim 2 of the grant. We have initiated two photon imaging experiments as proposed and will continue with these experiments in the next reporting period.

4. Impact

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

Our results so far point to metformin as a potential therapy for TBI. Since metformin has already been used in the clinic for decades and proven to be safe, our results can lead to “drug repurposing” and can potentially be quickly translated into the clinic.

What was the impact on other disciplines?

Our results have identified novel roles for Par1 in microglia activation, which will be of interest to the neuroimmunology field. Further, our results on HuD isoforms can offer insight into the mechanisms of neurodevelopment.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Our results on metformin may lead to new therapies in the clinic for TBI patients.

5. Changes/Problems

The COVID-19 pandemic significantly impacted the progress of this project. In March, we were directed to shutter laboratory operations and not initiate any new experiments. Students were mostly working remotely and no new experiments were being conducted. During the lab shutdown under the direction of the Rutgers Comparative Medicine Resources (CMR), we had to reduce our mouse colonies by 50% because of reduced staff support. Only essential activities such as mouse colony maintenance were allowed on campus during the shutdown period. The shutdown continued until late June when we were approved to re-open on a 50% capacity. We have been able to ramp up our research activities including re-expansion of mouse colonies and are currently functioning at 75% capacity.

6. Products

Publications, conference papers, and presentations

Journal publications

*DiBona, V.L., Zhu, W., Shah, M., Rafalia, A., Ben Cheikh, H., Crockett, D.P., and **Zhang, H.** Loss of Par1b/MARK2 primes microglia during brain development and enhances their sensitivity to injury. **Journal of Neuroinflammation,** 2019; 16(1):11.*
Status: Published.
Acknowledgement of federal support: Yes.

*DiBona VL, Shah MK, Krause K, Zhu W, Smith D, Crockett DP, and **Zhang H.** Metformin activates the Par1/MARK family kinases and improves cognitive functions after traumatic brain injury.*
Status: Under revision.
Acknowledgement of federal support: Yes.

Website(s) or other Internet site(s)
We have submitted our microglia morphology data (included in the Journal of Neuroinflammation publication) to NeuroMorpho.Org, an NIH-sponsored repository for data sharing of morphological data in neuronal and glial cells.

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?

Name: *Huaye Zhang*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): *0000-0002-4844-6111*
Nearest person month worked: *1*
Contribution to Project: *Supervise project, manuscript writing, editing and submission.*

Name: *Mladen-Roko Rasin*
Project Role: *co-PI*
Researcher Identifier (e.g. ORCID ID): *0000-0003-3063-6096*
Nearest person month worked: *1*
Contribution to Project: *Contribute to data analysis and interpretation of forebrain developmental markers.*

Name: *David Crockett*
Project Role: *co-PI*
Researcher Identifier (e.g. ORCID ID): *0000-0002-8137-755X*
Nearest person month worked: *1*
Contribution to Project: *Contribute to TBI experiments, manuscript editing.*

Name: *Tatiana Popovitchenko*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *12*
Contribution to Project: *IUE experiments, IHC and imaging of forebrain markers*

Name: *Mikayla Voglewede*
Project Role: *Graduate Student*

Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12
Contribution to Project: TBI experiments, IHC and imaging of forebrain markers

Name: Victoria DiBona
Project Role: Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID): 0000-0002-1914-2506
Nearest person month worked: 2
Contribution to Project: Analysis of effects of Par1 on neuroinflammation and the effect of metformin on injury recovery.

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

N/A

What other organizations were involved as partners?
Nothing to report.

8. Special Reporting Requirements

Quad Chart: Updated Quad Chart attached.

9. Appendices