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Genetics and Neural Circuitry of Sleep Homeostasis in *Drosophila melanogaster*

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Lawrence Kendall Satterfield

Committee in charge:

Professor William Joiner, Chair
Professor Rolf Bodmer
Professor Gabriel Haddad
Professor Chih-ying Su
Professor Jing Wang

2020

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University of California, San Diego

2020

EPIGRAPH

I love deadlines. I love the whooshing noise they make as they go by.
~ Douglas Adams

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LIST OF ABBREVIATIONS

AD	Activation domain
AMMC	Antennal mechanosensory and motor center
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CD8	Cluster of differentiation 8
cDNA	Complementary DNA
Cha	Choline acetyltransferase
CKII	Casein Kinase II
CREB	cAMP response element-binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CNS	Central nervous system
Da	dendritic arborizing neurons
DAMS	Drosophila activity monitoring system
DBD	DNA binding domain
DEG/ENaC	Degenerin/epithelial sodium channels
dFB	Dorsal fan-shaped body
DMSO	Dimethyl sulfoxide
DN1p	Posterior dorsal neurons 1
DNA	Deoxyribonucleic acid
EBR2	Ellipsoid-body Ring 2 neurons

EDTA	Ethylenediaminetetraacetic acid
EKO	Electrical knockout channel
GFP	Green fluorescent protein
GRASP	GFP reconstitution across synaptic partners
gRNA	Guide RNA
Hsp83	Heat shock protein 83
IHC	Immunohistochemistry
IP	Immunoprecipitation
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen-activated protein kinase
MARCM	Mosaic analysis with a repressible cell marker
MB	Mushroom bodies
MBONs	Mushroom body output neurons
mRNA	Messenger RNA
Myr	Myristoylation
Nf1	Neurofibromatosis 1
NREM	Non-rapid eye movement sleep
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PER	Proboscis extension reflex
PER/TIM	Period/Timeless complex
pH	Potential of hydrogen
PNS	Peripheral nervous system

ppk	pickpocket
qPCR	Quantitative polymerase chain reaction
REM	Rapid eye movement sleep
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-seq	Ribonucleic acid-sequencing
rp49	Ribosomal protein 49
S.E.M.	Standard error of the mean
SEZ	Subesophageal zone
shRNA	Short hairpin RNA
sLNvs	Small ventral lateral neurons
TNT	Tetanus toxin light chain
TrpA1	Transient receptor potential cation channel A1
UAS	Upstream activation sequence
UTR	Untranslated region
VNC	Ventral nerve cord
WT	Wild type
ZT	Zeitgeber time

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I would like to acknowledge my advisor, Dr. William Joiner, for all of his guidance and patience during my graduate training. He has given me so many opportunities to grow as a scientist and a critical thinker, for which I am extremely grateful.

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Work presented in Chapter 2 with some modifications and additions will be submitted for publication. De, Joydeep; Satterfield, L. Kendall; Wu, Meilin; Barrall, Erika; Joiner, William J. “Neural circuitry of sleep homeostasis originates outside of the brain”. The dissertation author performed all IHC and imaging included in this chapter as well as a behavioral screen and some additional behavior with assistance from corresponding author William Joiner who performed or assisted with many behavior experiments.

Work presented in Chapter 3 will be submitted for publication. De, Joydeep; Satterfield, L. Kendall; Wu, Meilin; Barrall, Erika; Qiu, Tianhao; Joiner, William J. “Identification of a sleep homeostasis circuit driven by peripheral sensory inputs to the brain”. The dissertation author performed all IHC and imaging included in this chapter as well as a behavioral screen and some additional behavior with assistance from corresponding author William Joiner who performed or assisted with many behavior experiments.

Work presented in Chapter 4 with minor modifications will be submitted for publication. Satterfield, L. Kendall; Lambatan, Vanessa; Lin, Veronica; Wu, Meilin; Lin, Michelle; Joiner, William J. “Protein Kinase CKII is required for sleep homeostasis in *Drosophila*”. The dissertation author was the primary investigator and author of the paper.

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Satterfield LK, Lambatan V, Lin V, Wu M, Lin M, Joiner WJ. Protein Kinase CKII is required for sleep homeostasis in *Drosophila*. Manuscript in preparation.

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ABSTRACTS

A newly identified synaptic circuit drives sleep need in *Drosophila*. **Kendall Satterfield***, Joydeep De, Glen Seidner, James E. Robinson, Melin Wu, Erika Barrall, Vanessa Lambatan, Tianhao Qiu, William J. Joiner. CCB Symposium, San Diego February 2019

Genetic and Proteomic Analyses Reveal Novel Regulators of Sleep Need. **Kendall Satterfield***, Nicole Hoffner, Glen A. Seidner, James E. Robinson, Meilin Wu, William J. Joiner. Cold Spring Harbor Laboratory Meeting: Neurobiology of *Drosophila*, Long Island NY October 2017

Identification of neural circuits and molecules that control sleep need. **Kendall Satterfield***, Nicole Hoffner, Glen A. Seidner, James E. Robinson, Meilin Wu, Kurtresha Worden, Pavel Masek, Alex C. Keene, William J. Joiner. GSA Drosophila Research Conference, San Diego April 2017

Identification of neural circuitry and genes that control sleep need. **Kendall Satterfield***, Nicole Hoffner*, Glen A. Seidner, James E. Robinson, Meilin Wu, Kurtresha Worden,

Pavel Masek, Alex C. Keene, William J. Joiner. CCB Symposium, San Diego February 2016

A novel assay to screen for neural circuits and genes involved in sleep homeostasis. **Kendall Satterfield***, Glen Seidner, James E. Robinson, Meilin Wu, William J. Joiner. Cold Spring Harbor Laboratory Meeting: Neurobiology of *Drosophila*, Long Island NY October 2015.

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ABSTRACT OF THE DISSERTATION

Genetics and Neural Circuitry of Sleep Homeostasis in *Drosophila melanogaster*

by

Lawrence Kendall Satterfield

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2020

Professor William J. Joiner, Chair

Research in humans and other model organisms has established that the daily timing and amount of sleep are regulated by the circadian clock and sleep homeostasis respectively. In contrast to the circadian clock, the biological basis of how sleep homeostasis is regulated in the brain remains poorly understood. In this dissertation, I investigate the genetics and neuroanatomy of sleep homeostasis regulation using the model organism *Drosophila melanogaster*.

Building on previous work in our lab, I explored the nature of a rare population of sensory neurons (named ppk) whose activity has a privileged role in driving sleep homeostasis. I mapped where ppk neurons are located in the peripheral nervous system (PNS) and used a combination of thermogenetic behavioral studies and imaging to examine the neuroanatomical relationship between ppk and other previously described sleep homeostasis driving neurons. These results showed that neurons capable of driving waking and subsequent sleep need do not have cell bodies in the brain but instead localize to the ventral nerve cord and to the PNS.

To investigate putative postsynaptic effectors of ppk-driven sleep homeostasis I used thermogenetically-controlled sleep behavior and trans-synaptic mapping to identify and characterize neurons that satisfy predictions of a neural circuit model for sleep homeostasis. Further, I report evidence that suggests this sleep homeostasis circuitry could be part of the output of the circadian clock, which would mechanistically link how the clock and sleep homeostasis interact to determine the arousal state of the fly.

Through an unbiased forward genetic screen I identified a mutation in the Protein Kinase CKII subunit CKIIβ that has impaired sleep homeostasis. Using genetic complementation and rescue studies I show that two transcripts of CKIIβ are necessary and sufficient for sleep homeostasis. I also demonstrate that the CKIIβ mutant's effect on sleep can be phenocopied by pan-neuronal expression of dominant-negative CKIIα, and I map this effect to circadian clock neurons. I also demonstrate that CKII is required for sleep-dependent associative memory formation. This study supports a novel role for a protein kinase in sleep homeostasis and suggests that dysregulation of circadian function can potentially impact the function of the homeostat.

Chapter 1: Introduction

1.1 Sleep is highly conserved throughout evolution

Research suggests that the requirement for sleep may have arisen at least as early as the development of the earliest nervous systems [1, 2]. From nematodes to jellyfish, fruit flies to zebrafish, and up the evolutionary ladder of complexity to humans – any organism with a nervous system studied in detail exhibits a sleep-like state [1, 3-10]. The ubiquity of sleep is indicative that it serves one or more critical biological functions; however, from an evolutionary perspective, the requirement is counterintuitive, as one would expect there to be a strong selective pressure against the need to spend hours unconscious and vulnerable to predation. Indeed, some species that occupy environmental niches disadvantageous to sleep, such as long-distance migratory birds and aquatic mammals, have evolved adaptations such as unihemispheric sleep [11, 12] - allowing one side of the brain to sleep at a time while the animal maintains awareness and even flight - rather than dispensing with sleep altogether. Therefore, it is clear that sleep must satisfy critical biological requirements, though the identity of these functions remains unknown.

To study sleep it is important to have clear definitions of the features of the behavioral state that distinguish it from other types of immobility or forms of unconsciousness. First, sleep is characterized by consolidated bouts of immobility during which the sleeping organism has an elevated arousal threshold to environmental stimuli such as noise or gentle touch compared to when it is awake. A sleep state is also rapidly reversible when a stimulus does surpass the arousal threshold, which distinguishes it

from other forms of unconsciousness such as coma, seizure, or anesthesia. The final behavioral hallmark of sleep is that the daily amount required by organisms is homeostatically regulated, meaning that they have an average amount of sleep that is required every day and deviations, especially lost sleep, must be compensated for later. Sleep is also characterized by different patterns of brain activity compared to waking. In mammals, multiple types of polygraphic recordings of electrophysiological changes that occur between sleep and waking have established two major sleep states: rapid eye movement (REM) sleep and non-rapid eye movement (NREM) [13]. While multiple sleep states have not been found in most invertebrates, studies have demonstrated electrophysiological changes that occur in the brains of sleeping flies [14, 15], and a recent study has described oscillatory brain activity in flies during sleep [16]. In invertebrates the behavioral features alone are considered sufficient to define and study sleep behavior [4-7].

1.2 The two-process model of sleep regulation

The brain must integrate multiple signals in order to determine any behavior state. Sleep need accumulates through the day with sustained waking, but how does the brain decide between wake and sleep? The two-process model of sleep regulation is a useful and predictive framework that describes the activity of the two central processes in the brain that regulate the daily timing (circadian clock) and amount (sleep homeostasis) of sleep [17, 18]. In the model, process C represents the wake promoting activity of the circadian clock which cycles in strength over an ~24 hr period. Process S reflects the increase in sleep need that rises with time spent awake and decreases with sleep and is

therefore thought to be sleep promoting. Over the course of a day these two processes are in opposition and the difference between the strength of process S minus the strength of process C determines the net sleep pressure that influences the behavioral outcome – the sleep or wake state over time (Figure 1.1 A). For example, in the morning, the arousal promoting output from the clock is rising as the sleep promoting signal from sleep homeostasis is reaching its lowest point and the animal wakes up. As the day progresses, the wake promoting contribution from the clock ebbs as the sleep promoting signal from process S continues to grow in strength until it predominates, and the animal sleeps to discharge sleep pressure. How and where the brain integrates different signals that influence arousal and sleep remains one of many open questions.

In the decades since the first circadian clock mutants were described [19-21] there have been many significant advances in our understanding of the molecular and neuroanatomical underpinnings of how the clock regulates the daily timing of sleep and other important biological rhythms in flies [22-29] and mammals [30-36]. In contrast, neither the molecular nor neuroanatomical basis of sleep homeostasis have been identified. Since process S reflects sleep need, any mechanistic understanding of how sleep homeostasis functions in the brain should offer more direct insights into what needs sleep fulfills. Therefore, sleep homeostasis has been the subject of great interest for years.

One significant obstacle to studying sleep homeostasis is that sleep behavior can be difficult to interpret. In theory, if process S is, at least in part, responsible for the total amount of daily sleep, genetic mutations that reduce overall sleep may result from either a reduction in sleep need or as a result of elevated baseline arousal. Over the last decade

multiple genes have been discovered that influence baseline sleep in flies [37-42], but to date, these discoveries have not advanced our understanding of sleep homeostasis regulation.

The standard method to study sleep homeostasis is to measure the amount of recovery or rebound sleep an animal recovers following a period of sleep deprivation [6, 7]. In this experiment, an animal is prevented from sleeping during its normal rest period. As a result, sleep need accumulates until the sleep deprivation ends. At this point elevated sleep need causes the animal to engage in recovery sleep during a period when it would normally be awake (Figure 1.1 B). Therefore, a mutation that reduces sleep need would be expected to reduce recovery sleep following sleep deprivation. To date, genetic screens for such a phenotype have not been successful.

Studies of sleep homeostasis and the circadian clock are generally conducted by attempting to isolate one process to minimize its influence on sleep/wake behavior. However, processes S and C must be interacting along with other sensory information in the brain to determine the arousal state of the animal both at baseline, and when the system is responding to sleep deprivation. The interactions between the two processes are important to consider when interpreting the results of sleep deprivation experiments in genetic screens. During sleep deprivation experiments, the output of an elevated process S is operating in direct opposition to the strong arousal-promoting signal from process C when the animal engages in recovery sleep following sleep deprivation (Figure 1.1 B). In wildtype (WT) animals process S suppresses arousal in the morning; however, it is difficult to predict what behavioral outcome following sleep deprivation might be expected when one process is significantly impaired. For example, if the strength of the

arousal signal is greatly diminished in circadian clock mutants, one might expect to see an exaggerated rebound phenotype measured following sleep deprivation, likely because an elevated process S is not opposed by process C [43].

I explore how processes C and S interact to influence behavior in Chapters 3 and 4. In Chapter 3, I examine how process S may overcome the peak pro-arousal signal from process C during rebound sleep. One possibility is that the signals could converge on an as-yet unidentified sleep/wake state integrator downstream of clock output and sleep homeostasis neurons. An alternative possibility could be through direct interactions between arousal-promoting clock output neurons and sleep promoting neurons. In Chapter 4, I examine whether an impaired sleep homeostasis phenotype results from an increase in the strength of the arousal-promoting clock output rather than a reduction in the sleep-promoting signal from process S.

1.3 *Drosophila* as a model organism for sleep research

How the brain senses sleep need has resisted mechanistic understanding because researchers have not been able to identify genes required for the process or structures in the brain where this process takes place. Historically, forward genetic screens in model organisms have been a powerful tool to identify genes necessary for processes that are still a black box [44]. For over a century, genetic studies in *Drosophila* have opened new fields by identifying the first genes involved in numerous biological processes [44-47]. As a model organism, flies are ideally suited for high-throughput forward genetic screens due to the powerful genetic tools and large stock collections that have been built up by the research community [48-54]. In addition, their short generation times and low

maintenance costs facilitate large scale screens, and their low genetic redundancy decreases the chance that related genes may compensate for a loss of function mutation, as often occurs in mammals. Their utility is further exemplified by genetic studies from the Benzer lab, which conducted screens for the first genes that influence behavior and established the field of behavioral genetics with their discoveries of the first circadian clock gene and the first genes that affect complex behaviors like courtship and memory [19, 55-58]. Comparative genetics is also a powerful tool for genetic discovery, and studies in *Drosophila* have been the starting point for genetic discoveries in other organisms. For example, the discovery of multiple core clock genes predicted the discovery of their homologues in mammals [31, 32].

Sleep in *Drosophila* is still a relatively new field. In fact, the first demonstrations that flies satisfy all of the behavioral hallmarks of sleep were published less than 20 years ago [6, 7]. Since then it has been established that, in addition to the conservation of many core molecular clock genes, the functions of arousal and sleep-promoting neurotransmitters as well as at least one gene that regulates sleep, are largely conserved between flies and mammals [1, 59-67].

Therefore, research using *Drosophila* is well-suited to advance our understanding of sleep homeostasis, just as research in *Drosophila* led to fundamental discoveries about the nature of the circadian clock over the last few decades.

1.4 Neural circuitry of sleep homeostasis in *Drosophila*

It has generally been thought that all forms of prior waking are sensed by sleep homeostasis and therefore contribute to increases in sleep need, but until recently this

assumption has been technically challenging to test. Previously, our lab directly activated distinct arousal systems in the brain to determine whether they contribute equally to sleep homeostasis [68]. To do this, the lab used the Gal4/UAS system [48] to express transgenic temperature sensitive TrpA1 cation channels [69] in different populations of neurons. TrpA1 channels remain closed at room temperature but open and allow cations to flow through and depolarize neurons when the temperature is mildly elevated until the temperature is lowered again. Thus, they can be used to thermogenetically test the roles of different neurons in behavior. Using this [70] approach, it was found that activation of cholinergic, dopaminergic or octopaminergic neurons wakes animals up when they would normally be asleep,. However, among these three groups, activation of only cholinergic neurons elicits subsequent recovery sleep following sleep deprivation. This result suggests that specific wake-promoting neural circuits, rather than waking itself, contributes to sleep homeostasis.

Next, the lab undertook a large thermogenetic screen of Gal4 drivers to identify more specific neurons that drive sleep homeostasis. The lab confirmed that most drivers that caused sleep deprivation did not elicit subsequent recovery sleep. As part of the screen, they tested drivers that expressed in different types of sensory neurons and found one, ppk-Gal4, that robustly caused thermogenetic sleep deprivation and subsequent recovery sleep. In combination with another Gal4 that had the same effects, they made a split-Gal4 [71] and found that this rare population of neurons originated in the periphery and made synaptic contacts with neuromeres in the thoracic ganglion and at the base of the central brain. The results of this study raised multiple questions, which I aim to examine in the research presented here. In Chapter 2, I aim to determine the location and

nature of these neurons. In Chapter 3, I examine what neural circuitry is downstream of these neurons and if they could be part of the sleep homeostat.

1.5 Rationale of the Thesis

The work presented in this thesis aims to further our understanding of the neuroanatomy and genetic regulation of sleep homeostasis. In chapter 2, building on previous work in our lab, I explore the nature of a rare population of sensory neurons whose activity has a privileged role in driving sleep homeostasis. And in Chapters 3 and 4 I describe the results of two complementary strategies to better understand how the brain regulates sleep need.

