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TITLE: **Therapeutic Sleep for Traumatic Brain Injury**

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

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14. ABSTRACT

To test the therapeutic effect of correcting sleep impairments caused by TBI, we developed and validated a *Drosophila* head-specific model for TBI where well-controlled, non-penetrating strikes are directly delivered to the head of unanesthetized flies. This assay recapitulates many TBI phenotypes, including increased mortality, decreased and fragmented sleep, impaired motor control and increased neuronal cell death. Using glial TRAP-seq, we detected substantial changes in gene expression, including a strong upregulation of the innate immune response as well as several wake-promoting genes. To test the in vivo functional role of these changes, we examined TBI-dependent behavior and lethality in mutants of the master immune regulator NF-κB and found that while lethality effects were still evident, changes in sleep and motor function were abolished, suggesting that TBI-induced changes in sleep are due to the immune response, rather than the injury.

15. SUBJECT TERMS

TBI, Traumatic Brain Injury, Sleep, TRAP-seq, Immune Response, NF-κB, Gene Expression, Drosophila

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Table of contents

INTRODUCTION:

To study how TBI causes sleep disorders, and to study whether restoring normal sleep patterns, post-injury, can have a therapeutic effect, we need a model organism where baseline sleep can be recorded accurately, and where tools exist to induce either sleep or wake in such a way that TBI-induced sleep disorders can be corrected, by promoting wake in hypersomniacs and by promoting sleep in insomniacs. This approach is only feasible in *Drosophila*, as this model organism comes with a wealth of genetic tools that have been proven useful to modulate sleep and wake with high precision. We have developed a paradigm to reliably induce TBI in *Drosophila*.

ACCOMPLISHMENTS:

Major goals

We have completed most Major Tasks for Aim 1, as outlined in the Statement of Work.
Task 1A) Develop and test single fly TBI setup – **COMPLETED on 07/01/2016**
Task 1B) Determine TBI-induced sleep changes – **COMPLETED on 11/01/2016**
Task 1C) Determine TBI-induced behavioral changes – **COMPLETED on 01/01/2017**
Task 1D) Determine TBI-induced cell death – **COMPLETED on 08/01/2017**

Task 2A) Test the effect of sleep intervention on subsequent behavior and sleep architecture – **50% COMPLETE**
Task 2B) Test the effect of sleep intervention on subsequent markers of cell death – ON HOLD

Keywords: TBI, Traumatic Brain Injury, Sleep, TRAP-seq, Immune Response, NF-κB, Gene Expression, *Drosophila*

What was accomplished under these goals?

Major Activity 1: to test the hypothesis that TBI causes either hypersomnia or insomnia in individual flies

Specific Objective 1A) Develop and test single fly TBI setup:
We have developed a *Drosophila* TBI paradigm. In this paradigm, individual flies are immobilized in a pipette tip and TBI is induced in a highly replicable manner using a solenoid. This paradigm is an improvement on previously published methods, where TBI is induced in groups of flies, using a vortexer or mechanical injury through a spring-driven contraption that. The drawbacks of these published methods are that 1) a wide variety of other injuries are induced as well as TBI and 2) the degree and severity of TBI is highly variable. By immobilizing individual flies in a 200µl pipette tip, each fly can be positioned in a highly replicable manner in front of a solenoid, a spring-driven metal pin. The solenoid itself allows precise control of the amount of force that is used to induce TBI. TBI induction resulted in reduced lifespan, where 50% of the TBI-induced flies had died 13 days after TBI induction, compared to 35 days for sham treated controls (Fig 1A).

