AWARD NUMBER: W81XWH-20-1-0211

TITLE: Uncovering New Therapeutics and Neuroprotective Mechanisms for TBI

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CONTRACTING ORGANIZATION: Northwestern University

REPORT DATE: May 2023

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MAY 2023	ANNUAL	1APR2022 - 31MAR2023

4. TITLE AND SUBTITLE
Uncovering New Therapeutics and Neuroprotective Mechanisms for TBI

5a. CONTRACT NUMBER
W81XWH-20-1-0211

5b. GRANT NUMBER
W81XWH-20-1-0211

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During the reporting period, we demonstrate the replicability of identified "hits" that mediate the in vivo effects of TBI in our novel model. Moreover, we have demonstrated that these effects are specific to TBI and not evident in sham controls. Taken together, these studies have leveraged the power of in vivo genetic screening in combination with transcriptomics to identify novel molecular pathways that mediate the effects of brain injury on lifespan, health and sleep.

15. SUBJECT TERMS
Drosophila, sleep, innate immunity, traumatic brain injury

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. **INTRODUCTION:** Sleep disorders are highly associated with traumatic brain injury and may contribute to adverse outcomes. Here we exploit a novel Drosophila TBI model to reveal the underlying molecular mechanisms linking TBI to behavioral impairments including sleep.

2. **KEYWORDS:** Drosophila, sleep, innate immunity, traumatic brain injury

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**
  - **Aim 1: To understand the cellular and molecular bases of the neuroprotective effect of NFkB**
  - **Aim 2: to uncover genes and pathways that are neuroprotective for TBI**
  - **Aim 3: to test FDA approved drugs for improving TBI-induced mortality and sleep disorders**

- **What was accomplished under these goals?**
  - We discovered potentially important candidate genes in mediating TBI effects on sleep, mortality and climbing (Fig. 2-7; Table 1&2)
  - We revealed specific locomotor impairments after TBI (Fig. 3 & 6)

- **What opportunities for training and professional development has the project provided?**
  - During the reporting period, an undergraduate received training in Drosophila genetics, circadian rhythms and sleep analysis, and scientific presentation of results.

- **How were the results disseminated to communities of interest?**
  - Some of these results have been published and were presented at the Chicago Society for Neuroscience meeting. We plan to publish the remaining results for the scientific community

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - We have requested a no cost extension and during this period we intend to capitalize on the discovery of novel molecular pathways to validate the findings by further examining their replicability, testing of sham controls, and most importantly, testing independent RNAi lines to ensure that observed effects are not due to off target effects. Given the potential therapeutic value of these discoveries, we intend to focus on these novel genes.