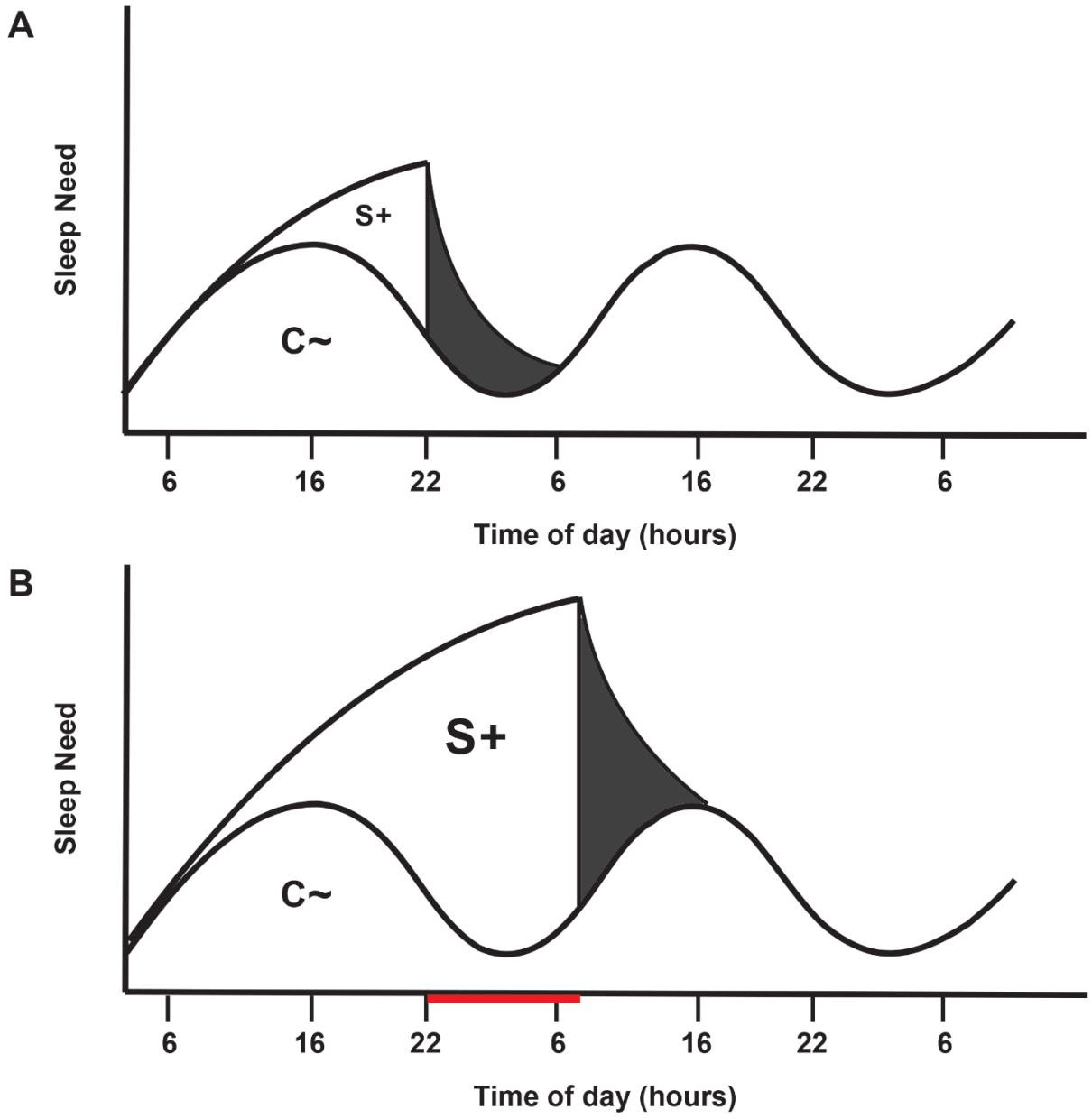


Figure 1.1 Diagram of the Two Process Model of sleep regulation. A) Process C represents an arousal promoting signal driven by the circadian clock that cycles with an ~24 hr period. The sleep promoting process S increases with prior waking time and dissipates with sleep. B) During sleep deprivation process S continues to accumulate sleep drive until sleep deprivation ceases. Process S discharges sleep pressure and suppresses waking while the arousal signal from process C is high. Red bar indicates period of sleep deprivation.

Chapter 2: Neural circuitry of sleep homeostasis originates outside of the brain

2.1 Introduction

Despite decades of study, the mechanisms by which the brain regulates sleep and sleep need remain a mystery. While the existence of a dedicated locus in the brain for sleep homeostasis remains a topic of debate, our mechanistic understanding would be greatly aided by identification of neural circuitry that influences sleep homeostasis. For example, if wake-promoting neurons that are coupled to sleep homeostasis are rare, then their identification might provide a starting point for determining additional relevant circuit components and ultimately a locus in which the cellular and molecular correlates of sleep need might be definable. Identification of rare, wake-promoting neurons that are coupled to sleep homeostasis could also offer insights into what type of information is conveyed to the brain that is relevant to sleep need.

With these ideas in mind, our lab previously conducted a large thermogenetic screen for Gal4>TrpA1 combinations that could elicit sleep deprivation and subsequent rebound sleep (SDR drivers). This screen identified a rare population of neurons that satisfy the criteria listed as potential inputs into a sleep homeostasis circuit [68]. Specifically, thermogenetic activation of these neurons causes acute sleep deprivation and subsequent recovery sleep when activation ends. These neurons are labeled by the ppk-Gal4 driver [72]. When the lab examined the distribution of these neurons in the central nervous system (CNS) they identified axonal projections that terminated in the ventral nerve cord (VNC) and in the subesophageal zone (SEZ) at the base of the brain but did not identify any cell bodies in the CNS. Based on these results as well as studies

in larvae, these neurons appear to be sensory neurons that originate in the peripheral nervous system (PNS). In the same study, they also identified a second Gal4, 24C10, which gives similar behavioral effects. The two drivers were used to generate a split-Gal4 [71] that recapitulated the sleep behavior of each parental Gal4>TrpA1 combination. The results of this study also suggested that sleep need could be dissociated from prior waking time and supported the hypothesis that dedicated neural circuitry exists in the brain to sense and discharge sleep need.

The peripheral neurons labeled by ppk-Gal4 are known as class IV dendritic arborization (da) neurons [72-74]. Ppk+ da neurons have been described in larvae, but little information is known about their localization and projection patterns in adults [75]. In larvae, class IV da neurons are positioned underneath the body wall in each hemisegment and extend complex dendritic arbors that extensively innervate each segment. They are believed to transduce different types of sensory information, including noxious chemical cues and mechanical stimuli [76-79]. The ppk gene encodes a subunit of a DEG/ENaC sodium channel that is thought to be mechanoreceptive [76]. In addition, in larvae the mechanosensing protein piezo shows overlapping expression with ppk [80]. Since it is likely that these neurons retain similar functions throughout development, we hypothesize that ppk neurons are mechanosensitive. It is interesting to note that Gal4s that express in other types of sensory neurons (auditory, gustatory, etc) were also tested in the screen that identified ppk, and activating neurons that convey these other sensory modalities did not have any effect on sleep homeostasis.

Two recent studies have identified additional Gal4s with similar capabilities to induce sleep homeostasis when activated thermogenetically [81, 82]. In each case the

study authors focused on using the driver as a thermogenetic tool for sleep deprivation and did not investigate the nature or expression of the identified neurons in detail. In both studies the authors found Gal4s which expressed in neurons in the central brain and did not examine expression elsewhere in the nervous system. In contrast, we know that the effects of ppk activity do not map to the central brain. Because neurons that can elicit sleep deprivation and subsequent rebound sleep (SDR drivers) are rare, we queried whether these drivers each represent distinct populations of neurons or if there is functional and anatomical overlap.

In this chapter I refine the identity of inputs into the sleep homeostasis circuit and locate where these neurons originate in the periphery. I establish where ppk cell bodies are located in adults and then use a combination of thermogenetic behavioral studies and imaging to map the neuroanatomical relationship between ppk and other SDR drivers that have been described.

2.2 Results

2.2.1 ppk sensory neurons originate in the periphery

Based on the well characterized expression pattern of class IV da neurons labeled by ppk-Gal4 in larvae, I hypothesized that the distribution of the ppk+ cell bodies along the body wall was likely conserved through development and could therefore be visualized through the cuticle to map their distribution in the body. To test this, we used fluorescence microscopy to image intact or freshly dissected flies expressing either UAS-CD8:GFP or a nuclear targeted RFP (UAS-Redstinger) [83]. As expected, the ppk cell bodies tile the body wall in the abdomen and, consistent with their identity as da sensory

neurons, send dense multidendritic arbors that fan out beneath each section of cuticle (Figure 2.1 A-B). In addition, I identified cell bodies in each leg, wing, the maxillary palp and labellum of the proboscis and in the antenna (Figure 2.1 C, and data not shown). We also expect there are cell bodies located under the cuticle in the thorax with a similar distribution to the abdomen, but the dense pigmentation of that body segment has made this difficult to confirm.

In order to colocalize the expression pattern of ppk with other sleep homeostasis Gal4s we cloned the ppk promoter from the Gal4 and generated a ppk-LexA transgenic line. We validated the ppk-LexA driver by determining that it can induce sleep deprivation when thermogenetically activated and elicit subsequent recovery sleep (data not shown). Next, I confirmed that ppk-LexA and ppk-Gal4 have overlapping expression in the CNS and periphery. To do this, I generated fly lines that combined ppk-LexA with dual UAS and LexAop fluorescent reporters. For co-labeling in the CNS I combined ppkLexA with UAS-sm:GFP:HA,LexAop-sm:GFP:V5 [84] (asterisk will denote LexA>LexAop throughout i.e. UAS-HA,ppk*>V5*) and for imaging in the periphery, I generated a UAS-RedStinger;ppk*>GFP* line to cross to the Gal4s. I crossed these lines to ppk-Gal4 and ppk∩24C10-Gal4 to confirm that the two ppk drivers have nearly identical expression patterns and that ppk∩24C10-Gal4 expresses in the expected subset of ppk neurons (Figure 2.1 D - E).

2.2.2 c584 neurons are a subset of ppk neurons

Next, we examined the relationship between ppk neurons and neurons labeled by c584-Gal4, a driver recently described for thermogenetic sleep deprivation [81]. We used

the Gal80 transcriptional repressor of Gal4 [85] under the control of the ppk promoter (i.e. ppk-Gal80) [68] and the VNC specific [86] tsh-Gal80 in combination with c584>TrpA1 to test if the behaviorally relevant c584 neurons express in those populations (Figure 2.2 A-C). We found that c584>TrpA1 could indeed induce sleep deprivation and recovery sleep, and that this effect was completely blocked by ppk-Gal80. This suggests that the behaviorally relevant c584 neurons overlap with ppk neurons. The inclusion of tsh-Gal80 did not block c584>TrpA1 from driving sleep deprivation and rebound, indicating that, the relevant neurons do not map to the VNC. As a control, we repeated these Gal80 experiments in combination with ppk-Gal4>TrpA1. We found that, as expected, combining ppk-Gal80 with ppk>TrpA1 completely abolished thermogenetic sleep deprivation and rebound (Figure 2.2 B), which confirms that the ppk-Gal80 recapitulates the expression pattern of ppk-Gal4. Next, we tested ppk>TrpA1 in combination with tsh-Gal80 and as expected, this did not block thermogenetic sleep deprivation and rebound (Figure 2.2 B). This finding indicates that the relevant neurons do not map to the VNC. These results suggest that while c584 expresses in multiple populations of neurons in the brain and the VNC, the behaviorally relevant neurons map to ppk cells.

2.2.3 52B10 neurons are behaviorally similar but anatomically distinct from ppk

Next, we examined the relationship between ppk neurons and those labeled by 52B10-Gal4, which was identified in a thermogenetic screen for drivers that could elicit sleep deprivation and subsequent rebound sleep [82]. In that study, the authors did not examine the expression pattern in the CNS in detail. The authors state, however, that they selected Gal4s for their screen with relatively sparse expression patterns in the brain

and limited or no expression in the VNC. To determine where the behaviorally relevant neurons are located, and to test if ppk neurons overlap with 52B10 neurons, we thermogenetically activated 52B10>TrpA1 either alone or in combination with ppk-Gal80 or tsh-Gal80 (Figure 2.3 A - B). We found that ppk-Gal80 did not block rebound sleep following thermogenetic activation of 52B10 neurons, but tsh-Gal80 did. These results suggest that the relevant neurons actually map to the VNC rather than the central brain or the periphery.

Since 52B10-Gal4 labels a population of SDR neurons that are distinct from ppk, we performed additional behavior experiments to determine their neurotransmitter identity. We had previously shown that ppk neurons are cholinergic [68], so we used Cha-Gal80 to block TrpA1 expression in both ppk>TrpA1 and 52B10>TrpA1 animals and found that SDR is blocked in both cases. Additionally, we found that SDR could be blocked by expressing transgenic tetanus toxin (TNT) in ppk and 52B10 neurons (Figure 2.3 C – D). We interpret these results to mean that inputs into the sleep homeostat require synaptic transmission to communicate information that is relevant to sleep need. These results also suggest that while some of the Gal4s express in different populations of behaviorally relevant neurons, these neurons have similar properties.

Next, to examine where 52B10-Gal4 expresses in relation to ppk neurons, I crossed 52B10 to UAS-HA,ppk*>V5* to label both populations of neurons and examined whole mounts of dissected brains by confocal microscopy (Figure 2.3 E-G). I found that the two drivers do not co-label any neurons in the brain or the VNC. In the VNC, 52B10 labels ~6-12 cells per thoracic neuromere [87]. These cells send processes throughout their respective neuromeres and appear to send projections up the midline towards the

brain. In the brain these processes appear to terminate in the SEZ in proximity to, but do not overlap with ppk axon terminals. These results suggest that both populations could converge on the same or a similar downstream target in the brain. Further study will be needed to determine how these neurons relate to each other anatomically.

2.2.4 ppk neurons overlap functionally and anatomically with 69F08 neurons

The study that identified 52B10-Gal4 also described a driver, 69F08-Gal4, which they concluded could elicit sleep homeostasis without prior sleep deprivation. They state that 69F08 must therefore express in the sleep homeostat itself and that this effect mapped to the Ring 2 (R2) neurons of the ellipsoid body (EB) [82]. In contrast, we observed sleep deprivation prior to rebound sleep and no expression in the EB using 69F08 (data not shown), so we examined the relationship between 69F08 and ppk neurons. We found that ppk-Gal80 significantly reduced, but did not eliminate the SDR capability of 69F08>TrpA1 (Figure 2.4 A - B). This result suggests that some of the functionally relevant 69F08 neurons are in fact ppk neurons, whereas other functionally relevant 69F08 neurons are probably distinct. That is, 69F08 neurons may represent a superset of ppk neurons that label additional SDR neurons. To determine if this is the case, we tested the ability of 69F08-driven synaptic blockade to block rebound driven by ppk*>TrpA1*. In this experiment we used ppk*>TrpA1* to drive SDR while inhibiting synaptic transmission from 69F08 neurons using temperature sensitive shi^{ts} [88]. If 69F08 neurons completely overlap with ppk neurons, we expected that 69F08>Shi^{ts} would completely block SDR. We found that SDR was significantly reduced but not completely blocked in this experiment (Figure 2.4 C - D). The results of these two behavior

experiments suggest that 69F08 and ppk partially overlap and also express in SDR neurons that are distinct to each driver (69F08+,ppk- and 69F08-,ppk+ subsets).

Next, I examined the anatomical relationship between ppk and 69F08 neurons. I crossed 69F08-Gal4 to UAS-HA,ppk*>V5* to label both populations of neurons and examined whole mounts of dissected brains by confocal microscopy (Figure 2.4 E - G). I found the expression in the CNS between ppk and 69F08 appears consistent with the behavior results – the drivers co-express in ~50% of ppk processes in both the SEZ and the VNC. The expression that is specific to 69F08 appears to consist of some processes which are likely projecting from the periphery and a small number of cells in the SEZ and VNC. It will be interesting to determine if any of the CNS cells labeled by 69F08 are involved in SDR or if the behavior maps exclusively to peripheral neurons. Imaging in the periphery also suggests that 69F08 expresses more broadly than ppk-Gal4 and co-expresses in a subset of ppk neurons in the legs and abdomen (data not shown). Taken together, these results suggest that 69F08-Gal4 expresses more broadly than was detected in the original study, and its ability to induce sleep homeostasis primarily maps to ppk neurons and a smaller population of 69F08-specific neurons.

2.3 Discussion

In this chapter I examined the anatomical distribution of ppk+ cell bodies in the periphery of the adult nervous system. Similar to what is known in larva, the morphology and distribution are consistent with mechanosensory/nociceptive functions, although I did not test this directly. Futures studies will be needed to directly test the stimuli that elicit responses from these neurons. It is also unclear what the baseline activity of these cells

is and how they might contribute to sleep homeostasis signaling when they are not being acutely activated thermogenetically. Because these cells are generally located near the surface it may be possible to use fluorescent Ca^{2+} reporters or electrophysiological recordings to measure neuronal activity at baseline and in response to stimuli.

Another interesting question is the extent to which each ppk neuron innervates the CNS. Axonal projections from ppk cells in the body clearly synapse on thoracic neuromere and send projections up the midline to the brain, but these processes are too densely clustered to map them back to individual points of origin. Future studies could use MARCM to label individual ppk neurons to determine where the processes project in the CNS [73, 75, 85]. This information could further clarify how sensory information is conveyed from the peripheral ppk neurons to the brain to drive sleep homeostasis.

We have determined that ppk neurons are cholinergic and that synaptic transmission from them is required to elicit SDR. If ppk neurons make direct synaptic contact with sleep promoting sleep homeostasis neurons, then they must have an inhibitory effect on their post-synaptic partners in order to deprive animals of sleep. Alternatively, they could be the beginning of a polysynaptic circuit that inhibits sleep homeostasis neurons. This question is explored in Chapter 3.

The results of multiple thermogenetic screens by different groups supports the conclusion that neural circuitry that can elicit sleep deprivation and subsequent rebound sleep is rare [68, 81, 82]. This study addressed an open question in the field by examining in detail the nature of multiple SDR Gal4s in relation to ppk neurons using a combination of behavioral genetics and neuroanatomical imaging. In the reports that described these drivers the study authors claim that the SDR effects map to cells in the central brain, but

our data indicate that this is not the case. Instead, the behavior of 52B10 maps to the VNC and the behavior of the other drivers can be explained completely or at least partly by overlapping expression with ppk neurons. The contribution of PNS sensory neurons to sleep homeostasis further suggests that sleep need is influenced by environmental information. In the future researchers may need to be careful to consider and characterize the full extent of nervous system expression of Gal4s that are used in sleep or other behavior experiments.