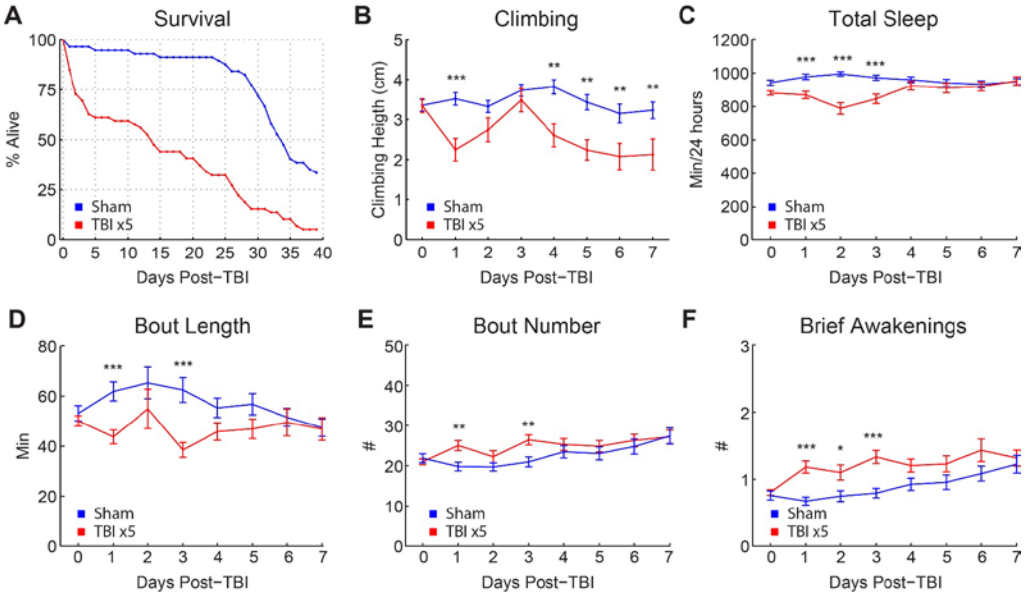

Figure 1 – TBI reduces lifespan and induces changes in climbing behavior and sleep architecture

Specific Objective 1B) Determine TBI-induced sleep changes:
To test whether TBI results in sleep changes (either insomnia or hypersomnia), individual flies were loaded into Drosophila Activity Monitors to quantify sleep and activity after TBI induction and compared to sham-treated controls. These activity monitors use a beam-break system to measure how active each individual fly is. Overall, TBI results in decreased sleep (Fig 1C) that is more fragmented (shorter bouts, more bouts; Fig 1D, E) and less deep (more brief awakenings, Fig 1E).

Specific Objective 1C) Determine TBI-induced behavioral changes:
To test whether TBI induction alters motor control, we performed a climbing assay. 24 hours after TBI induction, we measured how high individual flies can climb in 4 seconds. TBI induction results in a considerable decrease in climbing ability (Fig 1B).

Other Achievements: To further explore the *Drosophila* immune response after TBI, we performed Translating Ribosome Affinity Purification and Sequencing (TRAP-Seq), which allows for a tissue or cell-type -specific manner of looking at the transcriptome of a given organism. This has previously been used to look at translated mRNAs in *Drosophila*. Here, we look at the glial transcriptome - glial activation is a biomarker for TBI. We performed TRAP-seq at 1, 3 and 7 days after TBI. TBI results in a large increase in genes that are up- or downregulated on day 1 (Fig 2A). On day 3 and 7 post-TBI, the number of upregulated genes is much lower. Contrary to our experimental design we noted that we also observed significant expression of neuronal genes suggesting that our purification is not complete. There is modest overlap between the genes that are up/down regulated on day 1 and days 3 or 7 (Fig 2B). Genes associated with the defense response are upregulated 24 hours after TBI, suggesting that TBI induces the activation of immune-related genes in glia after injury. Seven days after injury, genes associated with cell development, cell motion and cell morphogenesis are upregulated in one replicate, suggesting that glial development and proliferation occurs after injury (data not shown). Together, this biphasic response of increased expression in immune-related genes, followed seven days later by increased expression in genes associated with cell development and morphogenesis serves as a validation of our head-specific TBI model.