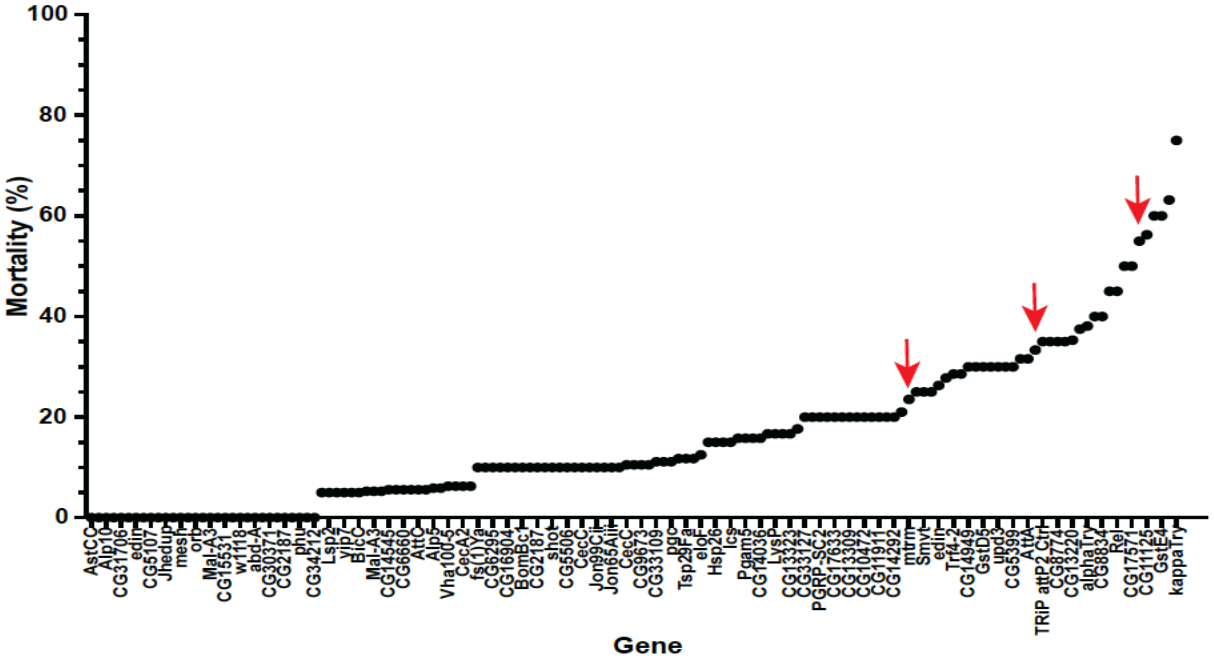

Figure 1. Knockdown of candidate genes with RNAi lines significantly suppresses TBI-induced mortality. We measured percentages of dead flies 24h after TBI induction. The X-axis indicates ranking of screened RNAi lines based on their average mortality scores. The red arrows indicate the average mortality scores of the controls (ISO31, TRiP att2 Ctrl, TRiP attp40 Ctrl). For instance, knockdown of AstCC significantly improves reduced survival. N=155 lines.

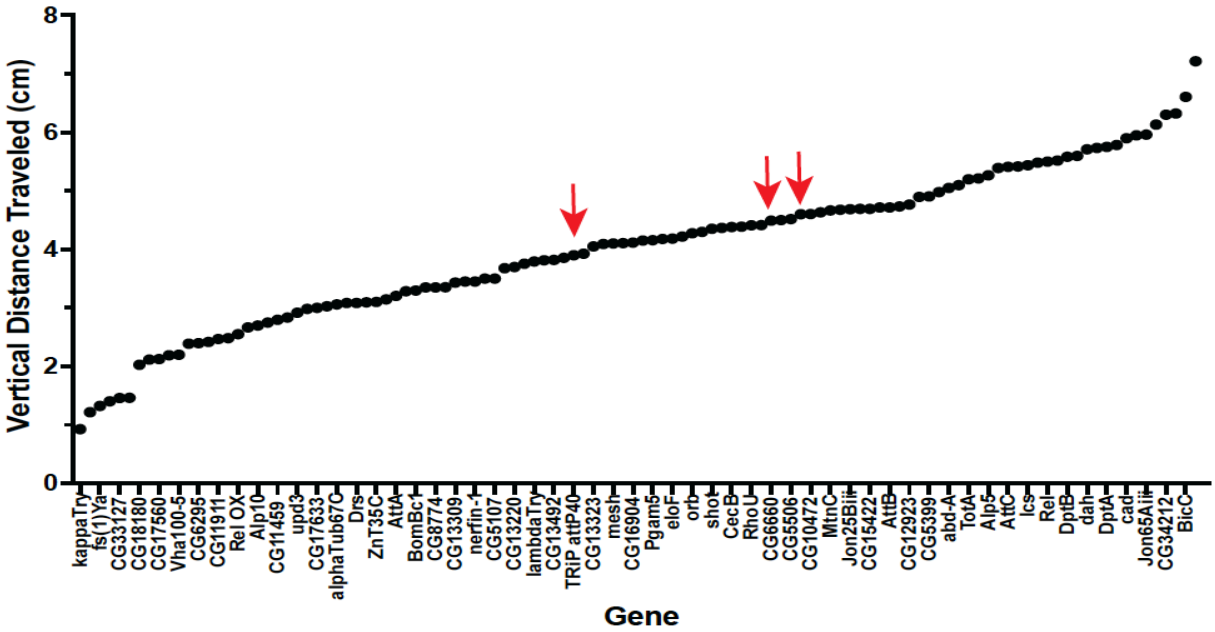

Figure 2. Knockdown of candidate genes with RNAi lines significantly rescues reduced climbing activity. We measured the distance single flies traveled in a 8-dram vial for 4 sec 24h after TBI induction. The X-axis indicates ranking of screened RNAi lines based on their average distance scores. The red arrows indicate the average distance scores of the controls (TRiP attP40 Ctrl, TRiP attP2 Ctrl and ISO31). For instance, knockdown of cad significantly improved reduced climbing behavior. N=155 lines.