In future experiments it will be interesting to further explore the relationship between 52B10 neurons in the VNC and ppk neurons since this driver labels the only SDR neurons studied that don't map to the periphery. This study did not address the possibility that these neurons could, at least partially, be in series. Future circuit epistasis experiments that hyperpolarize or otherwise silence 52B10 neurons while activating ppk could address this possibility. Additionally, if 52B10 neurons are distinct from a ppk circuit, then they could represent separate inputs into the sleep homeostat and mapping postsynaptic partners could help to triangulate onto the sleep homeostat. I observed that both ppk axon projections and 52B10 projections from the VNC appear to converge on a similar location at the base of the brain. In future experiments it may be possible to use techniques such as transsynaptic tracing [89] to determine if both populations of neurons share a postsynaptic target.

It will also be interesting to determine the identity of the 69F08+,ppk- cells that can also elicit SDR in order to more fully map and characterize the populations of neurons that elicit sleep homeostasis. It is possible that these cells are additional peripheral sensory neurons that may have a similar function to ppk neurons. Although 69F08 also

expresses in the VNC, preliminary data suggests that blocking VNC expression with tsh-Gal80 does not reduce 69F08 driven SDR. Future experiments will aim to address if 69F08 possibly co-expresses with 52B10 neurons in the VNC or if the behaviorally relevant, non-ppk, 69F08 neurons represent an as-yet unidentified additional population of SDR neurons. 69F08 was originally described as a driver that expresses in sleep homeostasis neurons in Ring 2 of the ellipsoid body – we believe that this behavior can at least partly be explained by 69F08's overlap with ppk. In addition, my efforts to image 69F08 in the brain found that the driver expresses at best only weakly in the EB.

2.4 Materials and Methods

Fly Stocks

All stocks were raised at room temperature (20-22°C) on cornmeal media. The following stocks were acquired from the Bloomington Drosophila Stock Center: 69F08 [34999], 52B10[38820], c584 [30842], UAS-TrpA1[26264], UAS-Redstinger [8546], UAS-Shi^{ts} [44222], UAS-TNT [28837], UAS-myr::smGdF-HA,LexAop-myr::smGdF-V5 [64092]. Ppk-Gal80 was generated as previously described [68]. cha-Gal80 was a gift from Toshihiro Kitamoto. tsh-Gal80 was a gift from Julie Simpson.

Transgenic fly lines were targeted to attP sites using PhiC31 integration (Rainbow Transgenics). ppk-LexA was cloned into pBP-LexA-p65UW and inserted into attP40 and attP2. All transgenic lines were outcrossed at least 2 generations into an isogenic background.

Behavioral assays

Female flies were used for all behavioral assays. Animals were entrained 2 days under 12 hr : 12 hr light:dark conditions at 22°C. Individual 1-4 day old flies were loaded into 5 mm x 65 mm Pyrex tubes containing 5% agarose with 2% sucrose at one end as a food source. Sleep and waking behavior were monitored using the Drosophila Activity Monitoring System (DAMS; Trikinetics).

For most thermogenetic sleep deprivation experiments animals were maintained at a baseline temperature of 22°C for 1 day, and sleep deprivation was achieved by elevating the temperature to 27.5°C from ZT 22-24 at the end of the second night. The temperature was then returned to 22°C and flies were monitored for 2 more days.

For experiments involving synaptic blockade with Shi^{ts} the baseline conditions were the same as described above but the temperature was elevated to 31°C from ZT 18 – 24 in order to achieve efficient inhibition of neurotransmission.

Sleep deprivation was calculated as the total amount of sleep during the deprivation period minus the amount from the same period during the baseline day. Rebound sleep was calculated in the same way based on sleep totals from ZT 0-6. Sleep analysis was calculated as previously described [6] using custom MATLAB (Mathworks) software.

Immunohistochemistry and confocal microscopy

For all experiments, female flies aged 3 – 7 days were dissected in cold PBS, brains were fixed for 30 mins in 4% paraformaldehyde, and blocked in PBST (PBS with 0.3% Triton X-100) with 5% normal donkey serum (Jackson Laboratory) prior to staining. Brains were incubated with 1:1000 rabbit anti-V5 (Thermo Fisher), 1:100 rat anti-HA

(Roche), and 1:100 mouse anti-nc82 (Developmental Studies Hybridoma Bank). Brains were incubated at 4°C with rocking overnight and then washed with PBST 4 times x 10 min. After washes, brains were incubated with 1:1000 Alexa 488 anti-rabbit (Life Technologies), 1:1000 Alexa 555 anti-rat (Invitrogen), and 3:1000 Alexa 647 anti-mouse (Jackson Laboratory) overnight at 4°C. Brains were washed again after secondary incubation 4 x 10 min. Brains were mounted in Vectashield (Vector Labs) and image stacks were taken on a Leica SP5 confocal microscope with 40x magnification using 1 um step intervals. Maximum intensity Z-projection images were generated, and any brightness/contrast adjustments were made, using Fiji [90].

Fluorescence microscopy

Animals were dissected in cold PBS and either pinned on SYLGARD (Dow Corning) coated dissection dishes or mounted in Vectashield (Vector Labs) for imaging PNS neurons. Native fluorescence of GFP and RFP were visualized and imaged with a Zeiss Axio Zoom.V16 equipped with a Zeiss Axio Cam 506 camera.

Statistical Analysis

Bar graphs depict mean ± SEM. Unpaired Student's t-test was used for comparisons between 2 groups. One-way ANOVA with Tukey's post hoc comparisons was used for comparisons between multiple groups. All statistical tests were two-sided and performed using Prism 8.0 for Windows 10 (GraphPad Software).

2.5 Acknowledgements

Work presented in Chapter 2 with some modifications and additions will be submitted for publication with coauthors Joydeep De, Meilin Wu, Erika Barrall, and William Joiner. Joydeep De performed additional peripheral and confocal imaging not included in this chapter. Meilin Wu performed cloning to generate new transgenic animals for this study. Erika Barrall performed brain dissections for IHC experiments. The dissertation author performed all IHC and imaging included in this chapter as well as a behavioral screen and some additional behavior with assistance from corresponding author William Joiner who performed or assisted with many behavior experiments.

I would also like to thank Vanessa Lambatan for additional assistance with brain dissections and Richard Daneman for use of microscopy equipment.

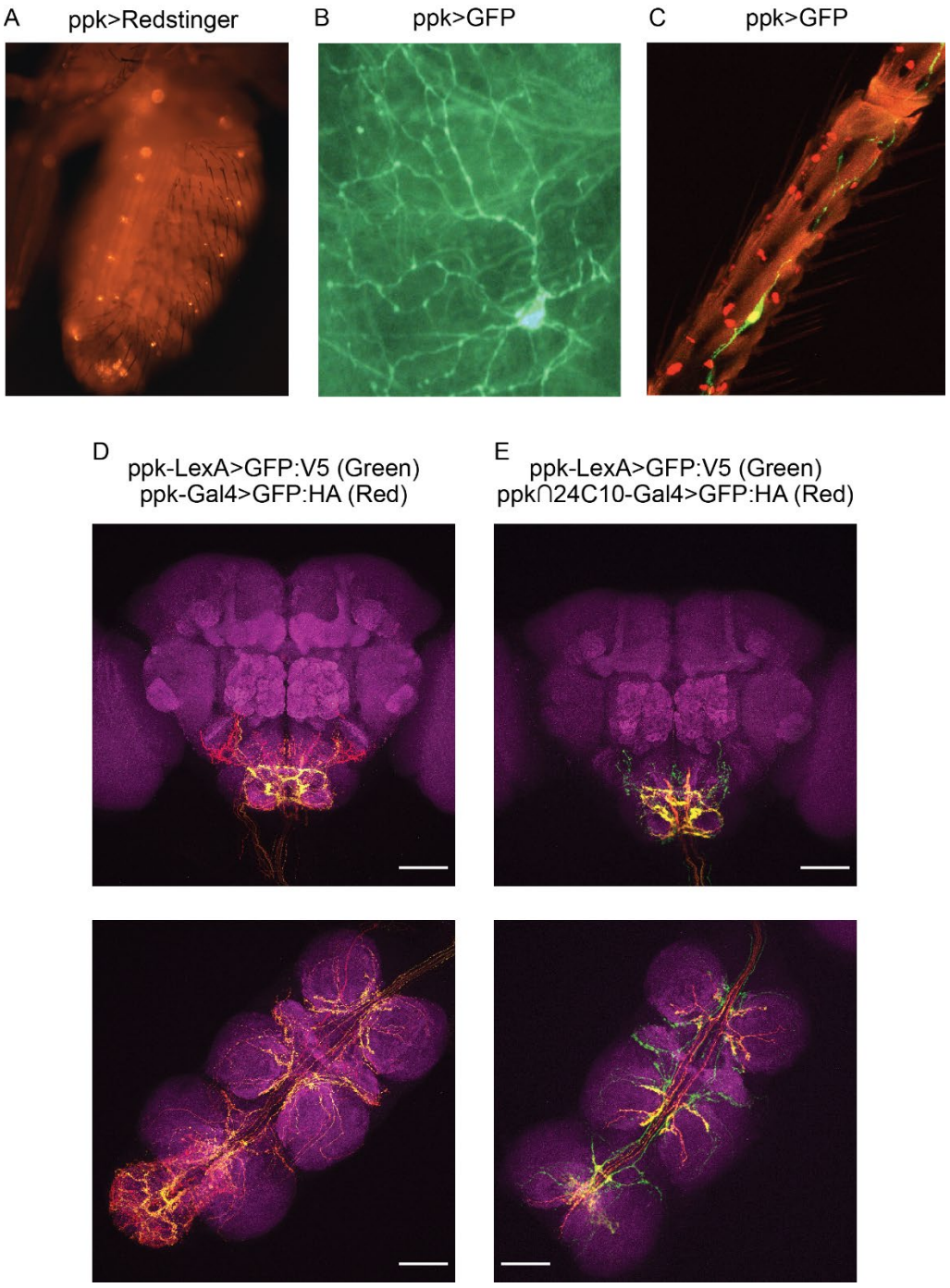


Figure 2.1 ppk sensory neurons originate in the periphery. A) ppk cell bodies are located underneath the cuticle in the abdomen. B) Close up of one cell in ventral abdomen shows dendritic arbors of class IV da neuron tiling the body wall. C) ppk neuron sends processes up and down the leg. D-E) Expression patterns of ppk-LexA>V5 co-labeled with ppk-Gal4>HA (D) and ppk∩24C10>HA (E). Top and bottom panels are brain and VNC, respectively. In CNS images ppk-LexA is labeled with anti-V5 (green); ppk-Gal4 is labeled with anti-HA (red) in (D) and ppk∩24C10 is labeled with anti-HA (red) in (E). Neuropil stained with anti-nc82 is shown in magenta.

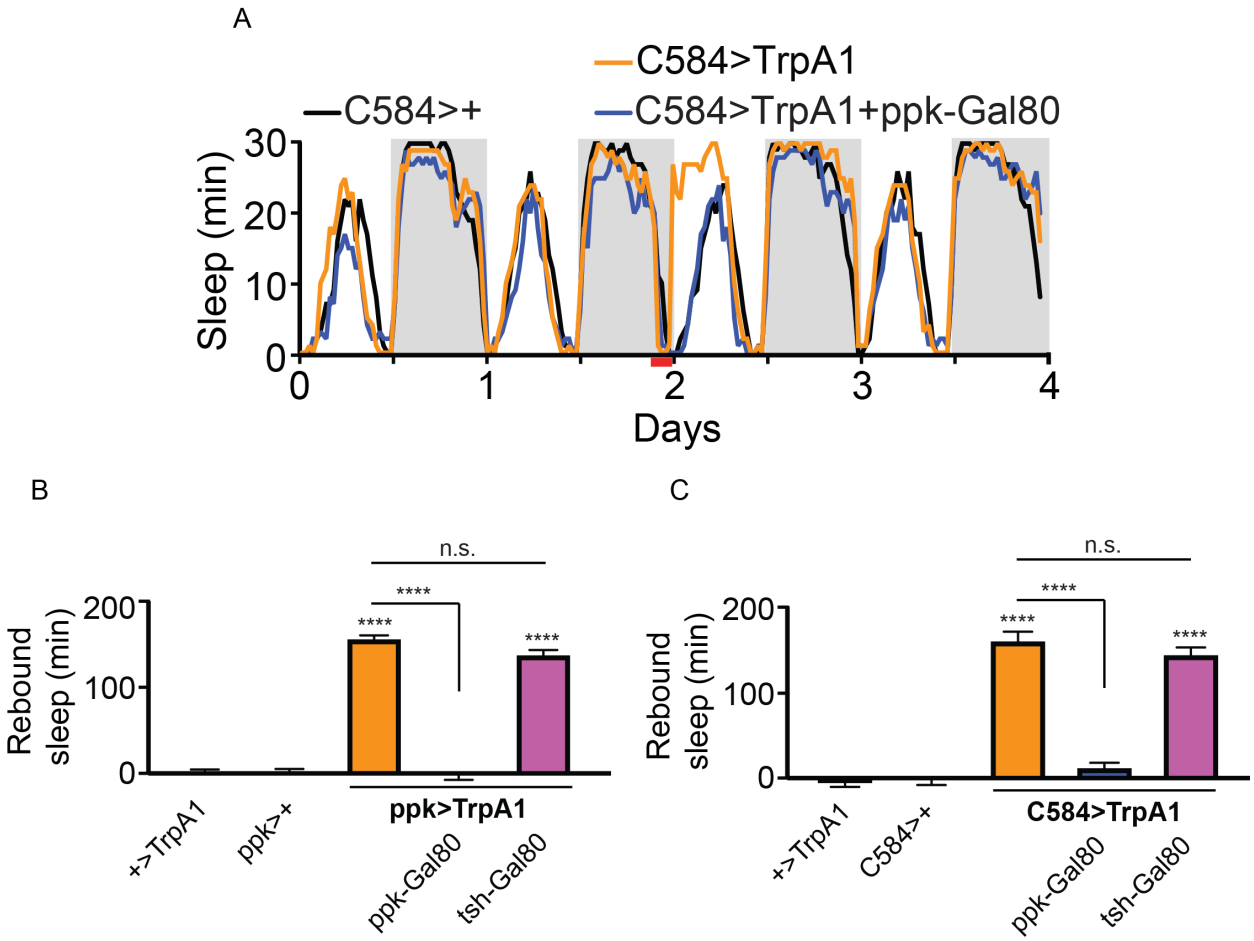


Figure 2.2 c584 neurons are a subset of ppk neurons. A) Sleep profiles of c584>TrpA1 and c584>TrpA1 + ppk-Gal80 and genetic control. In this and subsequent thermogenetic experiments, animals were maintained at 22°C for one baseline day, animals were pulsed for 2 hr at 27.5°C (red bar) until the end of the night and then returned to 22°C for two additional days of recording. B-C) ppk-Gal80 blocks rebound sleep in ppk>TrpA1 and c584>TrpA1 animals. Quantification of recovery sleep for ppk>TrpA1 + ppk-Gal80 and tsh-Gal80 (B) and c584>TrpA1 with same Gal80s (C). n ≥ 30 per group. In this and subsequent figures, *, **, ***, **** indicate $p < 0.05$, 0.01, 0.001 and 0.0001, respectively. Error bars indicate SEM.

Figure 2.3 52B10 neurons are behaviorally similar but anatomically distinct from ppk. Behaviorally relevant 52B10 neurons map to the VNC. A) Sleep profiles of 52B10>TrpA1 and 52B10>TrpA1 + ppk-Gal80 and genetic control. B) Quantification of rebound sleep in (A) with 52B10>TrpA1 + tsh-Gal80. C-D) ppk and 52B10 neurons are cholinergic and require synaptic transmission to elicit rebound sleep. Cha-Gal80 blocks rebound sleep in ppk>TrpA1 (C) and 52B10>TrpA1 (D). n ≥ 30 per group. E-G) Co-labeling of ppk and 52B10 neurons in the CNS shows non-overlapping expression patterns. Ppk neurons are labeled with anti-V5 (green); 52B10 neurons are labeled with anti-HA (red). Neuropil stained with anti-nc82 is shown in magenta. Top and bottom panels are brain and VNC, respectively.

