AWARD NUMBER: **W81XWH-16-1-0169**

TITLE: **Sleep Homeostasis and Synaptic Plasticity**

PRINCIPAL INVESTIGATOR: Ravi **Allada**

CONTRACTING ORGANIZATION: **Northwestern University**
Evanston, IL 60208

REPORT DATE: **June 2018**

TYPE OF REPORT: **Annual**

PREPARED FOR: U.S. Army Medical Research and Materiel 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
June 2018	Annual	1 Jun 2017-31 May 2018

4. TITLE AND SUBTITLE

Sleep homeostasis and synaptic plasticity

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0169

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Ravi Allada

Bart van Alphen email: r-allada@northwestern.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Northwestern University
Department of Neurobiology
2205 Tech Drive
60202
Evanston, IL

8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel 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

The molecular basis of sleep homeostasis remains one of the great mysteries of science with implications for health, performance, and disease. Here we combined genetics with circuit specific knockdown of genes implicated in sleep homeostasis. To date the studies do not yet reveal the circuit or genetic basis of sleep homeostasis and suggest a more complex process than previously thought.

15. SUBJECT TERMS

Sleep, Sleep Homeostasis, GRASP, frm1, Drosophila

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

Table of contents

INTRODUCTION:

After a busy day we are sleepy. Yet, how the brain translates this accumulated wake experience into sleep drive and eventually forces us to fall asleep remains a mystery. In this proposal, we aim to identify the neural circuitry that regulates this homeostatic sleep drive by mapping where in the brain sleep need is encoded and where it is translated into sleep drive.

Sleep pressure – the internal drive to sleep – is proposed to be regulated by the interaction of circadian and homeostatic processes. In this two-process model, circadian mechanisms synchronize sleep drive to the day-night cycle while homeostatic sleep pressure responds to wake experience, increasing in parallel with wakefulness and dissipating again during sleep. The homeostatic regulation of sleep remains shrouded in mystery. One of the most exciting recent hypotheses concerning the function of sleep homeostasis is the "synaptic homeostasis" hypothesis. The basic idea is as follows: everyday behavior and learning produce a net increase in synaptic weights in the brain, meaning that the chemical connections between neurons are strengthened. One function of sleep is therefore to downscale or "normalize" all synapses in the brain, while maintaining the relative synaptic strength differences that have accrued through learning.

But how is wake experience translated into sleep drive? Where in the brain does this occur? Is there a discrete sleep drive circuit (a homeostat) that operates in concert with the circadian circuitry or does sleep drive accumulate everywhere in the brain?

To answer these questions we need to study a brain that is highly accessible while still being similar enough to man to be a valuable model organism. The fruit *fly Drosophila melanogaster* is the best candidate, as it comes with a wide variety of genetic tools that allow precise control of gene expression and neuronal activity in discrete parts of the brain. At the same time, neuronal biochemistry is very similar – flies and man respond in a similar manner to wake and sleep promoting drugs.

This proposal aims to tackle these questions by studying where in the fly brain wake experience accumulates and how wake- and sleep promoting brain regions change their activity after sleep deprivation. This will result in a map of the inputs and outputs of the sleep homeostatic circuitry.

ACCOMPLISHMENTS:

Major goals

Task 1A: Determine homeostasis and arousal in null mutants
Task 1B: Attempt rescue of null phenotypes by expressing rescue construct in discrete regions
Task 1C: Verify rescue brain areas by RNAi knockdown (in wildtype) of gene in areas where rescue was successful

Task 2: Quantify wake experience dependent synaptogenesis

Task 3: Test the effect of synaptogenesis on sleep-wake

Keywords: Sleep, Sleep Homeostasis, GRASP, frm1, Drosophila

What was accomplished under these goals?