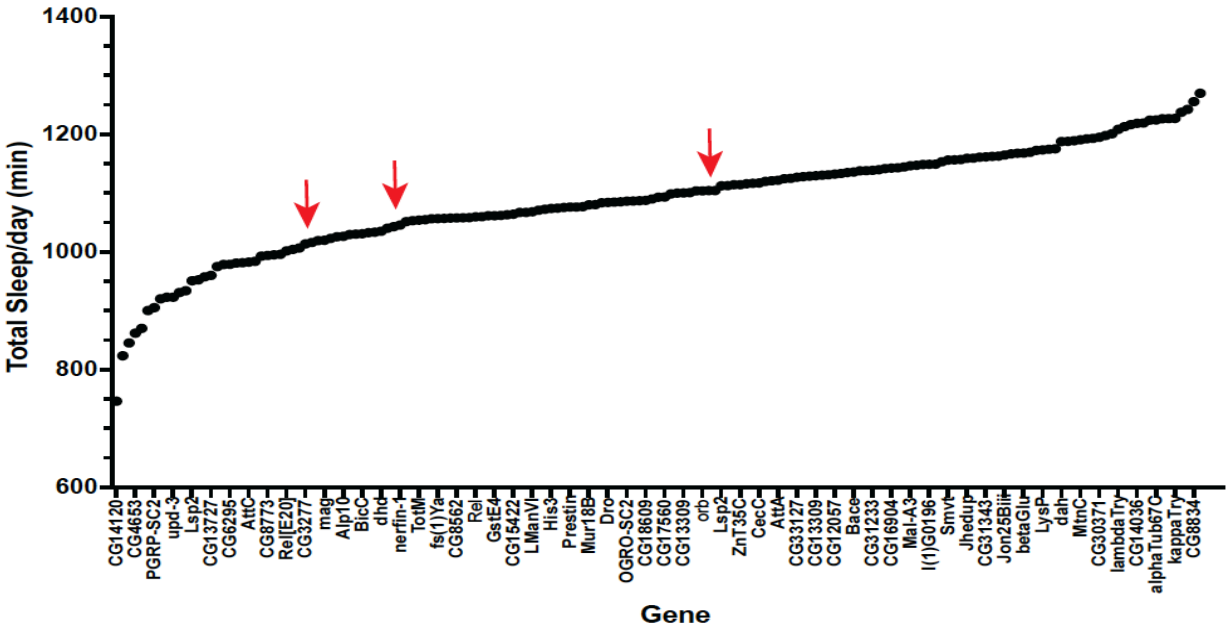

Figure 3. Knockdown of candidate genes with RNAi lines significantly increased sleep which was impaired by TBI. We identified ~350 genes differentially expressed in the glial cells using immunoprecipitation and TRAP-seq techniques. We inhibit expression of these genes with RNA interference methods and observed the inhibition effect on sleep during 12h daytime and 12h nighttime per day in the DAM system. The X-axis indicates ranking of screened RNAi lines based on their average sleep scores. The red arrows indicate the average sleep scores of the controls (ISO31, TRiP attP2 Ctrl, TRiP attP40 Ctrl). For instance, knockdown of kappaTry significantly improves reduced sleep behavior. N=141 lines.

Table 1. Screening results of candidate genes via RNAi in TBI-induced mortality, climbing and sleep phenotype. We pick 155 upregulated genes and knock down these genes with RNA interference to investigate changes in mortality, climbing activity and sleep behavior. Subsequently we prioritize testing of the candidate genes by repeating top and bottom 5% of RNAi lines that enhance or suppress TBI-induced reduced phenotypes.