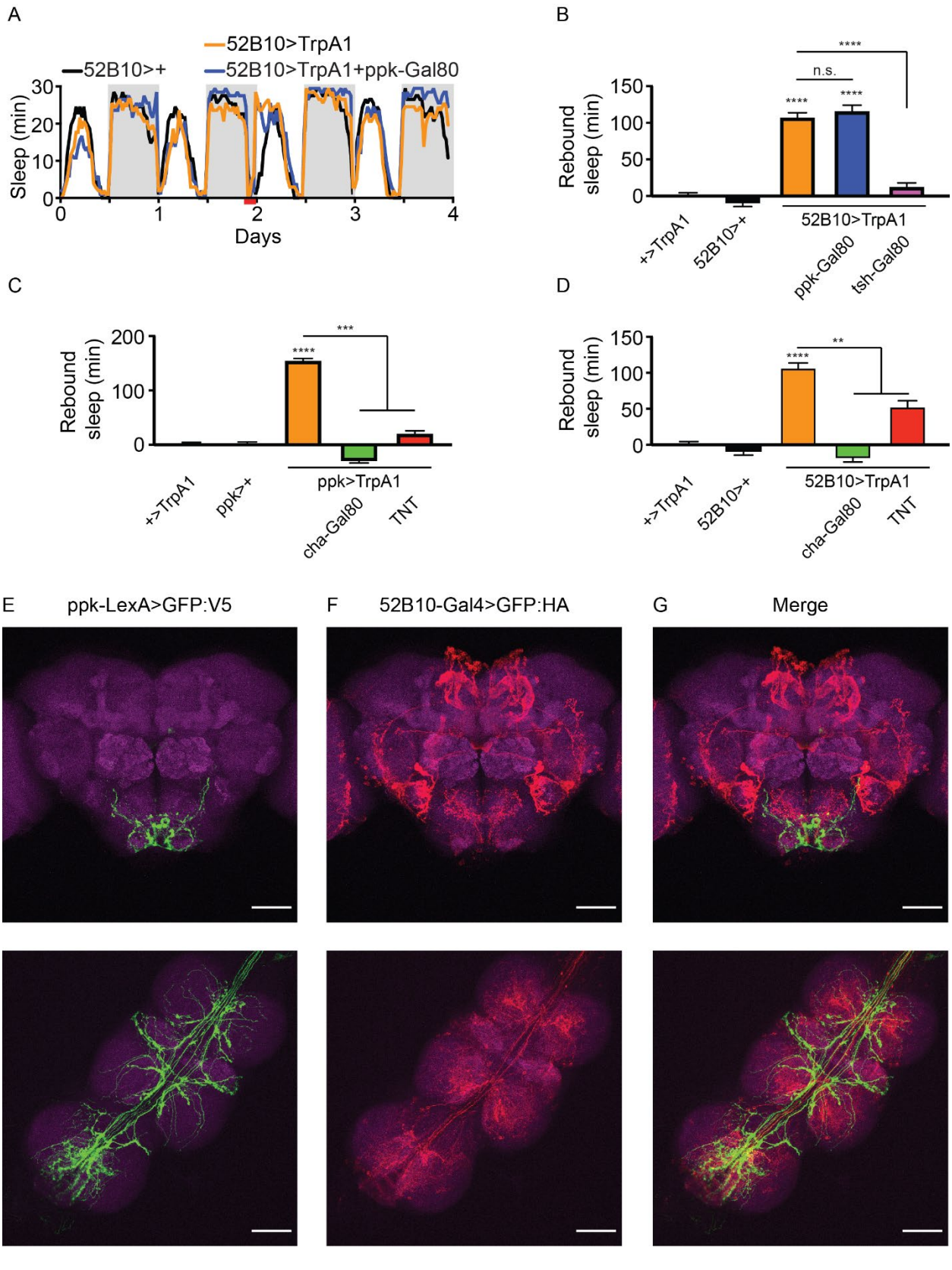
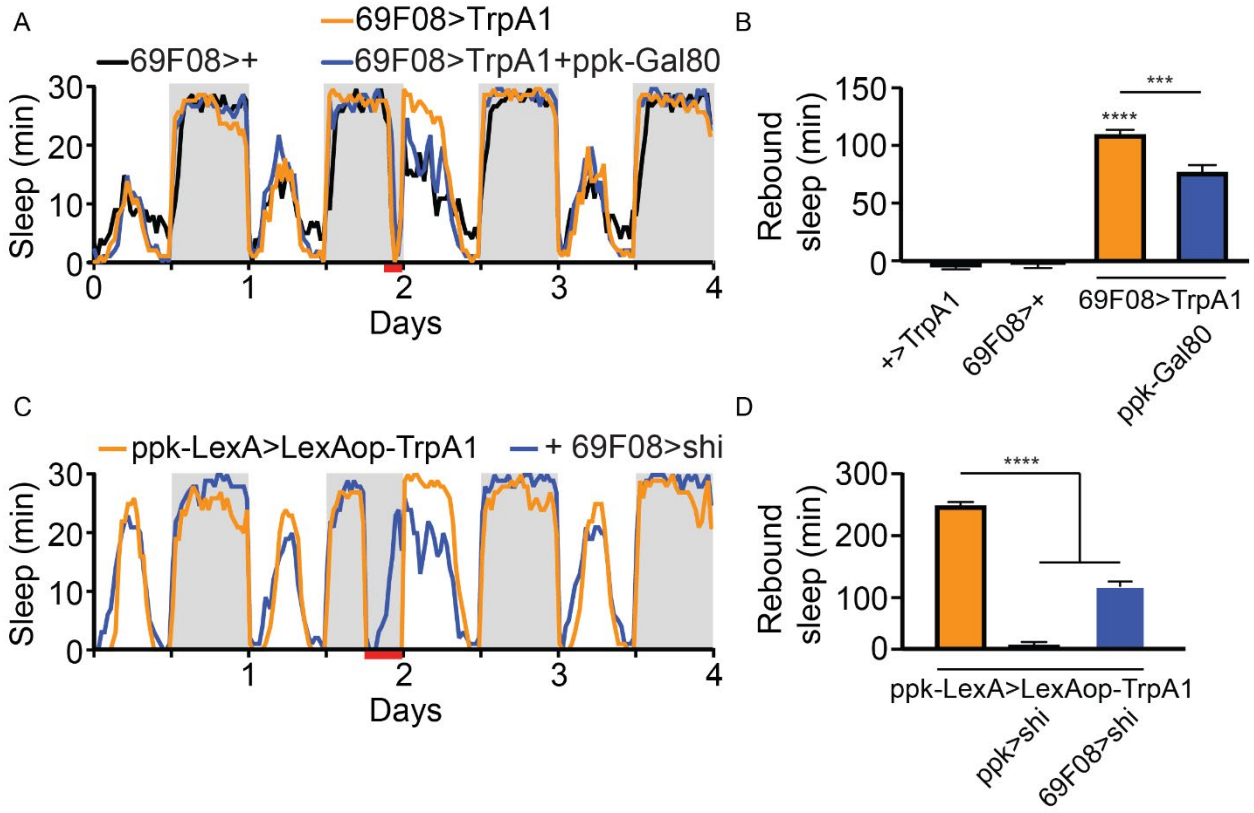
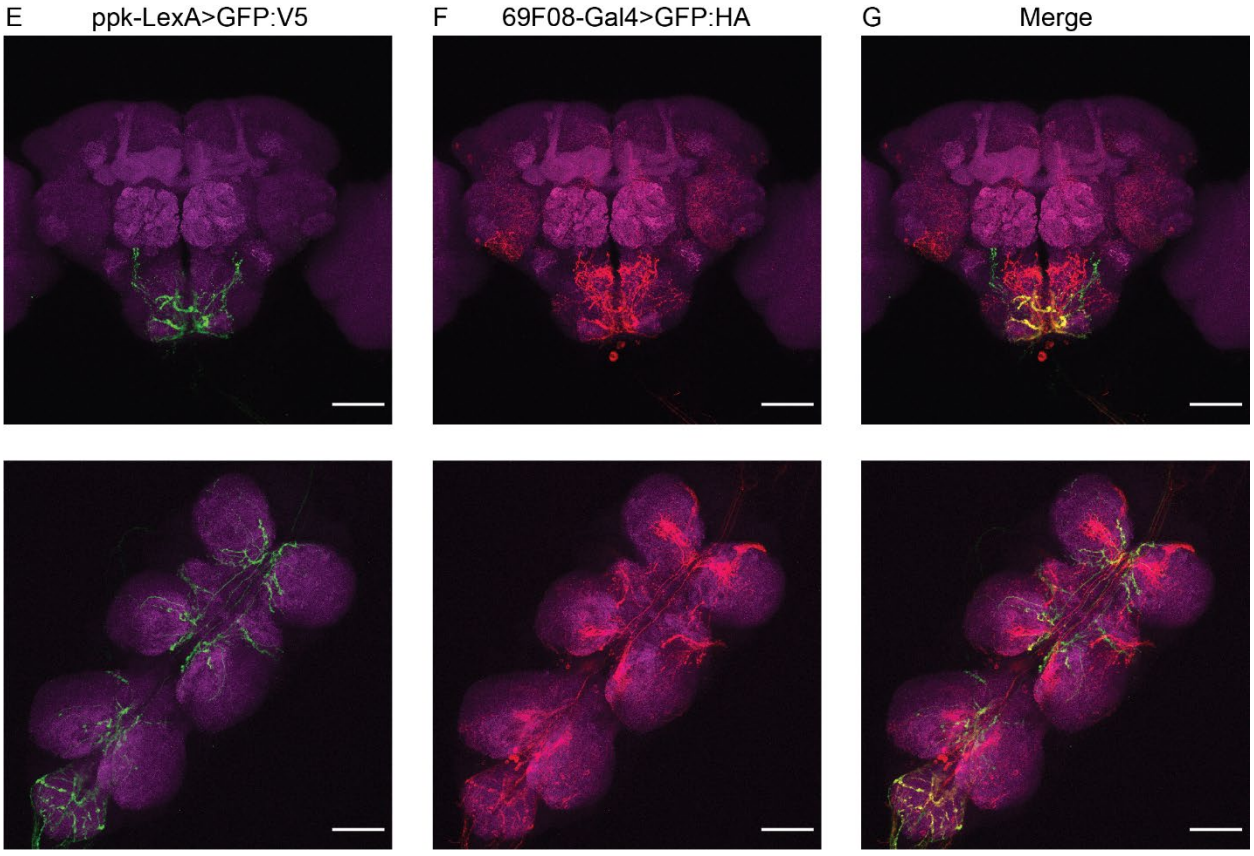

A
52B10>TrpA1
52B10>+
52B10>TrpA1+ppk-Gal80
Sleep (min)
Days
B
Rebound sleep (min)
n.s.
****
+>TrpA1
52B10>+
52B10>TrpA1
ppk-Gal80
tsh-Gal80
C
Rebound sleep (min)
***
****
+>TrpA1
ppk>+
ppk>TrpA1
cha-Gal80
TNT
D
Rebound sleep (min)
**
****
+>TrpA1
52B10>+
52B10>TrpA1
cha-Gal80
TNT
E
ppk-LexA>GFP:V5
F
52B10-Gal4>GFP:HA
G
Merge

Figure 2.4 ppk neurons overlap functionally and anatomically with 69F08 neurons. A-B) ppk-Gal80 reduces rebound sleep in 69F08>TrpA1 animals. A) Sleep profiles of 69F08>TrpA1 and 69F08>TrpA1 + ppk-Gal80 and genetic control. B) Quantification of rebound sleep in (A) with additional control. n ≥ 30 per group. C-D) Blocking synaptic transmission in 69F08 neurons impairs the ability of ppk neurons to elicit rebound sleep. C) Sleep profiles of ppk*>TrpA1* and ppk*>TrpA1* + 69F08>Shi^{ts} D) Quantification of rebound sleep in (C) with additional control. n ≥ 30 per group. E-G) Co-labeling of ppk and 69F08 neurons in the CNS shows overlapping expression patterns. Ppk neurons are labeled with anti-V5 (green); 69F08 neurons are labeled with anti-HA (red). Neuropil stained with anti-nc82 is shown in magenta. Top and bottom panels are brain and VNC, respectively.


A
69F08>TrpA1
69F08>+
69F08>TrpA1+ppk-Gal80
Sleep (min)
Days
B
Rebound sleep (min)
***
****
+>TrpA1
69F08>+
69F08>TrpA1
ppk-Gal80
C
ppk-LexA>LexAop-TrpA1
+ 69F08>shi
Sleep (min)
Days
D
Rebound sleep (min)
****
ppk-LexA>LexAop-TrpA1
ppk>shi
69F08>shi


E
ppk-LexA>GFP:V5
F
69F08-Gal4>GFP:HA
G
Merge

Chapter 3: Identification of a sleep homeostasis circuit driven by peripheral sensory inputs to the brain

3.1 Introduction

In the two decades since *Drosophila* was established as a model for sleep research, progress identifying molecules and neural circuitry involved in sleep homeostasis has been slow relative to advances in our understanding of the circadian clock and mechanisms that regulate baseline arousal [91, 92]. Although genes such as *CREB* and *Hsp83* have been reported to affect sleep homeostasis [43, 93, 94], these interpretations have often been complicated by additional effects on baseline sleep. For example, if a mutation causes animals to lose 50% of their sleep, then these animals can't be further sleep-deprived to the same extent as controls over the same time period. Therefore, rebound sleep is not directly comparable in the two groups under equivalent conditions (though such comparisons still appear in the literature). Furthermore, until recently, we have not had an anatomical context to investigate the function(s) of genes purported to affect sleep homeostasis. However, recent technical advances, such as the development of thermogenetics and the generation of a large collection of neuronally expressed Gal4 drivers [54, 70], have begun to accelerate the rate of discovery of molecules and neural circuitry involved in sleep homeostasis. For example, recent reports have implicated a population of mushroom body output neurons (MBONs), the ring 2 neurons of the ellipsoid body (EB-R2), and the dorsal fan-shaped body (dFB) in regulating sleep homeostasis [82, 95-101]. While these hypotheses are consistent with previous evidence that established roles for the mushroom bodies (MB) and dFB in promoting

sleep [96, 102-104], it is unclear how these structures fit into an overall sleep homeostasis circuit. Furthermore, consensus is lacking as to whether blocking activity of any of these neurons truly reduces rebound sleep, as would be predicted of a major locus for the sleep homeostat.

My lab has taken a different approach to identifying neural circuitry involved in sleep homeostasis. In previous work [68], it screened for and identified ppk neurons as having the rare ability to cause acute sleep deprivation when activated and subsequent rebound sleep when released from activation. Based on the rarity and strength of their phenotype, it was proposed that these neurons might lie upstream of the sleep homeostat, perhaps as close as a single synapse away. This concept led us to devise a neural circuit model that makes predictions about the nature of the homeostat which we then set about to test by screening for neurons that satisfied these predictions.

According to this model, ppk neurons inhibit sleep promoting neurons comprising the homeostat (Figure 3.1 A). Over time, however, prolonged inhibition of these neurons is predicted to cause gradual compensatory (i.e. homeostatic) upregulation of their activity. Finally, when ppk-driven inhibition is acutely withdrawn, the upregulated sleep homeostat is predicted to discharge, causing the animal to engage in rebound sleep (Figure 3.1 B - C). Testable predictions of this model include the following:

1) Sleep homeostasis neurons should be acutely sleep promoting when activated.

2) Inhibition of sleep homeostasis neurons should reduce rebound sleep after sleep deprivation.

3) Anatomically, sleep homeostasis neurons should be located near axon terminals of ppk neurons and make direct synaptic contact.

We conducted two behavior screens based on the predictions from our model. In the first we used a circuit epistasis design to screen for neurons which, when hyperpolarized, could block homeostatic recovery sleep following thermogenetic sleep deprivation. To do this we combined the ppk-LexA>LexAop-TrpA1 line with a transgenic hyperpolarizing potassium channel, UAS-EKO [105]. We crossed ~400 Gal4s that express in the nervous system [54] to UAS-EKO;ppk-LexA,LexAop-TrpA1 and tested progeny for reduced rebound following thermogenetic sleep deprivation. In the second screen we crossed Gal4s to UAS-TrpA1 and activated neurons during the day to test for acute sleep promoting effects. Each screen yielded one hit upon retesting candidate lines.

In this chapter I describe the physical relation between neurons labeled by these two drivers and how these neurons satisfy predictions of our neural circuit model for sleep homeostasis. Further, I report evidence that suggests this sleep homeostasis circuitry could be part of the output of the circadian clock, which would mechanistically link how processes S and C interact to determine the arousal state of the fly.

3.2 Results

3.2.1 42D11 and 53D10 satisfy behavioral criteria for sleep homeostasis neurons

We tested the candidate Gal4 lines to see if they satisfied all of the behavioral predictions for sleep homeostasis neurons from the circuit model. First, we tested the two candidate drivers, named 42D11 and 53D10, to see if they labeled neurons that acutely promote sleep when activated. We thermogenetically stimulated 42D11 and 53D10

neurons from ZT 0 – 6 when sleep is low, and we observed increases in sleep in both cases (Figure 3.2 A – D).

Next, we tested the second behavioral prediction that inhibition of sleep homeostasis neurons should reduce rebound sleep. We performed this experiment by blocking synaptic output from the candidate sleep homeostasis neurons during mechanical sleep deprivation and throughout the subsequent rebound period. To accomplish this, we coupled expression of the dominant-negative temperature sensitive dynamin mutant Shi^{ts} [88] to 42D11-Gal4 and elevated the temperature to 30°C from ZT 20 – 6 while mechanically sleep depriving the flies from ZT 20 – 0. We found that inhibition of synaptic transmission from 42D11 neurons significantly reduced rebound sleep (Figure 3.3 A - C). Taken together, these data are consistent with the behavioral effects predicted by the neural circuit model.

3.2.2 42D11 and 53D10 neurons satisfy neuroanatomical criteria for sleep homeostasis neurons

Based on the same model we hypothesized that sleep homeostasis neurons are located near ppk axon terminals in the subesophageal zone (SEZ). To determine where 42D11 and 53D10 express and to examine if they potentially share overlapping expression in any brain regions, I crossed both drivers to UAS-myr-GFP and examined whole mounts of dissected brains by confocal microscopy (Figure 3.4 A – B). 42D11 expresses more broadly than 53D10, including in the EB, which has been reported to undergo sleep need-dependent changes in activity that are both necessary and sufficient for generating sleep drive [82]. In contrast, 53D10 does not express in any central

complex neuropil. Notably, both drivers appear to share overlapping expression in a population of neurons between the antennal lobes and the SEZ known as the antennal mechanosensory and motor center (AMMC), as well as some sparse expression in the dorsal and lateral brain. The shared behavioral features of these two drivers as well as the apparent overlapping expression in the AMMC region suggested that those neurons might be the sleep-promoting neurons we are looking for. To test this hypothesis, we cloned the enhancer fragments from 42D11 and 53D10 and coupled them to the activation domain (AD) and DNA binding domain (DBD) of split Gal4 [71]. I imaged the expression of the 42D11∩53D10 split-Gal4 by confocal microscopy and found that, indeed, there is overlapping expression in a subset of the AMMC neurons labeled by each of the original drivers (Figure 3.4 C). While we haven't been able to visualize dendrites with 42D11∩53D10-Gal4, the AMMC neurons labeled by 53D10 and 42D11 both appear to send dendrites towards the SEZ, where ppk axons terminate. We also tested to see if 42D11∩53D10 recapitulates the sleep promoting properties of its parental drivers by crossing it to TrpA1 and activating the neurons from ZT 0 – 6. We found that it is sleep promoting, but the effect is weak (data not shown). This could be because the split Gal4 driver only expresses in a subset of behaviorally relevant neurons that are shared by both parental drivers, or it could be because split Gal4 is often weaker than Gal4 alone. Taken together, however, the sleep-promoting neurons we have identified satisfy the behavioral criteria we proposed for a discrete sleep homeostat.