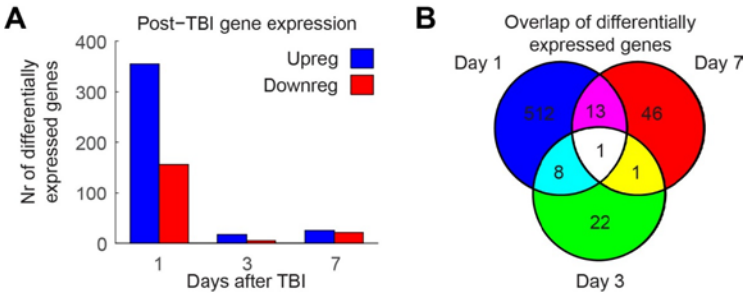

Figure 2 - TBI causes changes in glial gene expression

To test the role of the immune response in TBI survival and TBI-induced changes to behavior, we used the NFκB null mutant. This mutant lacks the master immune regulator NFκB Relish. This mutant is very sensitive to TBI induction, showing a mortality rate of 52% 24 hours after TBI (Fig 3A), much higher than the 15% 24 hour mortality rate showed in wild type flies (Fig 1A). Surprisingly, both climbing behavior and sleep are not affected by TBI in these flies (Fig 3B,C), suggesting that behavioral changes after TBI might be side effects of the immune response rather than the consequence of damage to the brain.

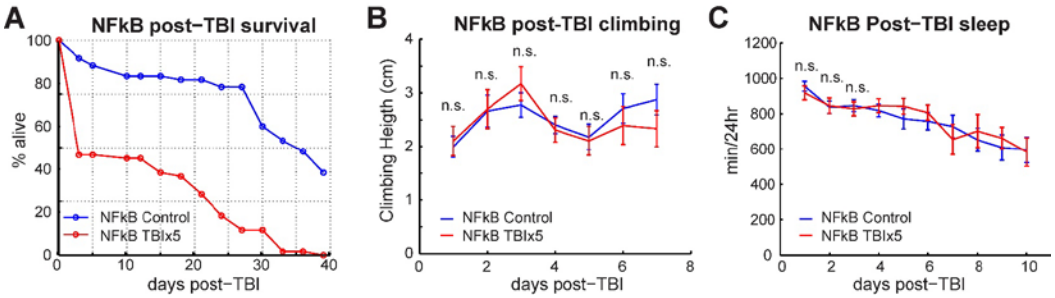

Figure 3 – TBI increases mortality in NFκB null mutants, without altering post-TBI behavior

Specific Objective 1D) Determine TBI-induced cell death

To test whether our TBI assay causes neuronal death, apoptosis was quantified using a TUNEL assay after inducing TBI by striking flies either 5 or 10 times and comparing the number of TUNEL positive cells at three different timepoints (4, 8 and 24 hours) between TBI-treated flies and sham-treated controls. Controls showed, on average, two to four TUNEL positive cells which may be spontaneous apoptotic cells (Fig. 4). Four hours after TBI induction we saw an increase in TUNEL positive cells in the TBIx10 condition, but not in the TBIx5 condition at this time point. Eight hours after TBI induction we also saw an increase in TUNEL positive cells in the TBIx10 condition, but not in the TBIx5 condition at this time point. 24 hours after TBI induction we saw an increase in TUNEL positive cells in both the TBIx5 and the TBIx10 condition at this time point (Fig. 4).

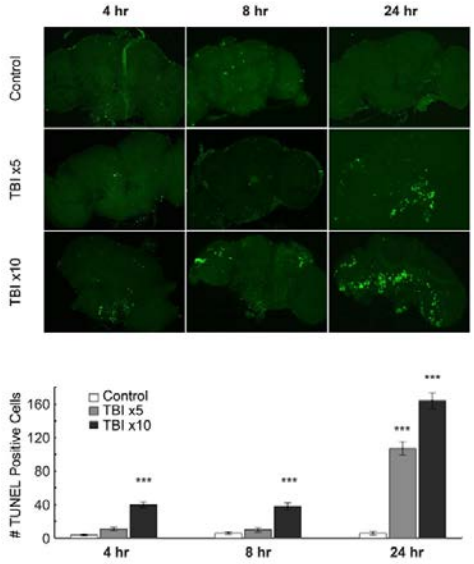

Figure 4 – TBI induces cell death

What opportunities for training and professional development has the project provided?
Nothing to Report.

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?
Nothing to Report.