Phenotype	Lines screened	Primary hits (Top and bottom 5%)	Primary hits replicates screened	Replicate hits ($p<0.05$)
Mortality	155	32	32	21
				cad, abd-A, lambdaTry, Mal-A3, PIG-Wb, Jhedup, edin, BicC, DptA, Lsp2, alphaTub67C, Mur18B, MtnC, Peritrophin-15a, CG10911, CG5107, CG31233, CG10472, CG33127, CG14949
Climbing	155	16	16	4
				phu, CG14990, cad, fs(1)Ya
Sleep	141	16	16	4
				CG8834, fs(1)N, kappaTry, CG15531

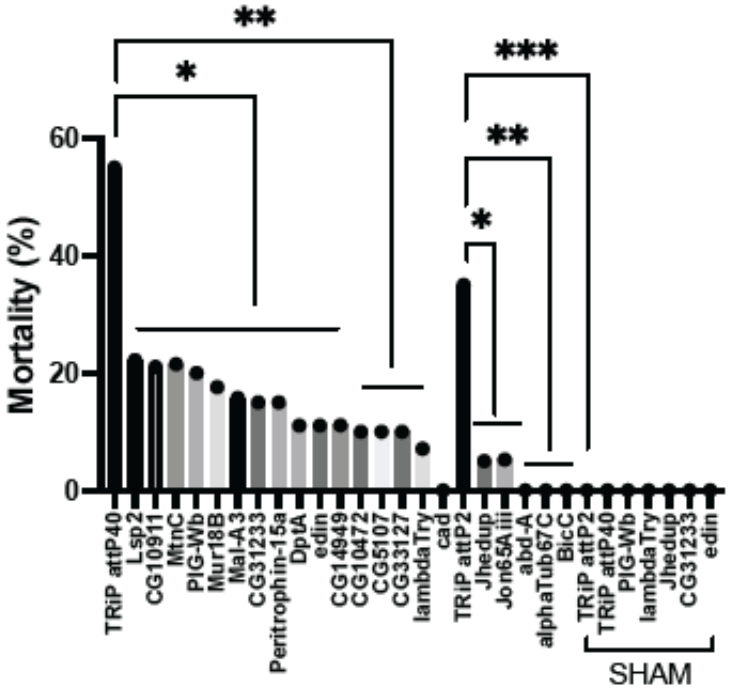

Figure 4. RNAi-mediated knockdown of replicate hits significantly reduces 24h mortality. Using the top and bottom 5% of candidate genes (32) we selected from primary screening we scored 24h mortality after TBI induction. Inhibition of expression of 21 genes with RNAi significantly increase survival compared to genetic controls, TRiP attP2 and TRiP attP40, respectively (* p<0.05, ** p<0.01, Fisher's exact test for significance). Non-TBI (sham) flies have 100% survival.