The AMMC neurons we identified are also located in a part of the brain where they could potentially form synapses with ppk neurons. To further test for this possibility we used a recently developed genetic tool for transsynaptic tracing called trans-Tango [89]

to label the population of neurons that are postsynaptic to SDR neurons covered by ppk∩24C10-Gal4. Confocal imaging of brains from ppk∩24C10>Tango flies revealed a population of postsynaptic neurons that cluster in the AMMC and send processes into the SEZ (Figure 3.4 D). The similar location of a subset of cell bodies labeled by Tango and the 42D11 and 53D10 drivers suggests to us that all 3 groups are likely to overlap in the AMMC and that these neurons are likely to be postsynaptic effectors of ppk activity. However, our interpretation is limited by the fact that all of this data is correlative. In the next experiment we took a more direct approach to establish an anatomical connection between ppk neurons and postsynaptic partners that might be part of the homeostat.

We used GFP reconstitution across synaptic partners (GRASP) [106] between ppk-LexA and 42D11-Gal4 to test for direct cell to cell contact between ppk axon terminals and 42D11 processes (Figure 3.4 E). Confocal microscopy of GRASP brains revealed a specific punctate signal, suggestive of synapses, in the SEZ where we see processes from both populations of neurons. These results demonstrate a direct connection between ppk and 42D11 neurons and satisfy the final prediction of our neural circuit model for sleep homeostasis.

3.2.3 Sleep homeostasis circuit may be part of the clock output pathway

As I discussed in Chapter 1, the mechanisms by which the arousal signal from the circadian clock interacts with the sleep signal from the sleep homeostat are unknown. Following sleep deprivation during the night, the increased sleep pressure from the homeostat is in opposition to the arousal signal from the clock in the morning. In *Drosophila*, the DN1p clock neurons have been shown to be involved in the morning

activity peak from the clock [25, 107-110]. Therefore, we hypothesized that these neurons might interact with sleep homeostasis neurons to influence the wake/sleep state during the morning. To test this, we used trans-Tango to map neurons postsynaptic to DN1p neurons by crossing the trans-Tango line to the DN1 driver Clk4.1M-Gal4 [108, 109] and examined labeled, dissected brains of progeny by confocal microscopy (Figure 3.5 A). We were surprised to find that the postsynaptic signal from DN1 clock neurons resembled ppk axons in the vicinity of where they make synaptic contact with 42D11 neurons. This result suggests that the sleep homeostasis circuit we have identified could be part of the clock output network.

To test this possibility further, we examined where the established clock output gene *Nf1* (*neurofibromatosis-1*) functions to control locomotor rhythmicity. Previous studies have shown that this gene is required for behavioral rhythms but not in core clock neurons [91, 111, 112]. We therefore hypothesized that *Nf1* might be required in 42D11/53D10 neurons for circadian locomotor rhythms. To test this hypothesis we knocked down Nf1 with our 42D11 and 53D10 drivers. In both cases reduction in *Nf1* abolished locomotor rhythms (Figure 3.5 B – C), suggesting that the neurons labeled by 42D11 and 53D10 may also be part of the output pathway of the circadian clock network.

3.3 Discussion

In this chapter, we identified two Gal4 drivers, 42D11 and 53D10, which label neurons that satisfy the behavioral and neuroanatomical criteria of our sleep homeostasis circuit model. For example, we have shown that thermogenetic activation of neurons labeled by both drivers increases sleep and that the expression patterns of both drivers

overlap in the AMMC. These results suggest that 42D11/53D10 neurons in the AMMC are responsible for sleep behavior in our assay. Furthermore, we have shown that 42D11/53D10 neurons have processes in the vicinity of ppk neurons, suggesting that synapses might be formed between them. Adding strength to this possibility, we have shown that ppk and 42D11 neurons make physical contact with each other and that cell bodies resembling 42D11/53D10 neurons lie postsynaptic to ppk neurons. One limitation with our anatomical mapping is that we cannot definitively assign the AMMC neurons a role as postsynaptic effectors of ppk-driven sleep homeostasis. Thus, one goal of future experiments should be to anatomically confirm that the postsynaptic cells labeled by ppk∩24C10>Tango are indeed the same AMMC cells labeled by 53D10 and 42D11.

Another limitation of our studies is that we have not determined whether ppk neurons acutely inhibit the activity of the 53D10/42D11 neurons but chronically cause their activity to undergo compensatory upregulation, as predicted by our neural circuit model. These experiments are ongoing by other members of the Joiner lab.

It will also be interesting to determine the mechanism by which ppk neurons (discussed in Chapter 2) inhibit the activity of sleep homeostasis neurons. Sensory neurons in *Drosophila* are usually cholinergic, which is consistent with our observation that cha-Gal80 blocks the sleep effects of ppk>TrpA1 [68]. Thus, we hypothesize that receptors in sleep-promoting 42D11/53D10 neurons that mediate wake-promoting ppk signaling are inhibitory muscarinic acetylcholine receptors (mAChRs). In *Drosophila* type-B muscarinic Acetylcholine Receptors (mAChR-B) have been shown to be inhibitory because, as in mammals, they are coupled to Gαi/o [113-115]. We have acquired RNAis against *Drosophila* mAChRs, and ongoing experiments by other members of the Joiner

lab are aimed at testing their function in our sleep homeostasis circuit. Another approach to test if 42D11 neurons express mAChR-B is to use a mAChR-B Gal4 driver to label all of the mAChR-B positive cells in the brain and test if those include the 42D11 neurons. In fact, a recent study published a mAChR-B gene trap line that expresses Gal4-DBD in mAChR-B neurons [53, 115, 116]. We could combine this mAChR-B-DBD hemidriver line with our 42D11-AD determine if our AMMC neurons of interest express mAChR-B.

The sleep homeostasis circuit components identified in this study are also compatible with previous studies that have implicated structures such as the R2 neurons of the ellipsoid body [82]. It is possible that the AMMC sleep homeostasis neurons could be upstream in that circuit, although we do not have evidence of an anatomical connection at this time. We certainly also considered the fact that 42D11 expresses in the EB as well and we can not rule out the possibility that 42D11 expresses in two separate components of a sleep homeostasis circuit, although this argument is weakened by the fact that 53D10 does not label ellipsoid body or any other central complex neuropil. Additionally, activating 42D11/53D10 neurons elicits a different sleep phenotype from activating R2 neurons. Activation of 42D11/53D10 neurons leads to an acute increase in sleep that ends when the thermogenetic activation ends, while activation of R2 neurons leads to increased sleep that outlasts the duration of the heat pulse.

My data also demonstrate a potential novel connection between sleep homeostasis neural circuitry and the output of the circadian clock. For example, using GRASP I have shown that neurons postsynaptic to DN1p neurons of the clock network lie in the vicinity of ppk axons and 42D11/53D10 dendrites. I have also shown that *Nf1* is required in 42D11/53D10 neurons for circadian locomotor rhythms. In this circuit, wake

promoting DN1p neurons could activate ppk neurons to suppress the homeostat activity in the morning, which could contribute to the accumulation of sleep need. The potential for neuronal circuit connections between output from the clock and neurons involved in sleep homeostasis is also supported by recent studies that proposed a connection between DN1 neurons and EB neurons [99, 100].

3.4 Materials and Methods

Fly Husbandry and Stocks

All stocks were raised at room temperature (20-22°C) on cornmeal media. Unless otherwise noted, all stocks were outcrossed 3 – 5 times into either an iso31 genetic background for behavior. The following stocks were obtained from the Bloomington Stock Center: 42D11-Gal4 [50156], 53D10-Gal4 [45347], Clk4.1M [36316], UAS-TrpA1 [26264], UAS-Shi^{ts} [44222], trans-Tango [77124], GRASP [64315], Nf1 RNAi (HMC03551) [52, 117] [53322]. ppk∩24C10 was generated as previously described [68].

Transgenic fly lines were targeted to attP sites using PhiC31integration (Rainbow Transgenics). ppk-LexA was cloned into pBP-LexA-p65UW and inserted into attP40 and attP2. 53D10-DBD was cloned into pBPZGal4DBDUw and inserted into VK00027. 42D11-AD was cloned into pBP-p65ADZpUW and inserted into attP40 and attP2. All transgenic lines were outcrossed at least 2 generations into an isogenic background.

Behavior

Female flies were used for all behavioral assays unless otherwise indicated. Animals were entrained 2 days under 12 hr : 12 hr light:dark conditions at 22°C. Individual

1-4 day old flies were loaded into 5 mm x 65 mm Pyrex tubes containing 5% agarose with 2% sucrose at one end as a food source. Sleep and waking behavior was monitored using the Drosophila Activity Monitoring System (DAMS; Trikinetics).

For the thermogenetic screen flies were maintained at a baseline temperature of 22C for 1 day, and sleep deprivation was achieved by elevating the temperature to 27°C from ZT 20-24 at the end of the second night. The temperature was then returned to 22°C and flies were monitored for 2 more days.

For the thermogenetic sleep promotion screen and experiments flies were maintained at a baseline temperature of 22C for 1 day, the temperature was then elevated to 29°C from ZT 0 - 6 at the start of the second day. The temperature was then returned to 22°C and flies were monitored for 2 more days.

For the thermogenetic rebound inhibition experiments flies were maintained at a baseline temperature of 20°C for 1 day, the temperature was then elevated to 30°C from ZT 20 - 6 beginning at the end of the second day. The temperature was then returned to 20°C and flies were monitored for 2 more days.

Rhythmicity experiments were conducted with male flies aged 1-4 days. Animals were entrained 2 days under 12 hr : 12 hr light:dark conditions at 25°C. Two baseline days in LD were recorded before animals were shifted to constant darkness for 6 days.

Sleep changes were calculated as the total amount of sleep during the thermogenetic activation period minus the amount from the same period during the baseline day. Rebound sleep was calculated in the same way based on sleep totals from ZT 0-6. All sleep and circadian rhythms analysis were calculated as previously described [6] using custom MATLAB (Mathworks) software.

Immunohistochemistry and confocal microscopy

Unless otherwise noted, 3-7 day old female flies were dissected in cold PBS, brains were fixed in 4% paraformaldehyde, and blocked in PBST (PBS with 0.3% Triton X-100) with 5% normal donkey serum (Jackson Laboratory) prior to staining.

For GRASP experiments, after blocking, brains were incubated with 1:100 mouse anti-GFP (Sigma) overnight at 4°C and then washed with PBST 4 times x 10 min. Brains were then incubated with 3:1000 Alexa 647 anti-mouse (Jackson Laboratory) overnight at 4°C. Brains were washed again 4 x 10 min with PBST prior to coverslip mounting in Vectashield (Vector Labs).

For trans-Tango experiments, females were aged 14 – 18 days post eclosion to allow the postsynaptic signal to develop [89]. Dissections were performed as described above. Tango Primary antibodies: 1:1000 rabbit anti-GFP (Invitrogen), 1:100 rat anti-HA (Roche), and 1:100 mouse anti-nc82 (Developmental Studies Hybridoma Bank). Brains were incubated at 4°C with rocking for two overnights and then washed with PBST 4 times x 10 min. Brains were then incubated with 1:1000 Alexa 488 anti-rabbit (Life Technologies), 1:1000 Alexa 555 anti-rat (Invitrogen), and 1:500 Alexa 647 anti-mouse (Jackson Laboratory) for two additional overnights with rocking at 4°C. Brains were washed again 4 x 10 min with PBST prior to coverslip mounting in Vectashield (Vector Labs).

Image stacks were taken on a Leica SP5 confocal microscope with 40x magnification using 1 um step intervals. Maximum intensity Z-projection images were generated, and any brightness/contrast adjustments were made using Fiji [90].

Statistics

Bar graphs depict mean ± SEM. Unpaired Student's t-test was used for comparisons between 2 groups. One-way ANOVA with Tukey's post hoc comparisons was used for comparisons between multiple groups. All statistical tests were two-sided and performed using Prism 8.0 for Windows 10 (GraphPad Software).

3.5 Acknowledgements

Work presented in Chapter 3 will be submitted for publication upon completion of follow-up studies with coauthors Joydeep De, Meilin Wu, Erika Barrall, Tianhao Qiu, and William Joiner. Joydeep De will be primary author and performed GCaMP6 and other imaging studies not included in this chapter. Meilin Wu performed cloning to generate new transgenic animals for this study. Erika Barrall performed brain dissections for IHC experiments. The dissertation author performed all IHC and imaging included in this chapter as well as a behavioral screen and some additional behavior with assistance from corresponding author William Joiner who performed or assisted with many behavior experiments.

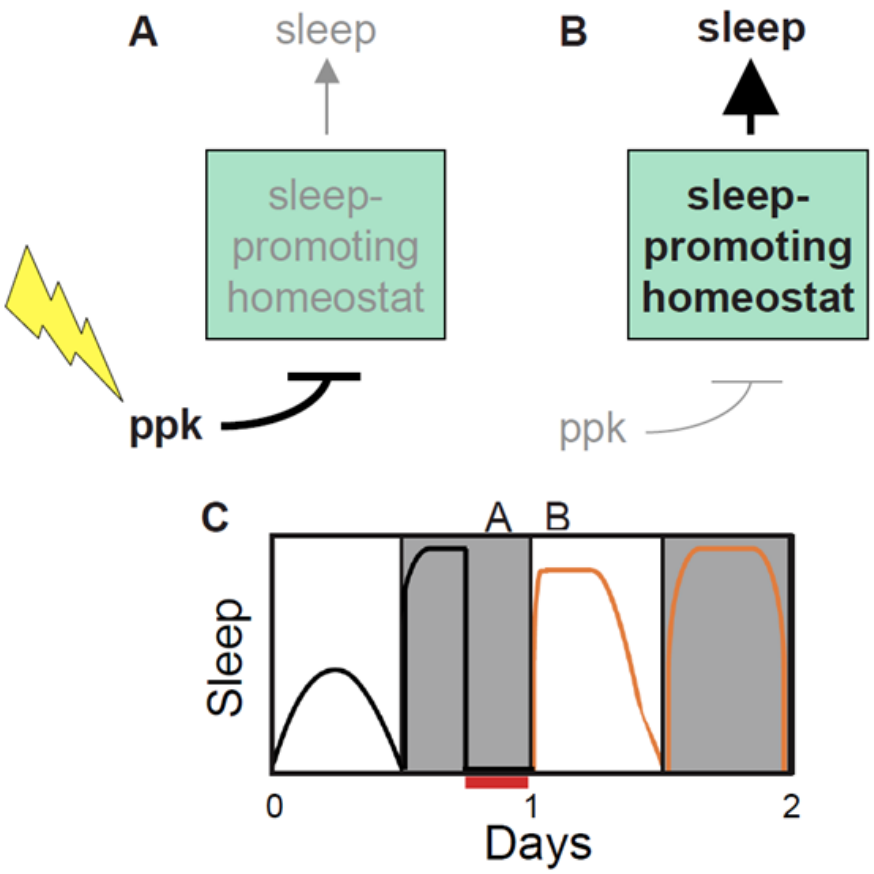


Figure 3.1 Model of ppk driven sleep homeostasis circuit. A-B) Neural circuit model for ppk driven sleep homeostasis. A) ppk neural activity inhibits the sleep promoting homeostat neurons to acutely drive waking. Prolonged inhibition of sleep homeostasis neurons leads to gradual compensatory upregulation of their activity. B) When ppk driven inhibition ends the upregulated homeostasis neurons discharge sleep pressure leading to rebound sleep. C) Cartoon sleep trace demonstrates time course of ppk driven changes to waking and sleep. Red bar indicates period of ppk driven waking.

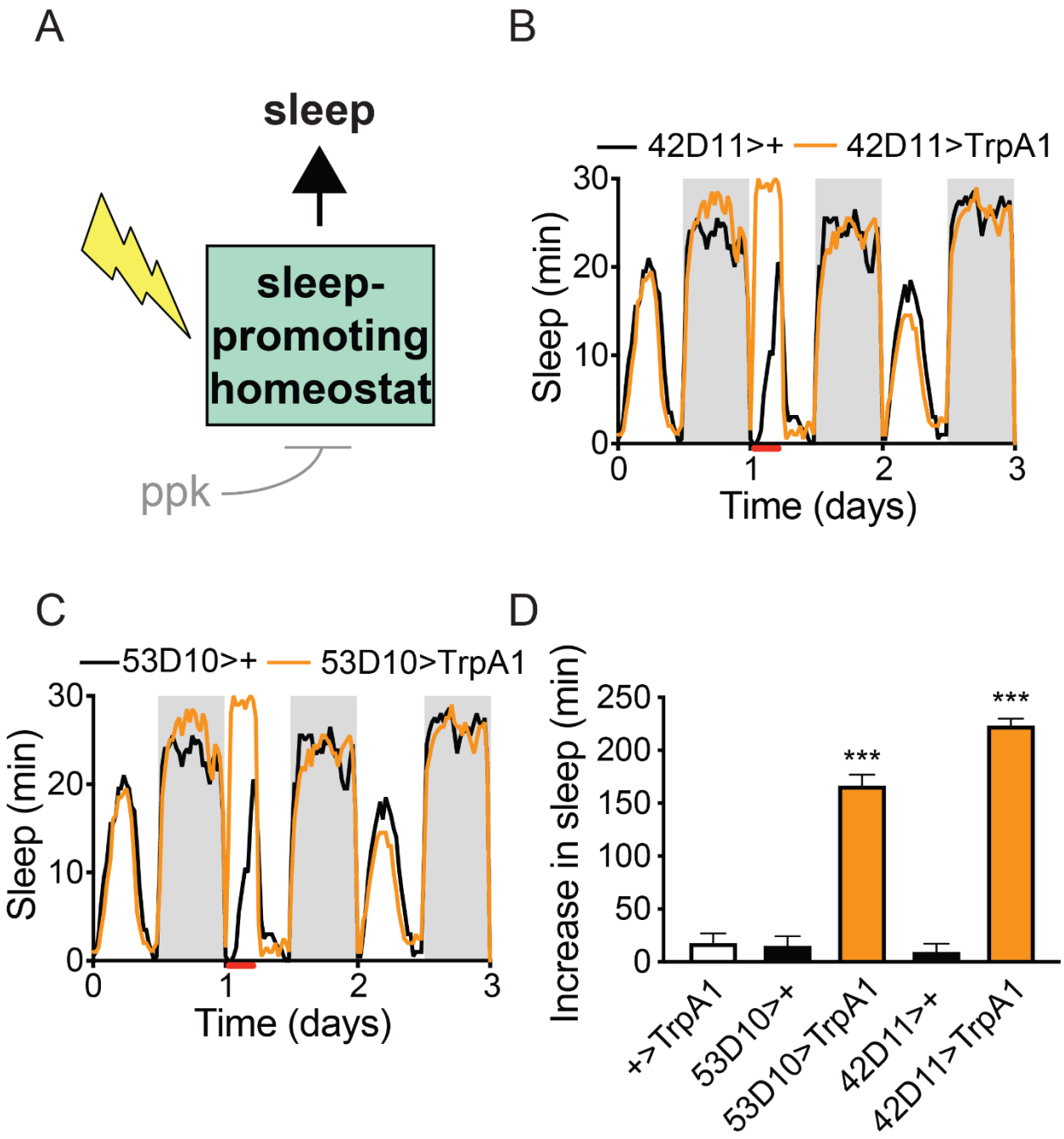


Figure 3.2 Thermogenetic activation of 42D11 and 53D10 neurons is acutely sleep promoting. A) Model of behavioral outcome from direct activation of sleep homeostasis neurons. B-C) Sleep profiles of 42D11>TrpA1 (B) and 53D10>TrpA1 (C) and genetic control. In these thermogenetic experiments, animals were maintained at 22°C for one baseline day, animals were pulsed for 6 hr at 29°C (red bar) from ZT 0 - 6 and then returned to 22°C for two additional days of recording. D) Quantification of change in sleep in (B) and (C) with additional control. n ≥ 30 per group. In this and subsequent figures, *, **, ***, **** indicate $p < 0.05$, 0.01, 0.001 and 0.0001, respectively. Error bars indicate SEM.