Major Activity 1: to identify circuits where known modulators of sleep homeostasis modulate rebound sleep after sleep deprivation

Approximately 15 neuromodulators of sleep homeostasis have been identified in *Drosophila*, where loss of function of a gene also impairs rebound sleep after sleep deprivation (reviewed in Bushey, 2011). However, it is not known where in the fly brain these neuromodulators act on sleep homeostasis. To explore where these neuromodulators act we first need to confirm that RNAi-mediated pan-neuronal knockdown of these genes impairs sleep homeostasis. Here, we test RNAi-mediated knockdown of *dFmr1* , the *Drosophila* homolog of the human Fragile X mental retardation gene, *Ecdysone receptor (EcR)* and *creb2*.

We crossed three RNAi lines for dFmr1 to elav-Gal4, a pan-neuronal driver. These are TRiP.JF02634 (BL 27484), TRiP.GL00075 (BL 35200) and TRiP.HMS00248 (BL 34944). All are attP2 lines. These lines are compared to a attP2 TRiP control line crossed to elav-Gal4.We also tested a dFmr1 null mutant, dFmr1$^{[\Delta 50M]}$. Female flies were loaded in Drosophila Activity Monitors. Baseline sleep was measured for 2 days, followed by 12 hours of sleep deprivation during the dark phase of day 3 (ZT 13-24). Rebound sleep was measured during the 24 hours after sleep deprivation (day 4). Sleep lost is calculated as sleep day 3 ZT13-24 – sleep day 2 ZT13-24. Sleep regained is calculated as sleep day 4 ZT1-24 – sleep day 2 ZT1-24 and expressed as a percentage of total sleep lost.

Rebound sleep in the dFmr1 null mutant, dFmr1$^{[\Delta 50M]}$ is impaired (Fig 1A, blue line). However, sleep in the control line (black) is also rather low. The three RNAi lines show rather different phenotypes. TRiP.JF02634 shows a large increase in rebound sleep, with almost 50% of sleep lost recovered over 24 hours (Fig 1B). However, the other two TRiP lines are not different from the control line (Fig 1C,D).

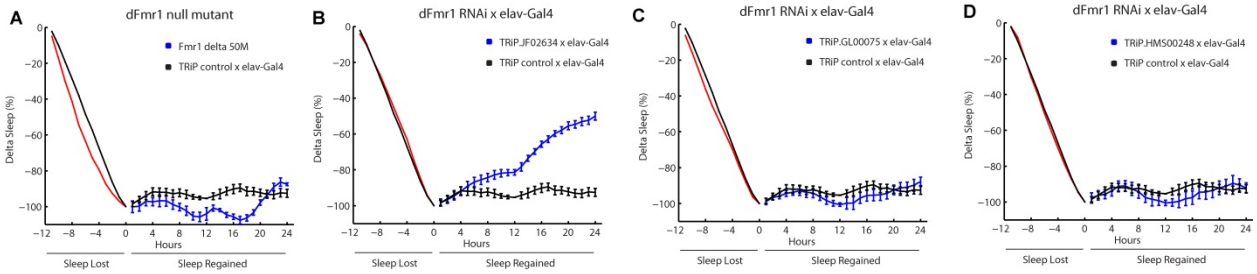

Figure 1 – dFmr1 knockdown

For creb2, we tested a null mutant CrebB[S162], (BL 4720) and two RNAi lines, TRiP.HMJ30249 (BL 63681) and TRiP.JF02494 (BL 29332) that were crossed to elav-Gal4. Rebound sleep is compared the same TRiP control as above. Rebound in the Creb2 null mutant is initially delayed, compared to the control line (Fig 2A). However, during the dark phase (hours 13-24) rebound sleep accumulates rapidly, resulting in approximately 60% sleep recoverd after 24 hours. The two RNAi lines show opposite results. Rebound sleep is impaired in TRiP.HMJ30249, resulting in 20% sleep recoverd after 24 hours, compared to 35% in the control line, where most of the lost sleep is recovered during the dark phase (Fig 2B, hours 13-24). However, rebound sleep is higher than controls in TRiP.JF02494, where over 40% of sleep lost is recovered (Fig 3C).