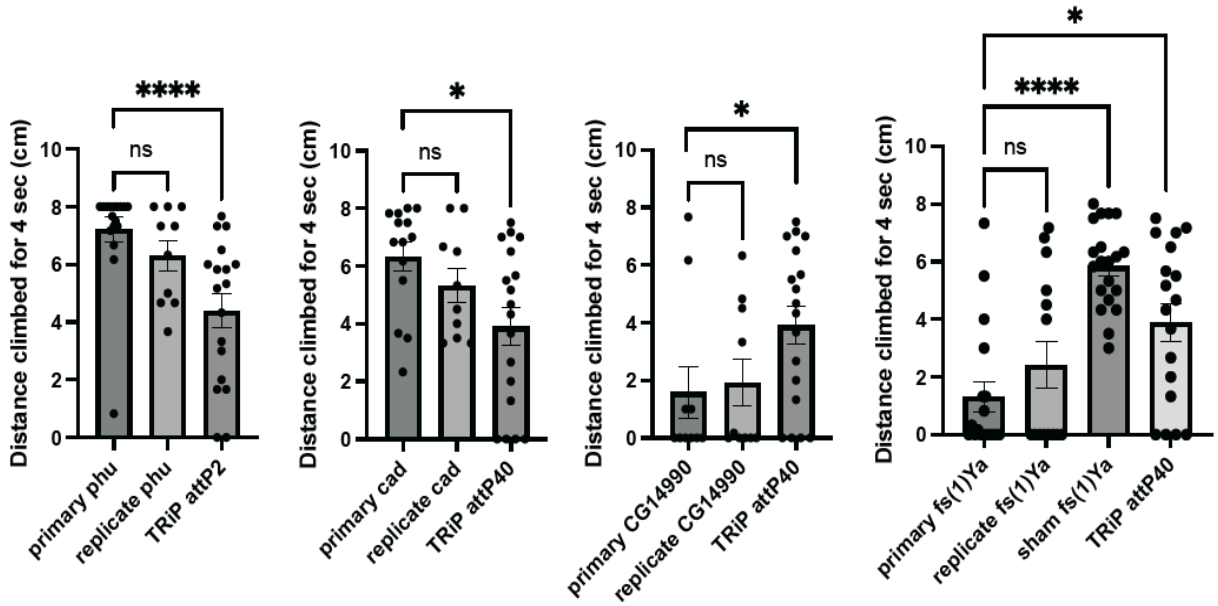

Figure 5. RNAi-mediated knockdown of replicate hits significantly enhance or suppress climbing activity. Using the top and bottom 5% of candidate genes (16) we selected from primary screening, we measured the distance single flies traveled in a 8-dram vial for 4 sec 24h after TBI induction. Inhibition of expression of 2 genes (phu, cad) with RNAi significantly enhances climbing activity while inhibition of expression of CG14990 and fs(1)Ya genes suppresses climbing activity (* p<0.05, ****p<0.0001, Dunn's multiple comparison for significance).

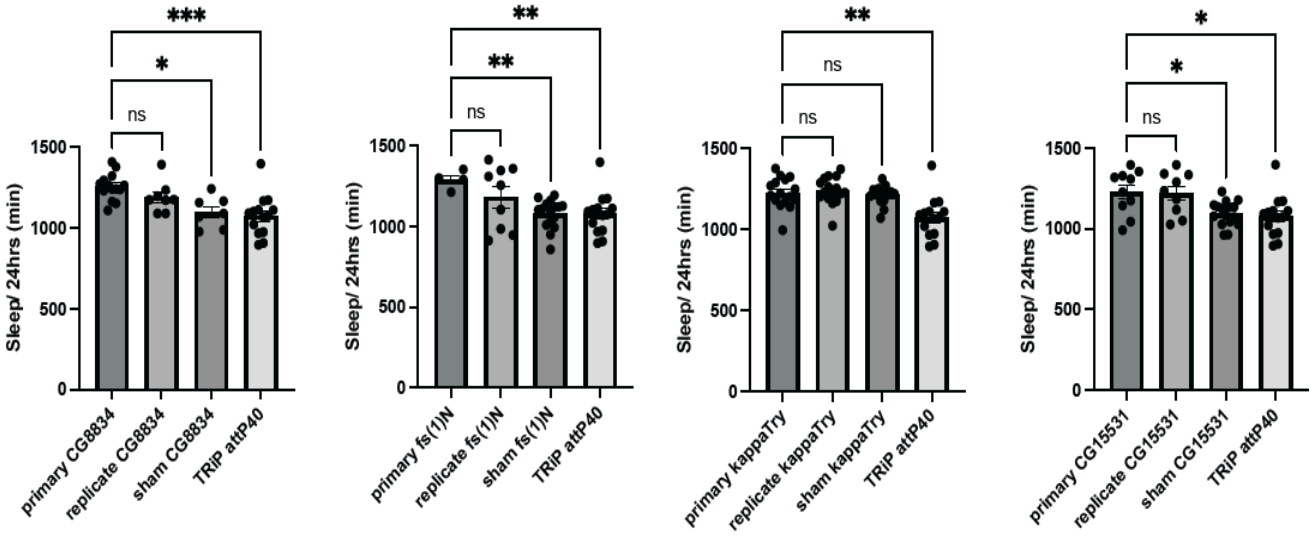

Figure 6. RNAi-mediated knockdown of replicate hits significantly enhance sleep. Using the top and bottom 5% of candidate genes (16) we selected from primary screening, we measured sleep during 12h daytime and 12h nighttime per day for 4 days in the DAM system after TBI induction. We confirmed that Inhibition of expression of 4 genes with RNAi significantly enhances sleep per day (* p<0.05, ** p<0.01, *** p<0.001 Dunn's multiple comparison for significance).

Table 2. Biological function and human orthologs of candidate genes identified in this study.