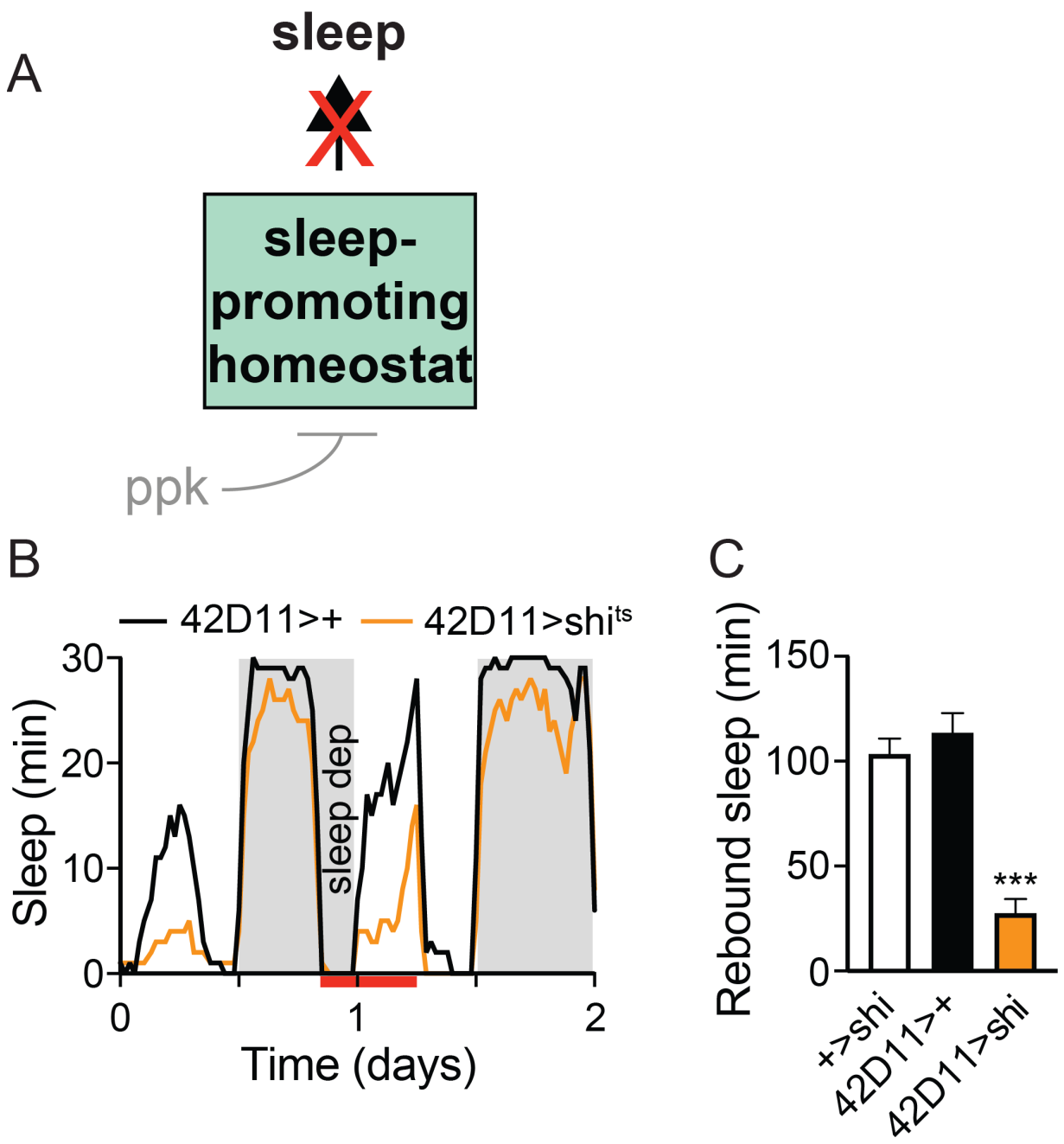
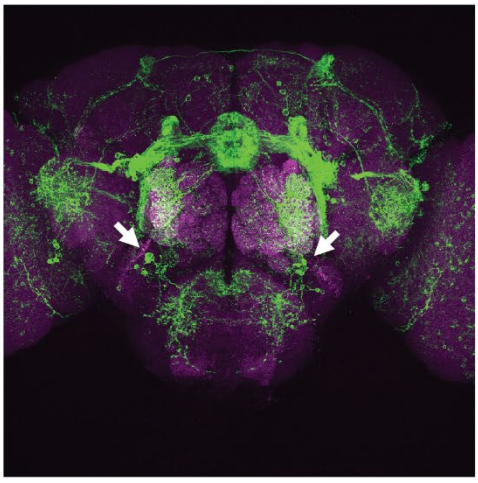


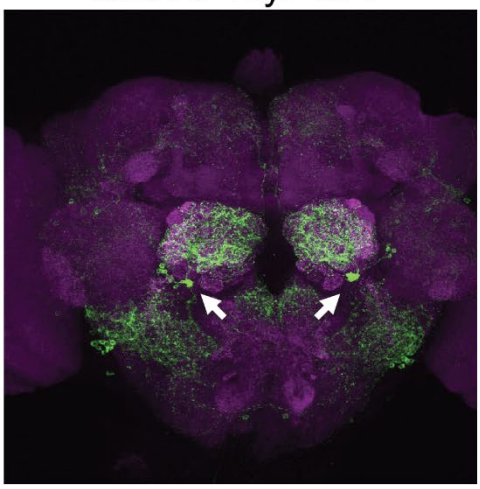
Figure 3.3 Inhibition of 42D11 neurons reduces rebound sleep. A) Model of behavioral outcome from inhibition of neurotransmission from 42D11 neurons during and after sleep deprivation. B) Sleep profiles of 42D11>Shi^{ts} and genetic control. In these thermogenetic experiments, animals were maintained at 20°C for one baseline day, animals were pulsed from ZT 20 - 6 at 30°C (red bar) with simultaneous mechanical sleep deprivation from ZT 20 – 24. At then end of the pulse, the temperature was returned to 20°C for two additional days of recording. C) Quantification of rebound sleep in (B) with additional control. n ≥ 30 per group.

Figure 3.4 42D11 and 53D10 neurons satisfy neuroanatomical criteria for sleep homeostasis neurons. A-C) Expression patterns of 42D11 (A), 53D10 (B), and 42D11∩53D10 split-Gal4 drivers. D) Transsynaptic tracing of ppk∩24C10 neurons reveals cells resembling 42D11/53D10 as postsynaptic partners. White arrows indicate relevant neurons. Gal4 expression patterns labeled with anti-GFP (green); anti-HA labels postsynaptic neurons in (D) (red). Neuropil stained with anti-nc82 is shown in magenta. E) GRASP reveals ppk neurons make cell-cell contact with 42D11 neurons. GRASP labeled with anti-spGFP (green).

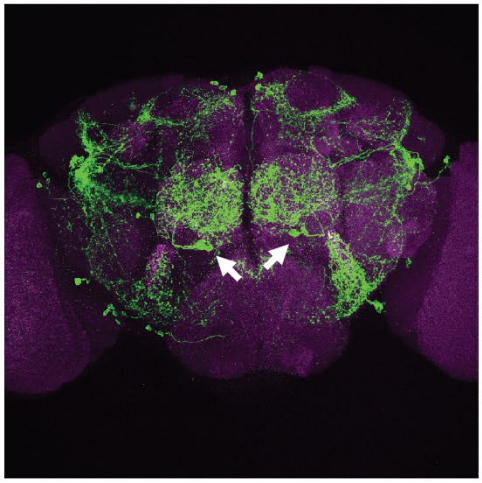
A 42D11>myr-GFP



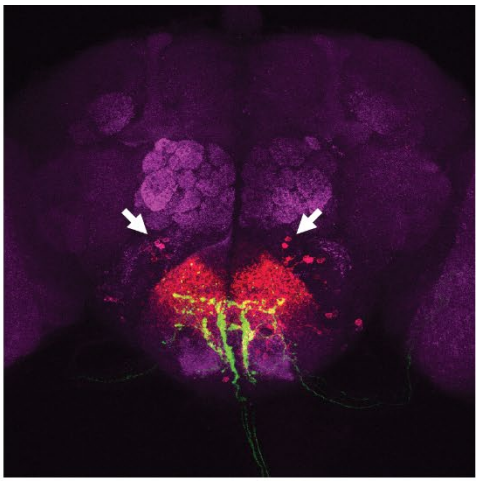
B 53D10>myr-GFP



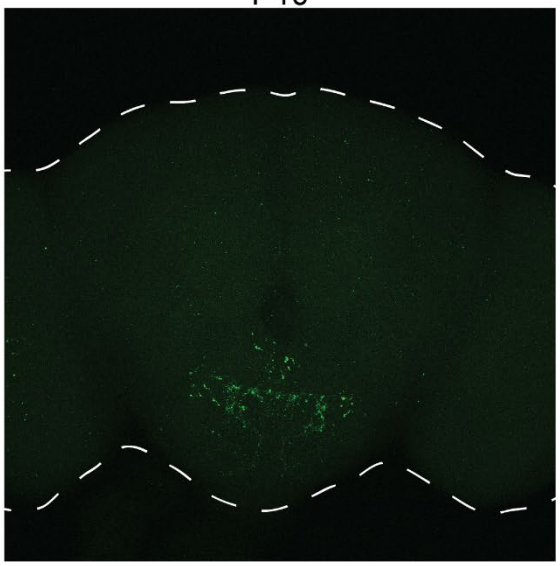
C 42D11∩53D10>myr-GFP



D ppk∩24C10>Tango



E ppk-LexA>spGFP$_{1-10}$+42D11>spGFP$_{11}$



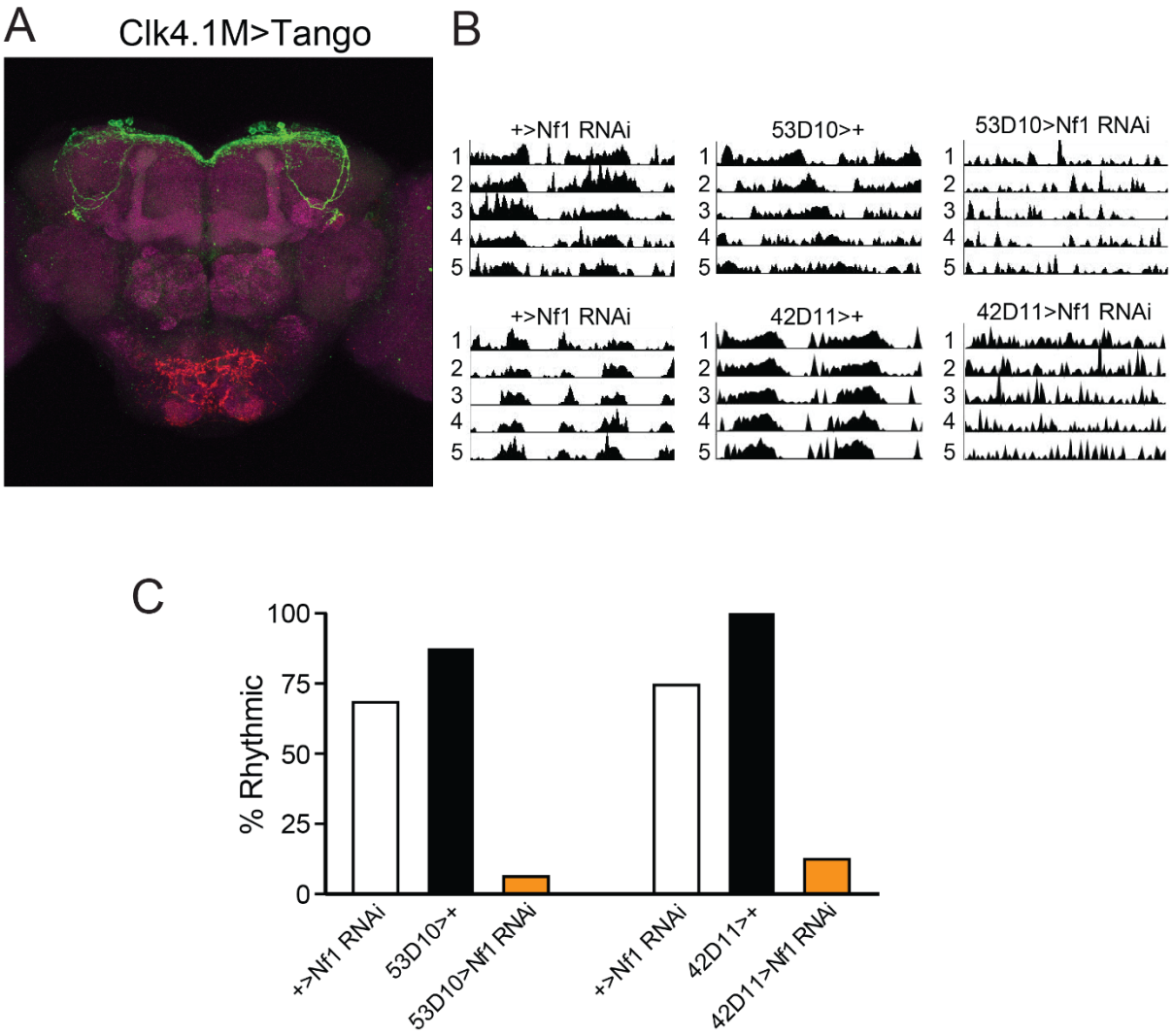


Figure 3.5 Sleep homeostasis circuit may be part of the clock output pathway. A) Trans-synaptic tracing of DN1 neurons with Clk4.1M-Gal4. Gal4 expression labeled with anti-GFP (green); postsynaptic neurons labeled with anti-HA (red). Neuropil stained with anti-nc82 is shown in magenta. B-C) Knockdown of *Nf1* in 42D11 and 53D10 neurons causes locomotor arrhythmicity. B) Representative actograms of 42D11>Nf1 RNAi and 53D10>RNAi with controls in constant darkness. C) Quantification of percent of animals rhythmic in constant darkness.

Chapter 4: Protein Kinase CKII is required for sleep homeostasis and sleep-dependent short-term memory formation in *Drosophila melanogaster*

4.1 Introduction

Spending hours per day unconscious and unresponsive to the environment would seem to be an evolutionarily maladaptive behavior, but sleep is highly conserved throughout evolution [1]. This evolutionary paradox implies that sleep must satisfy one or more critical biological functions, but what those functions are remains poorly understood despite decades of study. As described previously in Chapter 1, the two process model describes the two processes that seem to regulate sleep timing and duration [17, 92]. Process C, controlled by the circadian clock, acts as a wake promoting daily oscillator such that the strength of arousal cycles over ~24 hr. Process S regulates the amount of sleep an animal undertakes on a daily basis, with deviations from a set point leading to subsequent compensation, a process known as sleep homeostasis. Since these deviations are almost always due to loss of sleep, the homeostatically-driven compensatory changes usually involve increases in sleep. Thus, process S is generally thought to be sleep promoting, with its influence gradually increasing over time spent awake. It is therefore likely that the process of sleep homeostasis senses important physiological changes which occur in the brain in order to keep track of prior waking time and accumulate sleep pressure. New insights into what changes the brain is sensing and responding to will offer new clues into what biological processes and needs sleep is critical for.

In fruit flies sleep homeostasis is usually studied by forcing animals to stay awake at night and then measuring the amount of compensatory recovery sleep, also known as rebound sleep, that follows. Because of this requirement to sleep deprive animals in order to measure sleep need, sleep homeostasis has proven to be a challenging behavior to study. As a result, its molecular and neuroanatomical mechanism are also poorly understood. This intractability parallels results from genetic screens as well: so far these have revealed only genes involved in regulating baseline arousal [38-42].

One serious obstacle to screening for mutations that impair sleep homeostasis is the standard assay which is used in the field for sleep deprivation. In past studies, animals have been subjected to frequent mechanical agitation over hours to prevent them from sleeping during some or all of the night [6, 7]. This approach is time intensive, and low throughput. Because of these limitations, no mutants have been reported from forward genetic screens in which mechanical perturbation has been used to deprive animals of sleep.

In a previous study, our lab developed a thermogenetic approach [68, 70] (Chapter 2) to sleep deprive animals and induce subsequent rebound sleep. This approach allows for high throughput screening for mutants with reduced rebound sleep and therefore impaired sleep homeostasis. Using this approach, we then conducted a forward genetic screen of ~1300 lines with single transposon insertions on the X-chromosome. We identified one line carrying a mutation in the beta regulatory subunit of the serine/threonine kinase Protein Kinase CKII that had strongly reduced rebound sleep compared to genetic controls.