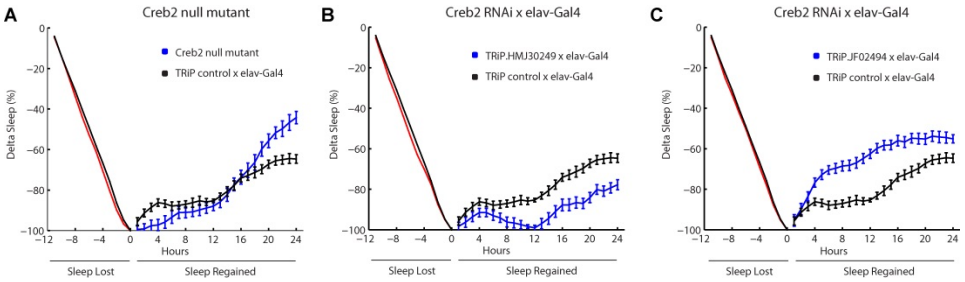

Figure 2 – Creb2 knockdown

We tested two null mutants for *Ecdysone receptor (EcR)*, EcR$^{[A483T]}$ (BL 5799) and EcR$^{[V559fs]}$ (BL 4901), two over-expression lines UAS-EcR.A and UAS-EcR.B1 and three TRiP RNAi lines, TRiP.HMJ22371 (BL 58286), TRiP. HMC03114 (BL 50712) and TRiP.JF02538 (BL 29374) that were crossed with elav-Gal4. For controls we used either w1118 x elav-Gal4 or TRiP control attP2 x elav-Gal4. Both null mutants show impaired sleep homeostasis (Fig 3A,B). However, rebound sleep is also impaired in the control line. One over expression line (UAS-EcR.B1 x elav-Gal4) is lethal after sleep deprivation, with 100% mortality, probably due to stress. Mortality in the other line (UAS-EcR.A x elav-Gal4) is also high (70%) after sleep deprivation. Surprisingly, the survivors show a strong negative rebound, where sleep loss further accumulates (Fig 3C), compared to the parental control w1118 x elav. The three RNAi lines show, again, mixed results. HMJ22371 and JF02538 show a strong, increased rebound compared to the TRiP control (Fig 3D,F) while HMC03114 shows no rebound at all.