Major Activity 2: to test the hypothesis that correcting impaired sleep patterns can facilitate post-TBI recovery

Specific Objective 2A) Test the effect of sleep intervention on subsequent behavior and sleep architecture – **IN PROGRESS**
After finding that TBI causes sleep loss and sleep fragmentations in the first three days after TBI, we tested whether pharmacologically correcting this sleep impairment would improve survival. We also tested whether further enhancing this sleep impairment would increase mortality further. Surprisingly, we found that administration of Gaboxadol, a sleep-promoting GABA receptor agonist, decreased post-TBI survival while administering caffeine, which is wake promoting in flies as well as mammals increased post-TBI survival (Fig 5).

Specific Objective 2B) Test the effect of sleep intervention on subsequent markers of cell death – **ON HOLD**

We did not manage to perform this part of the project.

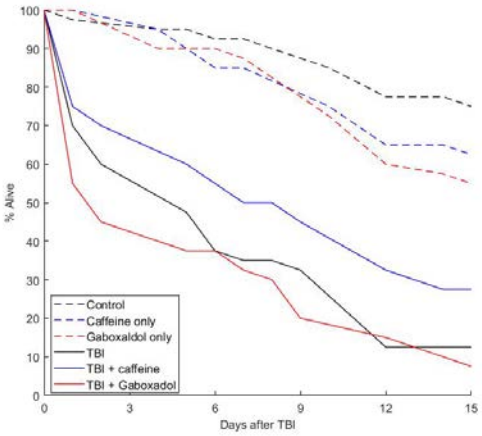

Figure 5 – feeding flies wake promoting caffeine improves TBI survival while feeding flies sleep promoting Gaboxadol decreases survival

What opportunities for training and professional development has the project provided?
Nothing to Report.

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?
Nothing to Report.

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

CHANGES/PROBLEMS
Aim 1: we initially planned to use a published assay (HIT method, (Katzenberger et al. 2013)) to induce TBI in groups of flies. However, we found that this assay causes many other types of injury as well. This led us to develop our single fly assay.
Aim 2: We had difficulty replicating published sleep promoting GAL4 drivers, so we used a pharmacological approach to induce wake or sleep instead.

PRODUCTS

Publications, conference papers, and presentations
Publications: We are currently preparing a manuscript describing our results. A preprint has been uploaded on BioRxiv (posted: Sept 20, 2018).
Glial immune-related pathways as mediators of closed head TBI effects on behavior in *Drosophila*
van Alphen B, Stewart S, Iwanaszko M, Xu F, Bang E, Rozenfeld S, Ramakrishnan A, Itoh T, Allada R.

Presentations: This work was presented at the Society for Neuroscience meeting in San Diego:
Glial immune-related pathways as mediators of closed head TBI effects on behavior in *Drosophila*
van Alphen B, Stewart S, Iwanaszko M, Xu F, Bang E, Rozenfeld S, Ramakrishnan A, Itoh T, Allada R.

Progress updates were also given at DoD satellite sessions of the 2017 and 2018 SLEEP meetings

Other products: a novel animal model to study the role of sleep in TBI
To test the therapeutic effect of correcting sleep impairments caused by TBI, we developed and validated a *Drosophila* head-specific model for TBI where well-controlled, non-penetrating strikes are directly delivered to the head of unanesthetized flies. This assay recapitulates many TBI phenotypes, including increased mortality, decreased and fragmented sleep, impaired motor control and increased neuronal cell death. Using glial targeted translating ribosome affinity purification in combination with RNA sequencing (TRAP-seq), we detected substantial changes in gene expression, including a strong upregulation of the innate immune response as well as several wake-promoting genes.

To test the in vivo functional role of these changes, we examined TBI-dependent behavior and lethality in mutants of the master immune regulator NF-κB and found that while lethality effects were still evident, changes in sleep and motor function were abolished, suggesting that TBI-induced changes in sleep are due to the immune response, rather than the injury.

These studies validate a new head-specific model for TBI in *Drosophila* and identify glial immune pathways as candidate in vivo mediators of TBI effects. In addition to genes involved in immunity, we also identified dozens of other genes that are up or down regulated after TBI with not prior functional connection to TBI.