Protein Kinase CKII is a heterodimer of two catalytic alpha and two regulatory beta subunits [118-120]. It is known to be a promiscuous priming kinase involved in many cellular processes during development and adulthood in flies and mammals [121-129]. Interestingly, in contrast to the CKIIα subunit gene, the β subunit has 10 alternate transcripts which are thought to confer tissue and temporal specificity of CKII activity and targets [130-133]. While these transcripts have been cloned and sequenced, little else is currently known about the specific roles of any of the CKIIβ isoforms. Protein Kinase CKII is also known to have a role in the molecular circadian clock. It has been shown to be one of the kinases that phosphorylates the PER/TIM complex while it is in the cytosol to promote nuclear entry [24, 134-140].

In this chapter, I confirm that the sleep homeostasis phenotype maps to the CKIIβ mutant by genetic complementation studies, demonstrate that the mutation reduces the expression of two alternate transcripts of CKIIβ, and show that restoration of expression of these transcripts rescues the sleep homeostasis phenotype of the mutant. I also demonstrate that the CKIIβ mutant's effect on sleep can be phenocopied by pan-neuronal expression of dominant-negative CKIIα, and I map this effect to circadian clock neurons. Additionally, we also demonstrate that CKII is required for sleep-dependent associative memory formation. This study supports a novel role for a protein kinase in sleep homeostasis and suggests that dysregulation of circadian function can potentially impact the amount of recovery sleep that is typically ascribed to the homeostat.

4.2 Results

4.2.1 Identification of sleep homeostasis deficient CKIIβ mutant

To date, forward genetic screens for mutations that impair sleep homeostasis have not been successful. We designed a screen to test hemizygous male progeny from a single cross between virgins bearing homozygous viable transposon insertions on the X chromosome to ppk∩24C10>TrpA1 males. Using this approach, we screened ~1300 transposon lines by thermogenetically sleep depriving them during the last 4 hours of the second night and recording subsequent recovery sleep (Figure 4.1 A - B). After retesting promising leads, we identified one mutation with impaired sleep homeostasis, named CKIIβ^{GT1} (GT1) (Figure 4.1 C - D) [141]. Importantly, the GT1 mutation impairs recovery sleep without affecting baseline sleep relative to the genetic controls. To further validate the phenotype, I outcrossed the GT1 mutant 5 times into an isogenic background [51] and tested homozygous females using the standard mechanical sleep deprivation assay. Again, I found that homeostatic rebound sleep was impaired (Figure 4.1 E - F). Although the CKIIβ gene has been implicated in circadian clock function, I found that the majority of the GT1 animals are rhythmic (Figure 4.1 G). Therefore, I initially hypothesized that despite CKIIβ's previously described role in the clock, the sleep homeostasis phenotype was possibly independent of the clock.

4.2.2 Genetic analysis of CKIIβ^{GT1} mutant

Next, I tested whether the sleep homeostasis phenotype mapped to the CKIIβ locus. First, I acquired a deficiency line that specifically covered the CKIIβ locus [132]. As expected, the GT1 mutant crossed to the CKIIβ deficiency failed to complement (Figure 4.2 A - B). This result indicated that the sleep homeostasis phenotype indeed maps to the GT1 mutation. I also acquired two additional mutant lines bearing transposon insertions

in the CKIIβ locus that had been previously described [50, 53] and tested them in both homozygous and complementation crosses using mechanical sleep deprivation (Table 4.1). Of all these combinations only the GT1 mutant and GT1/Df animals had a sleep homeostasis phenotype which indicated that the phenotype mapped to a unique feature of the GT1 mutant.

In parallel to the complementation study, I also undertook an RNAi based anatomical screen to map where CKIIβ functions to facilitate sleep homeostasis in the CNS. I combined a CKIIβ RNAi with ;ppk-LexA,LexAop-TrpA1 flies and used thermogenetic sleep deprivation to test well characterized Gal4 drivers that express in published sleep promoting regions of the brain, circadian clock neurons and a collection of ~50 Gal4s that had been selected from a thermogenetic screen for sleep homeostasis neurons (Chapter 3). Although several lines gave me initially promising results, upon retesting the results were too variable to draw any conclusion (data not shown).

4.2.3 Characterization of genetic lesion

The CKIIβ locus codes for up to 10 alternative transcripts (Figure 4.2 C). The core exons of CKIIβ are shared between all of the transcripts, but 4 of the transcripts have been shown to have unique C-terminal sequences encoded by alternatively spliced final exons [130-133]. Based on the known insertion site of the GT1 transposon I hypothesized that the splicing of the final, unique exon in 2 - 3 of the transcripts had been disrupted and that the expression of these transcripts was likely to be greatly reduced. To test this hypothesis, I designed transcript specific qPCR primers and compared transcript levels in heads from WT and GT1 mutants (Figure 4.2 D). We found that, as expected,

expression of two transcripts, E and I, was almost undetectable in the GT1 mutant. Unexpectedly I also found an increase in expression of transcript J in the mutant which could be because it shares its 5' UTR sequence with transcript E.

Next, I tested whether the affected transcripts were sufficient for sleep homeostasis. We cloned the E and I transcripts of CKIIβ from WT cDNA, generated transgenic animals, and found that each UAS-transgene was able to rescue the sleep homeostasis phenotype in the GT1 mutant background when expressed in all cells using tub-Gal4 (Figure 4.3 A - D). Based on these results I combined additional Gal4s of interest into the GT1 background to test for rescue, beginning with the neuronal driver nSyb-Gal4 and the glial driver repo-Gal4. These drivers failed to rescue the deficit in sleep homeostasis (data not shown). Due to the time intensive nature of combining many Gal4s into the GT1 mutant background I did not pursue an anatomical rescue screen.

I also tested whether the increased expression of transcript J in the mutant contributed to the sleep phenotype of GT1 mutants. We generated multiple transcript J specific shRNAs and validated their knockdown efficiency in S2 cells (Figure 4.4 A - B). Then I generated transgenic animals with the shRNAs under UAS control and crossed them to tub-Gal4 in a GT1 mutant background (Figure 4.3 E - F). I found that knockdown of transcript J did not rescue the deficiency in sleep homeostasis caused by the GT1 mutation. This did not appear to be due to an inability to knock down transcript J since we found that it was highly reduced in animals expressing our shRNA under tub-Gal4 control (Figure 4.4 C). These data strongly suggest that CKIIβ transcripts E and I are necessary and sufficient for sleep homeostasis and raise the question of where these two transcripts function in the brain.

4.2.4 CKIIβ transcripts E and I express broadly throughout the brain

While a prior study [135] had generated and used an antibody against CKIIβ for immunohistochemistry (IHC), it was not specific to any particular isoform and would therefore be of limited utility for localizing the sleep-regulating effects of transcripts E and I. I therefore pursued two complementary strategies to image the expression patterns of transcripts E and I in the brain: use CRISPR to epitope tag these transcripts for subsequent IHC and use RNAscope to detect expression patterns of these transcripts *in situ*.

My efforts to use CRISPR to epitope tag the CKIIβ transcripts failed to yield viable transgenic animals after multiple attempts. After corresponding with a lab that has been developing CRISPR techniques in the fly I think that the limited target area to make transcript specific modifications is not a good target for gRNA. For RNAscope analysis of transcript expression in the brain we developed transcript E and I specific probes. We performed RNAscope with brains from WT and GT1 mutant females and found that both transcripts appear to express broadly throughout the brain and that their expression is nearly undetectable in the GT1 mutant (Fig 4.5). While these results indicated that the technique and probes worked, the apparently broad expression of both transcripts did not help to determine where CKIIβ-E or -I is required in the brain for sleep homeostasis.

4.2.5 CKIIα is required for sleep homeostasis

I focused my efforts on studying the requirement of CKIIβ for sleep homeostasis because I had identified two specific alternative transcripts that are required for sleep

homeostasis. In contrast to CKIIβ, the CKIIα gene does not encode any alternative transcripts of the CKII catalytic subunit. Therefore, CKIIα expression would be expected to be everywhere that any transcript of CKIIβ is expressed, so each CKIIβ transcript is likely to have more restricted expression than CKIIα. While CKIIβ has been shown to interact with a few proteins besides CKIIα [142, 143], I hypothesized that reduced activity of protein kinase CKII was responsible for the sleep homeostasis phenotype of CKIIβ mutants. Although null mutations in CKIIα are developmentally lethal, two studies have shown that overexpression of a dominant negative UAS-CKIIαTik (Tik) [125, 137] transgene is viable and causes a reduction in CKII activity. To test my hypothesis, I overexpressed UAS-Tik in the CNS with elav-Gal4 and found that it phenocopied the GT1 sleep homeostasis phenotype (Figure 4.6 A – B). This suggests that CKII kinase activity is necessary for sleep homeostasis. Next, I used the UAS-Tik transgene to conduct a small Gal4 screen to anatomically map where CKII is required in the brain (Figure 4.7). I focused on well characterized Gal4s that express in neurons that use specific neurotransmitters and other classes of neurons that have been described to be involved in sleep regulation (Figure 4.7). The screen did not map the phenotype to neurons based on the neurotransmitter they express, but I found that overexpressing Tik in circadian clock neurons using tim-Gal4 strongly suppresses rebound sleep (Figure 4.6 C - D). In contrast, overexpression in other sleep related neuropil such as fan-shaped body and ellipsoid body did not. Taken together these results suggest that CKII functions in clock neurons to facilitate rebound sleep.

4.2.6 Reducing PER/TIM accumulation in clock neurons does not rescue CKII sleep homeostasis phenotype

There are two possible explanations for the surprising result that overexpressing CKIIα-Tik in circadian clock neurons phenocopies the GT1 mutant. The first is that CKII affects sleep homeostasis by an unknown mechanism that is independent of the clock. The second is that CKII is required to suppress clock-driven waking. In this second scenario, reduction in CKII activity would increase clock-dependent arousal, thus enhancing antagonism of the sleep-promoting homeostat and reducing rebound sleep. It has been previously shown that disruptions in CKII activity in clock neurons leads to an increase in cytosolic PER/TIM accumulation and a delay in nuclear entry of the PER/TIM transcriptional repressor complex [24, 135-140]. I therefore hypothesized that this misregulation of the molecular clock could be responsible for the suppression of recovery sleep. I further hypothesized that if this is the case, the sleep phenotype should be rescuable by reducing the levels of PER/TIM in the cytoplasm. I used two genetic approaches to test this hypothesis. I acquired RNAi lines against both *per* and *tim* and conducted sleep deprivation experiments where I knocked down each gene in animals overexpressing Tik pan-neuronally. I also tested elav>Tik and tim>Tik animals in either a per^{0} or tim^{01} null genetic background. Both approaches did not rescue the reduced rebound sleep homeostasis phenotype (data not shown). These results suggest that CKII may function in clock neurons downstream of the molecular clock to reduce arousal, at least in the morning. Additional experiments to test this hypothesis are underway and described below.

4.2.7 Consequences of impaired sleep homeostasis on sleep-dependent short-term learning and memory.

Since sleep homeostasis restores lost sleep, I hypothesized that it would also restore associative learning and memory after sleep loss [104, 144, 145]. In this case the GT1 mutation, which reduces sleep homeostasis, might be expected to cause memory loss after sleep deprivation and a normal rebound period. To test these hypotheses, we assayed CKIIβ mutants and control animals for gustatory associative memory using the Proboscis Extension Reflex (PER) assay [146-148] immediately after sleep deprivation and after 3 hr recovery (Figure 4.8 A - D). First, we established that the GT1 mutant does not have an impaired sense of taste using a taste discrimination assay between the fructose solution used for PER and fructose mixed with quinine (data not shown). As expected, when we tested WT and GT1 animals immediately following sleep deprivation both groups had similarly impaired performance compared to rested controls. Importantly, this experiment also established that the GT1 mutant does not have any associative memory impairment at baseline (Figure 4.8 C). Next, we repeated the experiment with animals that had 3 hr to rest after sleep deprivation and found full recovery of memory in WT but not in GT1 mutants (Figure 4.8 D - E). These data suggest that recovery sleep can substitute for baseline sleep in facilitating memory formation. By extension, these data also suggest that CKII mutants cannot recover from memory loss after sleep deprivation because they are impaired in sleep homeostasis. Thus, identification of brain circuitry and molecular mechanisms that are required for sleep homeostasis may inform our understanding of memory formation.

4.3 Discussion

In this chapter I identified CKIIβ as a gene required for sleep homeostasis through a large forward genetic screen and demonstrated that two transcripts of CKIIβ are necessary and sufficient for recovery sleep following sleep deprivation. I also show that the catalytic subunit CKIIα is required in clock neurons for recovery sleep. Lastly I show that CKIIβ is required for sleep-dependent short-term memory formation. Together, these results support a novel role for Protein Kinase CKII in sleep homeostasis and cognition.

Gal4 based anatomical screens can be a very powerful way to map where a gene is required either using RNAi or rescuing a mutation with a transgene. In this study I used both strategies to map the requirement of CKIIβ for sleep homeostasis with mixed results. In the anatomical screen using CKIIβ RNAi I did not observe a sleep homeostasis phenotype when knocking down CKIIβ in clock neurons. One possible explanation is that the shRNA did not reduce CKIIβ expression levels in clock neurons adequately to disrupt CKII function. An alternative explanation is that the RNAi was assayed using thermogenetic sleep deprivation, which may be more powerful or have different effects than mechanical sleep deprivation. Additional studies using multiple RNAi's against CKIIβ will determine if knockdown in clock neurons phenocopies the GT1 mutant. Additionally, restoring expression of CKIIβ-E and/or CKIIβ-I throughout the CNS was not able to rescue the GT1 phenotype. This could be because the pan-neuronal Gal4 used in the experiment, nSyb-Gal4, is too weak to express UAS-CKIIβ to physiologically relevant levels. A future experiment to determine if restoring expression of CKIIβ-E or -I using tim-Gal4 is sufficient to rescue the GT1 phenotype is underway now.

Two previous studies focused on the role of CKII in the clock showed that CKIIα/β are only expressed in ventral lateral neurons of the clock [24, 135]. However, our RNAscope data indicate that both transcripts E and I are expressed far more broadly throughout the brain than only clock neurons, and the transcript specific qPCR results indicate that multiple CKIIβ transcripts are expressed in the head. To date, no other studies have investigated which transcripts of CKIIβ are expressed in the brain, either in the circadian context or otherwise. Additional studies to endogenously epitope tag CKIIα and different transcripts of CKIIβ could help to resolve this discrepancy. While my efforts to use CRISPR to tag transcripts E and I were not successful the technology is rapidly advancing, and I anticipate it will be possible to overcome this technical hurdle in the near future. It will be interesting as well to determine which transcripts of CKIIβ are expressed in clock neurons. My genetic rescue and complementation data strongly support a specific requirement for transcripts E and I in sleep homeostasis. Additional studies in the lab are aimed at combining IHC with RNAscope to colocalize CKIIβ-E and CKIIβ-I transcript signal in brains from animals expressing GFP under the control of tim-Gal4. Previous studies have used microarray and RNA-seq to assess transcriptional profiles of different populations of clock neurons, including the LNv's [149, 150], but these studies focused on changes in cycling gene expression and generally did not compare alternative transcripts in the data sets.

The s-LNv neurons have been shown to be responsible for the morning activity peak [25, 107] and are therefore an interesting candidate population of neurons where CKII might be required for the sleep homeostasis phenotype. CKII is known to phosphorylate PER/TIM and promote translocation from the cytosol to the nucleus, and

it has been previously shown that CKII clock mutants have elevated levels of PER/TIM in the cytoplasm [24, 135-140]. We hypothesized that loss of CKII activity in these cells could lead to mistimed or excessive arousal signal from the clock during the morning that overrides the increase in sleep pressure following sleep deprivation. In order to test this hypothesis, I used RNAi and null alleles to reduce the amount of PER/TIM in the cytosol in animals overexpressing CKIIalphaTik. While these initial experiments did not rescue the sleep homeostasis phenotype, more nuanced genetic manipulations might be required, as it may be necessary to restore the timing of PER and TIM nuclear translocation rather than just reducing their levels to achieve genetic rescue.

Future studies are also aimed at further refining the population of neurons where CKII is required. Based on the role of the s-LNv neurons in the morning activity peak I hypothesize that CKII is required in those cells for the sleep homeostasis phenotype. One would therefore expect that overexpressing Tik with clock neuron Gal4s that do not express in the pacemaker neurons would not phenocopy the sleep homeostasis phenotype. Indeed, preliminary results indicate that overexpressing Tik in DN1 neurons using Clk4.1-Gal4 does not phenocopy the sleep homeostasis impairment seen with tim>Tik animals. Further refining the population of neurons where CKII activity is required will also enable more targeted mechanistic experiments. For example, a study has shown that p38 MAP kinase may activate CKII in the circadian clock as part of daily response to stress and influence the phosphorylation of Period [151] but this relationship has not been investigated further. Additionally, a recent study has shown that the voltage-gated potassium channels, Shaw and Shal, mediate daily changes in clock neuron excitability [152]. A mistiming in excitability could also be responsible for the morning activity

suppressing sleep homeostasis, and I hypothesize that this might be observable in the GT1 mutant. In this case manipulating the expression levels of potassium channels might also rescue the phenotype.

4.4 Materials and Methods

Fly Stocks and Transgenic Fly Lines

All stocks were raised at room temperature (20-22°C) on cornmeal media. Unless otherwise noted, all stocks were outcrossed 3 – 5 times into either an iso31 or RC1 genetic background for behavior. The UAS-TrpA1;ppk∩24C10 fly line was described previously [68]. All other fly lines used in this study were obtained from the Bloomington Stock Center. CKIIβ: GT1 [12739], TP2 [22488], TP3 [41538], Df [35825], CKIIβ RNAi's [42943, 34939, 31254]. CKIIa: UAS-CKIIaTik [24624]. Gal4s: tub-Gal4 [5138], elav-Gal4 [8760], cha-Gal4 [6793], OK371-Gal4 [26160], GAD-Gal4 [51630], ChAT-Gal4 [60317], Tdc2-Gal4 [9313], Trh-Gal4 [38388], 69F08-Gal4 [39499], 23E10-Gal4 [49032], tim-Gal4 [40864], and all X chromosome transposon mutants used in the screen.

Transgenic fly lines were generated using pUAST-attB or pWALIUM20 targeted to attP sites using PhiC31integration (Rainbow Transgenics). UAS-CKIIβ-E was integrated into VK00027, UAS-CKIIβ-I into VK00033, and UAS-CKIIβ-J shRNAi's into attP5 and attP40. All transgenic lines were outcrossed at least 3 generations into an isogenic background.

