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TITLE: **Sleep Homeostasis and Synaptic Plasticity**

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14. ABSTRACT In this proposal, we aim to identify the neural circuitry that regulates homeostatic sleep drive by mapping where in the brain sleep need is encoded and where it is translated into sleep drive. Our initial attempt of using RNAi to knock down genes proposed to be involved in sleep homeostasis in discrete circuits was not successful. We also had difficulty replicating published methods that quantify synapse formation. We are now using a thermogenetic approach to activate discrete circuits in the brain to find brain regions that encode sleep homeostasis. To date, we have screened about 200 lines and have found a number of interesting effectors. Perhaps the most compelling finding is that prolonged wakefulness is not necessarily a strong driver of homeostatic buildup. Likewise, prolonged sleep does not necessarily dissipate sleep drive. As a result, we now have access to a unique tool set of drivers to manipulate acute behavioral state as well as homeostatic drive, which we can use to dissociate sleep/wakefulness from subsequent rebound (ie. homeostatic drive).					
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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*^[Δ 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*^[Δ 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).

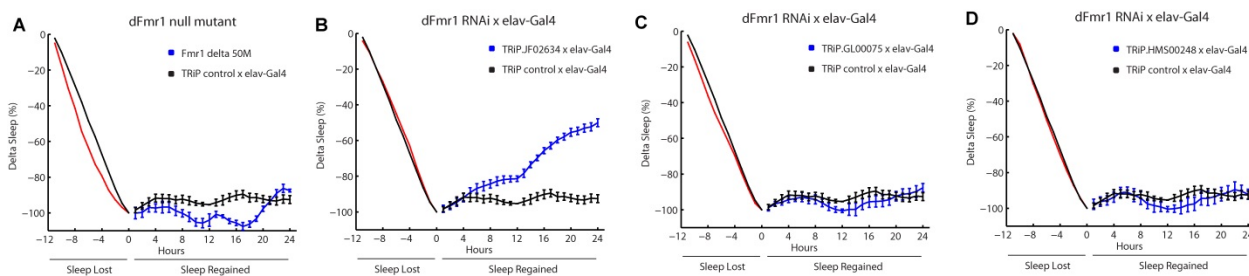


Figure 1 – *dFmr1* knockdown

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).

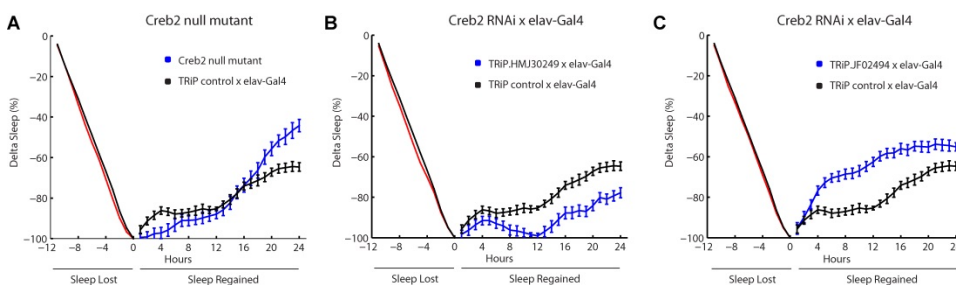


Figure 2 – *Creb2* knockdown

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.