We used pharmacological interventions to test whether correcting TBI-induced sleep decrease had a therapeutic effect, and whether exacerbating sleep fragmentation would exacerbate TBI phenotypes. Surprisingly, we found that feeding flies caffeine after TBI, which further decreased and fragmented sleep, increased TBI survival. Increasing sleep by feeding flies a GABA receptor agonist had the opposite effect, decreasing TBI survival.

Other products: data
We identified 500+ genes that are up or down regulated 24 hours after TBI, including genes involved in immunity, stress responses and sleep. We also identified dozens of other genes with no prior functional connections to TBI. This valuable resource forms one of the foundations for our proposed follow-on research.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Bart van Alphen*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*?*
Nearest person month worked:	*7*
Contribution to Project:	*Dr van Alphen designed the project, developed the TBI paradigm, performed some sleep experiments and analyzed sleep data*
Funding Support:	*This award*

Name:	*Samuel Stewart*
Project Role:	*Master student*
Researcher Identifier (e.g. ORCID ID):	*?*
Nearest person month worked:	*12*
Contribution to Project:	*Mr Stewart performed sleep experiments, the lifespan experiment, the climbing assay and the TRAP-seq experiments*
Funding Support:	*N/A*

Name:	*Anujaianthi Ramakrishnan*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*?*
Nearest person month worked:	*9*
Contribution to Project:	*Dr Ramakrishnan developed and performed the cell death assays*
Funding Support:	*This award*

Name:	*Melanie Zhang*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*?*
Nearest person month worked:	*2*
Contribution to Project:	*Ms. Zhang performed the cell death assays*
Funding Support:	*This award*

Name:	*Ravi Allada*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*?*
Nearest person month worked:	*4*
Contribution to Project:	*Dr Allada supervised the design, execution and analysis of the experiments*
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?

NEW SUPPORT

W81XWH1810594 (Allada) 0.30 academic
09/01/18-2/29/21 Dept. of the Army -- USAMRAA 1.20 summer
Discovery of Novel Therapeutics for Disordered Sleep in Fragile X Syndrome
This proposal performs a chemical library screen to restore robust sleep and circadian rhythms in a Fragile X animal model

DMS-1764421(PI:Carthew/co-I:Allada) 0.00 academic
07/01/18-06/30/23 NSF & Simons Foundation 1.00 summer
Northwestern University Quantitative Biology Center (NUQuB)
This center is to stimulate the application of mathematics to the study of developmental biology

1R01NS106955-01A1 (Allada) 1.50 academic
05/01/18-02/28/23 NIH/NINDS 2.50 summer
Molecular Mechanisms Integrating Circadian Timing and Photic Signaling
The goal of this proposal is to study the molecular and neuronal mechanisms involved in photoperiodic behavior

AARG-17-532626 (Allada) 03/01/18-02/28/21 0.00 academic
Alzheimer's Association 0.24 summer
Discovery of Novel Mechanisms by which Sleep Modulates AB Toxicity
The goal of this proposal is to study the effect sleep deprivation on the toxicity of Alzheimer's related Abeta

COMPLETED SUPPORT

C-074 Catalyst Award (PI: Allada; PI: Rust) 08/01/16-07/31/18 0.00 academic
Chicago Biomedical Consortium 0.06 summer
Transplanting a prokaryotic oscillator to animals to restore circadian clock function
The goal of this award is to transplant a bacterial clock into animals and engineer this system to drive molecular, physiological and behavioral rhythms.

PR151747 (PI Allada) 1.00 academic
06/01/16-11/30/18 Dept. of the Army -- USAMRAA 1.40 summer
Therapeutic Sleep for Traumatic Brain Injury
This proposal investigates the correlation between TBI-induced sleep disorders and TBI-induced behavioral changes and evaluates whether induced changes in sleep architecture rescue or worsen these behavioral changes.

PR152258 (PI Allada) 1.00 academic
06/01/16-11/30/18 Dept. of the Army -- USAMRAA 1.40 summer
Sleep homeostasis and synaptic plasticity
The goal of this proposal is to study where in the fly brain wake experience accumulates and how wake- and sleep promoting brain regions change their activity after sleep deprivation

What other organizations were involved as partners?

Nothing to Report

SPECIAL REPORTING REQUIREMENTS

Not applicable