Genes	Type	Biological Process	Human Orthologs
cad	Transcription factor	Organ morphogenesis; innate immune homeostasis	CDX, DLX, EMX
abd-A	Homeobox transcription factor	Developmental regulatory system	HOX
lambdaTry	Trypsin-like serin protease	Proteolysis	PRSS36, PRSS53
Mal-A3	Protein coding gene	Carbohydrate metabolic process	SLC3A
PIG-Wb	Protein coding gene	GPI anchor biosynthetic process	PIGW
Jhedup	Protein coding gene	Juvenile hormone catabolic process	CES
edin	Protein coding gene	Humoral immune response	n/a
BicC	Protein coding gene	RNA binding, Oogenesis	BICC1
DptA	Protein coding gene	Immune response; stress response	n/a
Lsp2	Protein coding gene	Motor neuron axon guidance, Synaptic target inhibition	n/a
alphaTub67C	Protein coding gene	Central nervous system development	TUBAL3
Mur18B	Protein coding gene	Chitin binding	n/a
MtnC	Protein coding gene	Cellular detoxification of metal ion	MT1B, MT1E
Peritrophil-15a	Protein coding gene	Chitin binding	n/a
phu	Protein coding gene	Response to nicotine & hypoxia, dephosphorylation	ALPG, ALPI, ALPP
fs(1)Ya	Protein coding gene	Biogenesis, cellular process	n/s
fs(1)N	Protein coding gene	Torso signaling pathway, terminal region determination	BSPH1, ELSPBP1
kappaTry	Protein coding gene	Protein metabolism	PRSS36, PRSS53

CG10911	Protein coding gene	Not known	AHNAK2, NPIPA1
CG5107	Protein coding gene	Not known	n/a
CG31233	Protein coding gene	Proteolysis, peptide catabolic process	ANPEP, ERAP1
CG10472	Protein coding gene	Proteolysis	CTRB2, CTRC
CG33127	Protein coding gene	Proteolysis	KLS1, PRSS21, PRSS33
CG14990	Protein coding gene	Proteolysis	TPSAB1, TPSD1
CG14949	Protein coding gene	Not known	n/a
CG8834	Protein coding gene	Fatty acid biosynthetic process	SLC27A6, ACSF2
CG15531	Protein coding gene	Lipid metabolic process	SCD5, SCD

Future Work:

We will follow up identified genes by retesting with independent genetic reagents and validate identified phenotypes. Candidate genes showing similar effects for both RNAi lines will be considered to be successful hits.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
  - The discovery of candidate genes in mediating TBI effects provides important in vivo evidence for TBI effects on behavior and health
- **What was the impact on other disciplines?**
  - These studies may also highlight novel treatments for TBI
- **What was the impact on technology transfer?**
  - N/A
- **What was the impact on society beyond science and technology?**
  - If successful, new treatments for TBI could improve the lives of affected individuals.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**
  - We have focused our energies on the discovery of novel genes that mediate TBI effects on sleep, behavior and health
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - None during the reporting period
- **Changes that had a significant impact on expenditures**
  - We have focused our energies on the discovery of novel genes that mediate TBI effects on sleep, behavior and health
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - Not applicable

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  - **Journal publications.**

van Alphen B, Stewart S, Iwanaszko M, Xu F, Li K, et al. (2022). Glial immune-related pathways mediate effects of closed head traumatic brain injury on behavior and lethality in Drosophila. PloS Biol 20(1): e3001456. https://doi.org/10.1371/journal.pbio.3001456

Zhang MY, Lear BC, Allda R. (2022). The microtubule-associated protein Tau suppresses the axonal distribution of PDF neuropeptide and mitochondria in circadian clock neurons. Hum Mol Genet 31(7), 1141-1150. DOI: 10.1093/hmg/ddab303

- **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 AND OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	*Clark Rosensweig*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	2
Contribution to Project:	*Dr. Rosensweig analyzed sleep*
Funding Support:	*This award*

Name:	*Yong-Kyu Kim*
Project Role:	*Senior Research Associate*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	6
Contribution to Project:	*Dr. Kim analyzed behavioral and genetic effects of TBI*
Funding Support:	*This award*

Name:	*Ravi Allada*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	2
Contribution to Project:	*He supervised this project*

Funding Support:	*This award*

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

  735135 (PI: Allada), Simons Foundation (SFARI), Defining Behavioral Gene networks for Autism Spectrum Disorder Genes Using Sleep and Circadian Rhythms (active to past funding)

  W81XWH2210217 (PI: Allada), US Army Medical Research and Material Command, Discovery of In Vivo Molecular Pathways Mediating Tau Induced Sleep and Circadian Disruption (pending to active funding)

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

  - Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**
   - Not applicable
9. **APPENDICES**
   - Not applicable