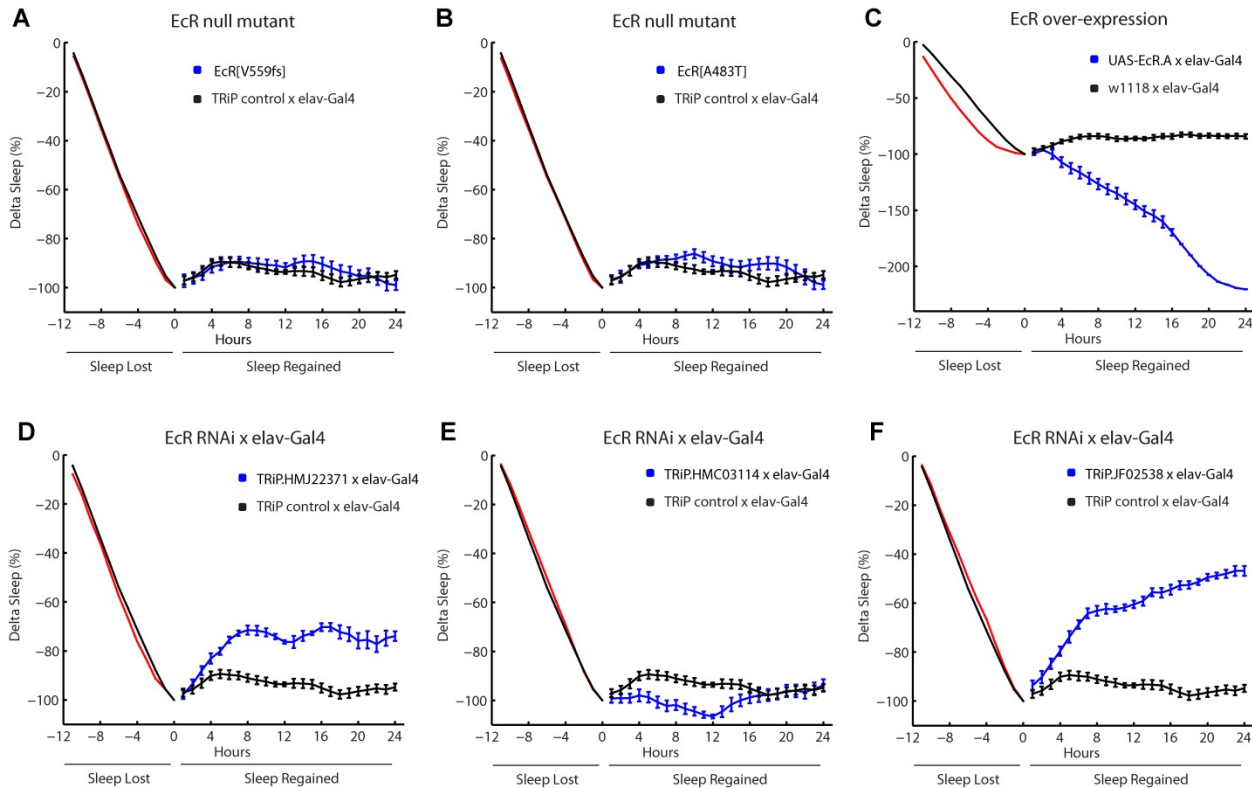


Fig 3 - Ecdysone receptor knockdown

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 the 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.

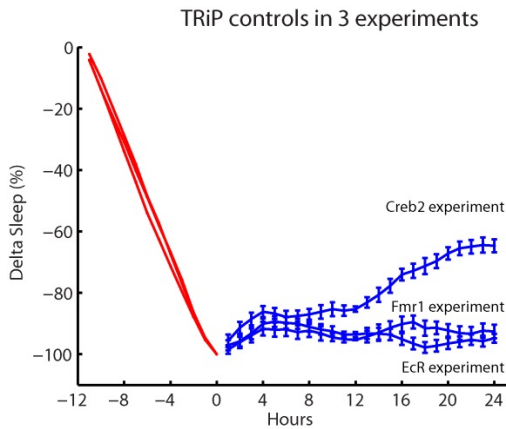


Figure 4 – TriP controls in 3 experiments

We tested sleep homeostasis in *insomniac*, a short sleeping mutant with impaired sleep homeostasis. Wild type flies recover 20-40% of sleep lost during the 24 hours after sleep deprivation. *Insomniac* does not recover any sleep lost. To rescue the short sleeping phenotype, we used *inc⁰⁰²⁸⁵*. This is an *insomniac* null mutant with a UAS-*inc* construct. By crossing *inc⁰⁰²⁸⁵* with a library of GAL4 lines, we can test whether this rescues sleep and/or sleep homeostasis. To test for rescue of the short sleeping phenotype, we crossed *inc⁰⁰²⁸⁵* to Mushroom Body output neurons (MBONs). The *Drosophila* mushroom body is an important sleep regulating region and consists of sleep promoting and wake promoting subdivisions. When we rescued *inc* in wake promoting regions, this decreased total sleep while rescuing *inc* in sleep promoting regions this increased total sleep. We used RNAi-mediated knockdown on *insomniac* to verify our rescue experiments. Knockdown of *inc* in wake promoting MBONs resulted in increased sleep while *insomniac* knockdown in sleep promoting MBONs resulted in decreased sleep. In 2016, Mark Wu's lab identified a dedicated circuit in the *Drosophila* central brain that encodes sleep homeostasis. This subset of R2 ellipsoid body neurons is capable of generating sleep drive. RNAi-mediated knockdown of *insomniac* in R2 neurons abolished sleep homeostasis without affecting baseline sleep.

Selecting vortexer method and exploring parameters

Identifying the optimal method of sleep deprivation is central to understanding sleep homeostasis in the fly. Such a method should robustly deprive flies of sleep and induce rebound sleep the following day. In *Drosophila* there are three predominant methods to induce sleep deprivation; The rotator, sleep nullifying apparatus (SNAP), and the vortexer. To identify the best deprivation device, we tested all automated methods. Our work showed that the vortexer serves as the most effective method of depriving flies. Flies deprived using the vortexer method demonstrated a 2-fold increase in recovery sleep compared to the other two devices and was more effective at depriving flies of sleep, thus demonstrating that it was the most effective means of testing sleep homeostasis.