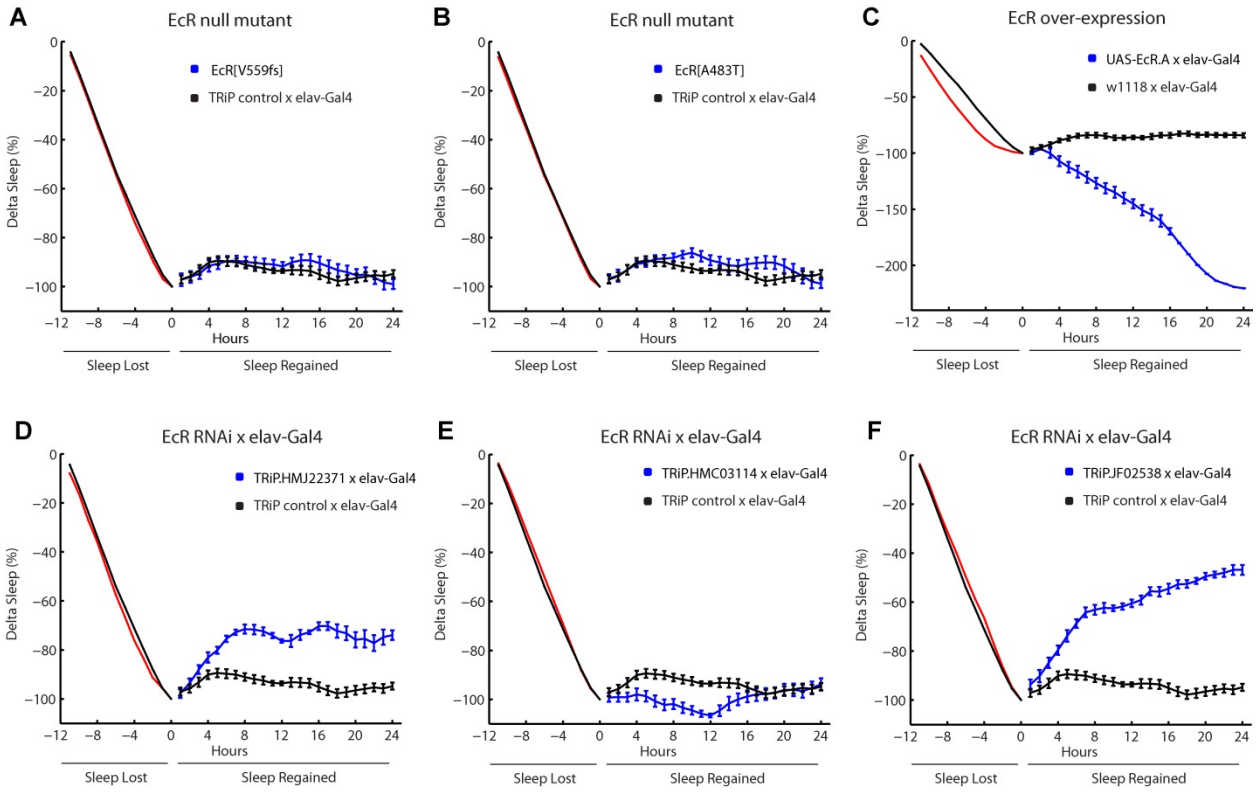

Fig 3 - Ecdysone receptor knockdown

Unfortunately, RNAi knockdown of Fmr1, Creb2 and EcR showed variable results in each experiment. However, protein levels should be quantified to confirm that RNAi-mediated knockdown of rhe gene of interest was successful. Another confounding factor is that the control line shows considerable variability from experiment to experiment (Fig 4). Rebound sleep in TRiP control x elav-Gal4 is almost 40% in the Creb2 experiment. However, in the other two experiments rebound sleep is low, with no more than 10% sleep

recovered over 24 hours (Fig 4). The most interesting trend is the opposite effects of EcR knockdown and over expression on rebound sleep, where 2 out of 3 TRiP lines show increased rebound sleep while over-expression shows strong negative rebound. It would be worth repeating this experiment, but with a lower dose of sleep deprivation to reduce mortality. We also tried over expression for dFmr1 and Creb2 but encountered technical difficulties.

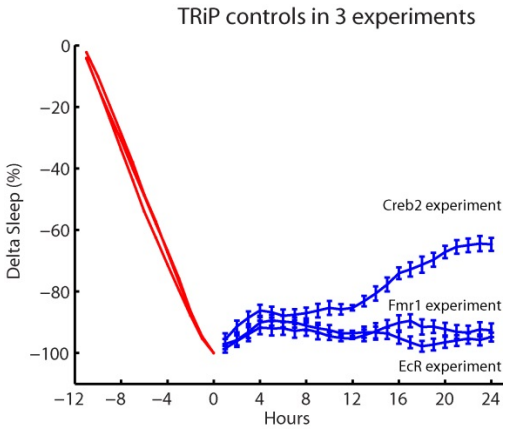

Figure 4 – TriP controls in 3 experiments

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?
We are currently trying to optimize our sleep deprivation protocols. Numerous devices have been used to induce sleep deprivation through mechanical stimulation (Pffeifenberger et al 2012; Shaw et al 2002; Guo et al 2016). These methods are varied not only in the diversity of frequency of stimulus, length of deprivation or timing of deprivation but also they deliver unique sensory experiences to the fly (flipping, jarring or shaking). Published work indicates that even when selecting for flies that are deprived of at least 90% of sleep, the rebound induced by these methods are not equivalent. Preliminary data in the testing three of the canonical methods support this observation. This has led us to believe that not all wake is created equally and that there must be certain aspects of wake inducing mechanical stimuli that result in homeostatic drive to sleep. We have found that flies that are deprived using a randomly generated stimulus will show greater rebound compared to those that are deprived using a fixed interval stimulus when controlling for wake during the stimulation. Additionally, more frequent stimulation will induce a greater rebound than less frequent stimulation. This is true to the extent that 20 seconds of repeated stimulation will be more effective at driving sleep need compared to one minute of continuous stimulation even though fly sleep is believed to be 5 minutes or more of inactivity. Lastly, flies vary their rebound depending on the time of day when recovery occurs indicating that homeostatic drive to sleep is modulated by the intrinsic clock. Together this demonstrates that homeostatic drive to sleep is dependent on when wake occurs and the character of a wake.

Major Activity 2: To identify neural circuits where wake experience results in increased synapse formation
Specific Objective 2) Quantify wake experience dependent synaptogenesis

We've had inconsistent results with getting synaptic GRASP to work. On average, we only saw GFP staining in 25% of the attempts, but sometimes we also saw GFP expression in flies that were not exposed to odor. We were not able to troubleshoot these problems.
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?

Work over the last decade in *Drosophila* has begun to point toward the notion that homeostatic sleep drive is encoded in specific circuits rather than as a defined global brain state. As a result, we hypothesized that identification of genes involved in sleep homeostasis would require a targeted approach in which defined neuronal populations with a demonstrable role in sleep homeostasis are isolated and subjected to RNA-sequencing. To this end, we are first seeking to identify relevant populations of neurons for in-depth profiling by screening sparse GAL4 drivers primarily from the Janelia Farms collection. In this screen, drivers are used to express either the temperature-sensitive TRPA1 ion channel or the temperature-sensitive dynamin mutant *shibire*. Flies are subsequently allowed to sleep normally for several days in a 12h:12h light:dark cycle at 21°C. After an acclimation period, the flies are subjected to a 12h temperature pulse of 29°C during the night, which will either activate neurons expressing TRPA1 or silence neurons expressing *shibire*. Following the temperature pulse, flies are returned to 21°C and allowed to sleep without intervention. Rebound sleep is measured and compared to baseline and relevant controls. The two screens are designed to identify neurons that either promote homeostatic drive when activated or block the dissipation of homeostatic drive when silenced. Both outcomes are judged on the basis of the quantity and quality of rebound sleep following intervention. Through this screening we have identified several subclasses of neurons with unique effects on sleep homeostasis, including lines that promote wakefulness with significant subsequent rebound, lines that promote wakefulness with no significant subsequent rebound, and lines with no effect on wakefulness but a significant subsequent rebound. Using these lines as tools, our immediate plan is to interrogate gene expression in these circuits during and following sleep deprivation to identify genes that are significantly up or downregulated as homeostatic drive mounts and dissipates.

Major Activity 3: To test the hypothesis that altering synapse formation anywhere in the brain alters sleep

Specific Objective 3) Test the effect of synaptogenesis on sleep-wake

We have tested several dFmr1 modifier lines (uas-dFmr1 and EP3517 (overexpression) and three RNAi lines(knock down)) for their ability to change dfmr1 expression and alter sleep architecture. dFmr1 is involved in synaptic pruning and plasticity. We hypothesized that, as published before, dFmr1 overexpression will result in loss of synapses and decreased sleep while dFmr1 knockdown has the opposite effect – increased synapse formation and sleep.

Crossing these lines with a pan-neuronal inducible driver (daughterless geneswitch) did not produce any phenotypes after one or two weeks of induction. When we crossed the RNAi lines with elav-Gal4, a pan neuronal driver, we found that RNAi knockdown of dFmr1 resulted in *decreased* sleep in two out of three lines. Overexpression seemed lethal.