Next, we examined parameters of the vortex stimulus to isolate the most effective for inducing rebound through modulating frequency and randomizing the timing of the stimulus. We demonstrated that randomly varying the timing of the vortex stimulus increased rebound sleep, suggesting that constant stimuli are not as effective at inducing wakefulness as randomize stimuli. In varying stimulus frequency, we demonstrated that a frequency of one stimulus every 20 seconds produced greater rebound than either 1- or 5-minute frequencies, suggesting that even below the threshold of 5 minutes of inactivity that is the accepted definition of sleep in the field, there is some form of rest.

Now that we established the best parameters to induce sleep deprivation, we have been investigating the circadian and homeostatic drives for sleep and wake are integrated. Some, but not all, clock gene mutants display altered homeostatic responses. But it is unclear if this is due to their role in the circadian clock or not. To address this question, we used the fruit fly which exhibits conserved circadian clock and homeostatic regulation of sleep as well as conserved core clock mechanisms. We demonstrated that the circadian clock selectively suppresses the sleep rebound response to sleep deprivation during the evening phase, suggesting the involvement of evening cells in modulating the homeostat or the behavioral response to the homeostat.

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 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 worked together with the Gallio lab, who created the synaptic GRASP lines, to resolve these issues but we were unable to improve our success rate.

In order to better understand the neural circuits underlying wake and sleep promotion, as well as the buildup and dissipation of homeostatic drive, we selected a large collection of Gal4 drivers with sparse expression in the fly's brain. Using this collection, we expressed the temperature sensitive ion channel TrpA1 in discrete sets of neurons and exposed these flies to an activating temperature pulse for twelve hours during the day and the night. We monitored the sleep behavior of these flies during and also following each exposure to the activating temperature. To date, we have screened about 200 lines and have found a number of interesting effectors. Perhaps the most compelling finding is that prolonged wakefulness is not necessarily a strong driver of homeostatic buildup. Likewise, prolonged sleep does not necessarily dissipate sleep drive. As a result, we now have access to a unique tool set of drivers to manipulate acute behavioral state as well as homeostatic drive, which we can use to dissociate sleep/wakefulness from subsequent rebound (ie. homeostatic drive). Additionally, because these lines were sourced from the Janelia collection of drivers, we have access to images of the expression pattern of each driver within the brain and ventral nerve cord. Thus, we have begun to compare the lines to look for anatomical regions with a significant role in promoting sleep/wake/homeostatic drive, which will help us to understand the circuitry underlying the various processes. So far, this analysis suggests that there are other regions critical for these processes beyond what has been reported in the literature.

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 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?

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

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). As a result, we re-evaluated and improved our sleep deprivation techniques

PRODUCTS

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

Name:	<i>Tomas Andreani</i>
Project Role:	<i>PhD student</i>
Researcher Identifier (e.g. ORCID ID):	?
Nearest person month worked:	6
Contribution to Project:	<i>Mr Andreani performed the insomniac experiments and the synaptic GRASP experiments and tested different sleep deprivation methods</i>
Funding Support:	-

Name:	<i>Nahee Park</i>
Project Role:	<i>Undergraduate student</i>
Researcher Identifier (e.g. ORCID ID):	?
Nearest person month worked:	12
Contribution to Project:	<i>Miss Park performed the sleep deprivation experiments</i>
Funding Support:	-

Name:	<i>Clark Rosensweig</i>
Project Role:	<i>Postdoctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	?
Nearest person month worked:	2
Contribution to Project:	<i>Dr. Rosensweig performed fly genetics experiments</i>
Funding Support:	<i>This award</i>

Name:	<i>Matthieu Flourakis</i>
Project Role:	<i>Postdoctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	?

ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Dr. Flourakis initiated synaptic experiments</i>
Funding Support:	<i>This award</i>

Name:	<i>Dae-Sung Hwangbo</i>
Project Role:	<i>Postdoctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	?
Nearest person month worked:	12
Contribution to Project:	<i>Dr. Hwangbo performed fly genetics and sleep homeostasis experiments</i>
Funding Support:	<i>This award</i>

Name:	<i>Mikhail Koksharov</i>
Project Role:	<i>Postdoctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	?
Nearest person month worked:	5
Contribution to Project:	<i>Dr. Koksharov examined metabolic homeostasis mechanisms</i>
Funding Support:	<i>This award</i>

Name:	<i>Ravi Allada</i>
Project Role:	<i>Principal Investigator</i>
Researcher Identifier (e.g. ORCID ID):	?
Nearest person month worked:	3
Contribution to Project:	<i>Dr. Allada supervised the design, execution and analysis of the experiments</i>
Funding Support:	<i>This award</i>

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)

09/01/18-2/29/21

0.30 academic

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) 07/01/18-06/30/23 0.00 academic
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) 05/01/18-02/28/23 1.50 academic
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) 06/01/16-11/30/18 1.00 academic
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) 06/01/16-11/30/18 1.00 academic
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