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?

n/a

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

We encountered several problems in this project:

1) The synaptic GRASP technique, which uses split GFP expressed at synapses to quantify synapse formation, is hard to get to work in our lab. One possibility, suggested by the Gallio lab, is that one or more lines were contaminated. Redoing the experiments with verified lines did not provide better results
2) RNAi-mediated knockdown of dfmr1 had the opposite result of what has been published – instead of increased sleep we found strongly decreased sleep.
3) We could not verify loss of sleep homeostasis in all tested null mutants
4) There was a lot of variation in the amount of rebound sleep detected in control lines (Fig 4)

PRODUCTS

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Bart van Alphen*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*?*
Nearest person month worked:	*5*
Contribution to Project:	*Dr van Alphen designed the project and analyzed data*
Funding Support:	-

Name:	*Dae Sung Hwangbo*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*?*
Nearest person month worked:	*5*
Contribution to Project:	*Dae Sung Hwangbo designed and performed the circuit genetics studies*
Funding Support:	-

Name:	*Clark Rosensweig*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*?*
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Rosensweig designed experiments to examine methods for sleep deprivation and sleep homeostasis*

Funding Support:	-

Name:	*Mikhail Koksharov*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*?*
Nearest person month worked:	*5*
Contribution to Project:	*Dr. Koksharov contributed to completion of genetics of sleep homeostasis experiments*
Funding Support:	-

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 Active Research Support

R01NS106955-01A1 05/01/18-02/28/23 1.5 academic, 2.5 summer
NIH/NINDS
Role: PI
Total R01: $1,728,125
Contact: Program Official Janet He, hey@ninds.nih.gov
Title R01: Molecular Mechanisms Integrating Circadian Timing and Photic Signaling
Project Goal: The goal of this proposal is to examine how circadian timing and photic signaling are integrated in the *Drosophila* clock neural network.
Specific Aims:
Aim 1. To identify the neuronal basis of PRL-1 effects on circadian and photoperiod-dependent diurnal behavior
Aim 2. To determine the relative roles of PRL-1 on the clock response to neural communication and on cell autonomous clocks
Aim 3. To test the hypothesis that PRL-1 functions in circadian and photoperiod-dependent diurnal behavior via TIMELESS

AARG-17-532626 03/01/18-02/28/21 0.25 summer
NIH/NINDS
Role: PI
Total: $150,000
Contact: Rita Freeman, Post Award Grant Specialist, rita.freeman@alz.org
Title*:* Discovery of Novel Mechanisms by which Sleep Modulates AB Toxicity
Project Goal: The goal of this proposal is to study the effect sleep deprivation on the toxicity of Alzheimer's related Abeta
Specific Aims:
Aim 1. To identify molecular pathways that mediate the effects of sleep deprivation on Aβ toxicity
Aim 2. To test the hypothesis that modifiers of sleep deprivation induced Aβ toxicity act via changes in Aβ levels, synapses, and/or cell death

2P01AG011412-18A1 09/15/17-05/31/22 0.45 academic, 0.15 summer
National Institute on Aging
Role: PI
Total: $22,308
Contact: Mack Mackiewicz, mackiewiczm2@mail.nih.gov

Title: Alterations of Sleep and Circadian Timing in Aging (Core C)
Project Goal: The proposal focuses on the interactions between peripheral tissue clocks, sleep and centrally regulated circadian rhythms in the age-related increase in metabolic disease.
Specific Aims:
Aim 1. To provide Projects 1 and 2 with reliable measurements and analyses of blood hormones appetitive, glucoregulatory, lipid, organic acid, and nucleotide blood constituents from human subjects who are affected by aging and/or sleep duration and quality.
Aim 2. To provide all projects with metabolic assays and gene expression analyses to investigate the role of the clock/NAD/sirtuin pathway in age-dependent decline in metabolic function and circadian behavior.
Aim 3. To provide genomic/transcriptome analyses using next generation sequencing (NGS) methods in animal models (Project 3)
Aim 4. To maintain and document assay quality and assure reliability of data produced from experiments required by the Projects.
Aim 5. To expand scope techniques as required for exploitation of new information and methods they become available.

What other organizations were involved as partners?

Nothing to Report

SPECIAL REPORTING REQUIREMENTS
Not applicable