Sleep Deprivation and Rebound Measurements

Female flies were used for all behavioral assays unless otherwise indicated. Animals were entrained 2 days under 12 hr : 12 hr light:dark conditions at 22°C. Individual 1-4 day old flies were loaded into 5 mm x 65 mm Pyrex tubes containing 5% agarose with 2% sucrose at one end as a food source. Sleep and waking behavior was monitored using the Drosophila Activity Monitoring System (DAMS; Trikinetics).

For the thermogenetic screen male flies were maintained at a baseline temperature of 22C for 1 day, and sleep deprivation was achieved by elevating the temperature to 27C from ZT 20-24 at the end of the second night. The temperature was then returned to 22C and flies were monitored for 2 more days. For mechanical sleep deprivation experiments flies in DAMS monitors were loaded into a VX-2500 multi-tube vortexer (VWR) with a custom base. Sleep was deprived by periodic shaking for 2 sec/min from ZT 20-24 with the vortexer set to the lowest setting.

Sleep deprivation was calculated as the total amount of sleep during the deprivation period minus the amount from the same period during the baseline day. Rebound sleep was calculated in the same way based on sleep totals from ZT 0-6. Sleep analysis was calculated as previously described [6] using custom MATLAB (Mathworks) software.

Quantitative PCR

For each sample at least 20 heads from 5-9 day old female flies were lysed in Trizol (Life Technologies) and cDNA was synthesized from extracted RNA using High Capacity cDNA Reverse Transcription (Life Technologies). Quantitative PCR was performed on each sample using the primers in Table 4.2. Results were normalized to

RP49 expression levels and fold change in gene expression was calculated using the 2(–ΔΔCt) method. All primer pairs designed for this study were validated for amplification efficiency (R^2) greater than 0.98.

RNAscope

Transcript E and I specific RNAscope probes were designed and tested in collaboration with the RNAscope kit manufacturer (ACD). Tissues samples were prepared as previously published. Brains were mounted in Vectashield (Vector Labs) and image stacks were taken on a Leica SP5 confocal microscope with 40x magnification using 1 um step intervals. Maximum intensity Z-projection images were generated and any brightness/contrast adjustments were made using Fiji [90].

Aversive Taste Memory Assay

Proboscis Extension Reflex assays were performed as previously described [68] with the following modifications. Four to seven day old mated females were entrained on a 12hr : 12 hr light:dark cycle at 22C for two days before experiments. Individual animals were loaded into 5 mm x 65 mm Pyrex tubes containing 5% agarose as a source of water 12 hr prior to the beginning of the behavior experiment and placed in DAMS monitors for mechanical sleep deprivation. Animals were either sleep deprived from ZT 20-24 or controls were left undisturbed. They were then either tested immediately post sleep deprivation or allowed to rest 3 hr before being tested. For PER suppression experiments, we performed one round of training consisting of 3 presentations of 100mM fructose solution to the tarsi paired with a presentation of 10mM quinine to the extended proboscis

allowing flies to drink up to 2 sec and PER response is recorded. During the test, flies were offered 100mM fructose alone to their tarsi and PER was recorded.

Taste Discrimination Assay

Four to seven day old mated females were entrained on a 12hr : 12 hr light:dark cycle at 22C for two days before experiments. Flies were transferred at ZT0 to an empty 25 mm x 95 mm plastic vial containing two half circles of 5% agarose with either 100 mm fructose or a mixture of 100mM fructose + 10mM quinine at the bottom of the vial. Each half disc of agarose was dyed with a different food coloring to be able to visualize feeding preferences. The flies were allowed to feed for 3 hr in the dark before their abdomens were examined and scored for color. The experiment was repeated with the colors reversed to control for any potential color preference.

Statistics

Bar graphs depict mean ± SEM. Unpaired Student's t-test was used for comparisons between 2 groups. One-way ANOVA with Tukey's post hoc comparisons was used for comparisons between multiple groups. All statistical tests were two-sided and performed using Prism 8.0 for Windows 10 (GraphPad Software).

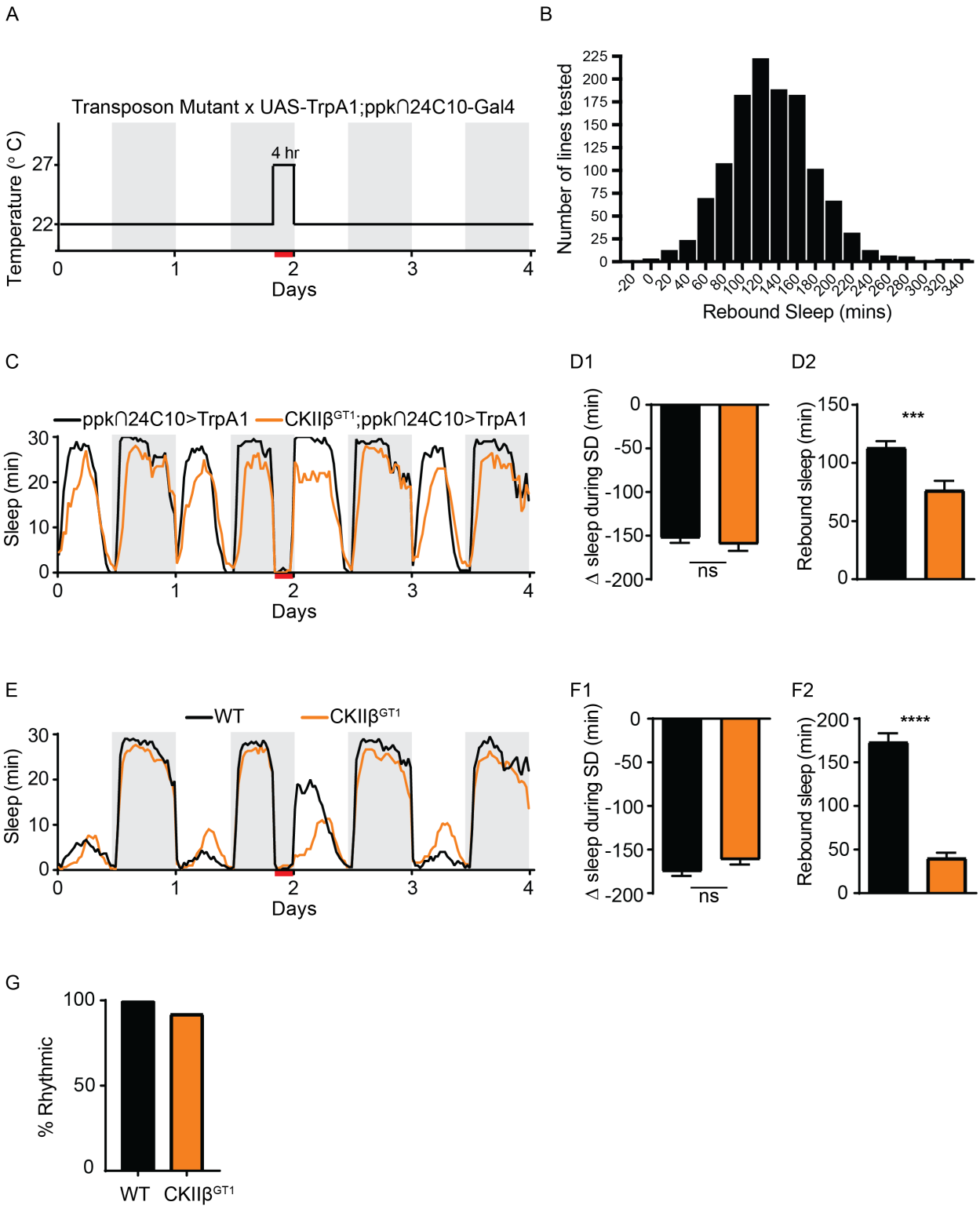
4.5 Acknowledgements

Work presented in Chapter 4 with minor modifications will be submitted for publication with coauthors Vanessa Lambatan, Veronica Lin, Meilin Wu, Michelle Lin, and William Joiner. The dissertation author was the primary investigator and author of the

paper. Vanessa Lambatan performed brain dissections and RNAscope experiments. Veronica Lin performed and analyzed PER experiments. Michelle Lin performed RNA extraction and qPCR on biological replicates. Meilin Wu performed S2 cell culture and western blots to validate CKIIβ-J RNAi as well as cloning the CKIIβ-E and -I sequences into UAS plasmids.

I would also like to thank Glen Seidner and Giovanni Hanna for training and assistance during the sleep homeostasis screen.

Figure 4.1 Identification of sleep homeostasis deficient CKIIβ mutant. A) Protocol for thermogenetic sleep deprivation: after being maintained at 22°C for one baseline day, animals were pulsed for 4 hr at 27°C (red bar) until the end of the night and then returned to 22°C for two additional days of recording. B) Results of forward thermogenetic transposon screen. C) Sleep profiles of hemizygous male CKIIβ^{GT1};ppk∩24C10>TrpA1 animals and control. D) Quantification of sleep deprivation (D1) and subsequent recovery sleep (D2) in (C). n ≥ 31 per group. E) Sleep profiles of mechanically sleep deprived (red bar) outcrossed female CKIIβ^{GT1} and control. F) Quantification of sleep deprivation (F1) and subsequent recovery sleep (F2) in (E). n ≥ 86 per group. G) CKIIβ^{GT1} males are rhythmic after 6 days in constant darkness. n ≥ 47 per group. In this and subsequent figures, *, **, ***, **** indicate $p < 0.05$, 0.01, 0.001 and 0.0001, respectively. Error bars indicate SEM.


A
Transposon Mutant x UAS-TrpA1;ppk∩24C10-Gal4
4 hr
Temperature (° C)
27
22
0
1
2
3
4
Days
B
Number of lines tested
225
200
175
150
125
100
75
50
25
0
-20
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
Rebound Sleep (mins)
C
ppk∩24C10>TrpA1
CKIIβGT1;ppk∩24C10>TrpA1
Sleep (min)
30
20
10
0
0
1
2
3
4
Days
D1
Δ sleep during SD (min)
0
-50
-100
-150
-200
ns
D2
Rebound sleep (min)
150
100
50
0
***
E
WT
CKIIβGT1
Sleep (min)
30
20
10
0
0
1
2
3
4
Days
F1
Δ sleep during SD (min)
0
-50
-100
-150
-200
ns
F2
Rebound sleep (min)
200
150
100
50
0
****
G
% Rhythmic
100
50
0
WT
CKIIβGT1

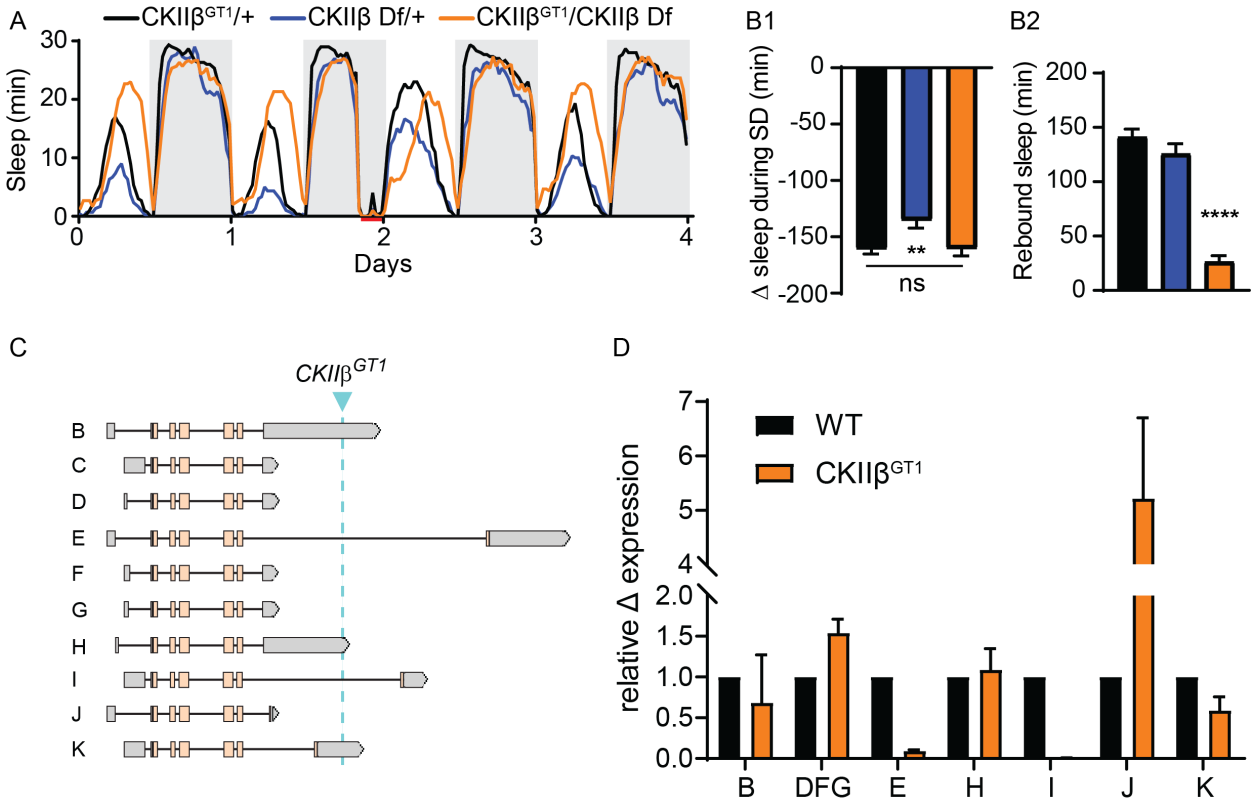


Figure 4.2 Genetic analysis of CKIIβ^{GT1} mutant. A) Sleep profiles of mechanically sleep deprived CKIIβ^{GT1}/Df females and genetic controls shows failure to complement. B) Quantification of sleep deprivation (B1) and subsequent recovery sleep (B2) in (A). n ≥ 90 per group. C) Diagram of alternative transcripts of CKIIβ and the insertion site of the GT1 transposon. Exons are denoted by tan boxes and UTR is denoted in grey. D) Quantitative PCR demonstrates that expression of transcripts E and I are greatly reduced, and expression of transcript J is increased in the GT1 mutant relative to WT. In this and subsequent figures all animals tested are female unless otherwise noted.

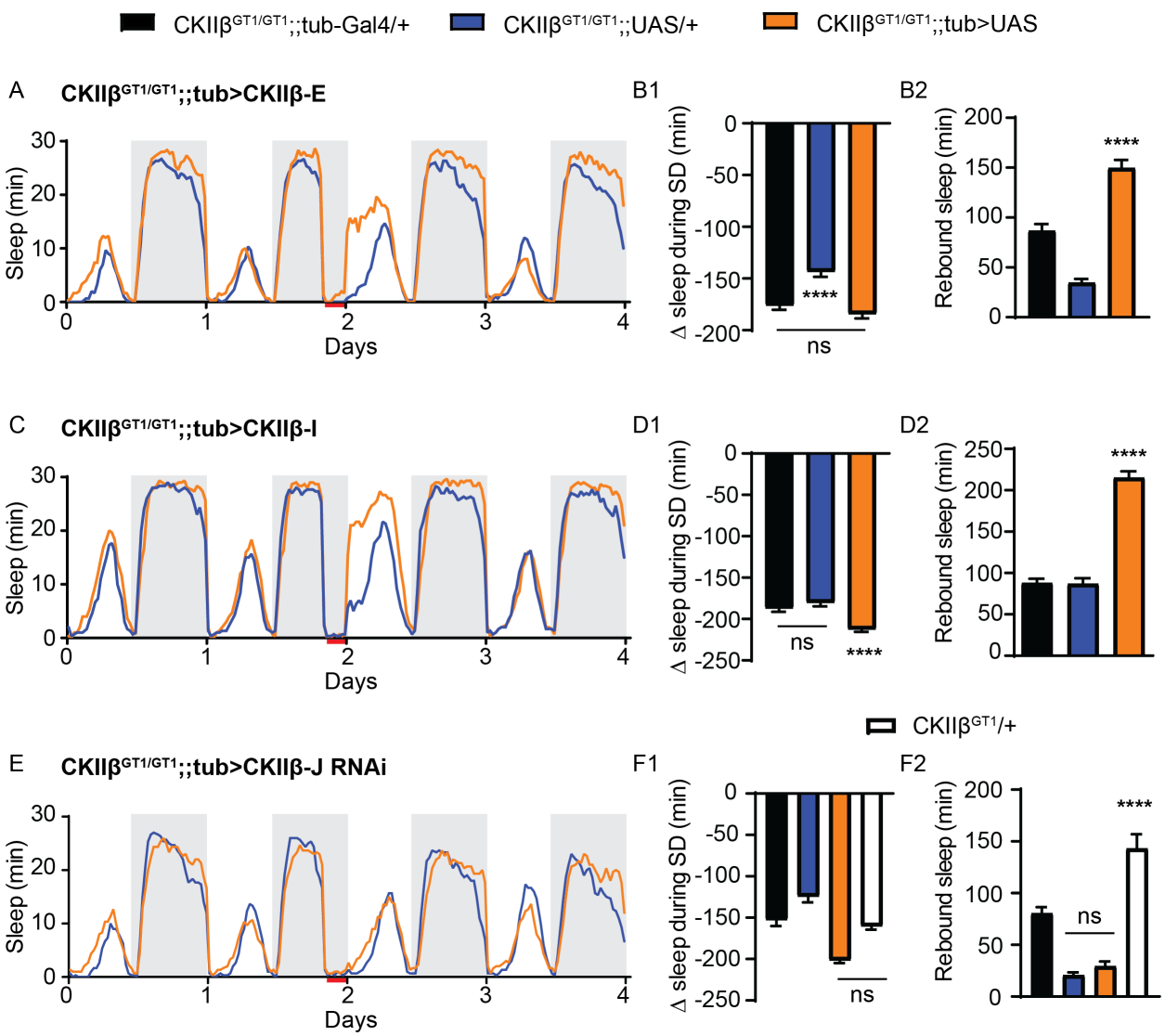


Figure 4.3 Restored expression of isoforms E and I are sufficient to rescue sleep homeostasis phenotype. A) Sleep profiles of genetic rescue with a transgene for transcript E and genetic control. B) Quantification of sleep deprivation (B1) and subsequent recovery sleep (B2) in (A). n ≥ 95 per group. C) Sleep profiles of genetic rescue with a transgene for transcript I and genetic control. D) Quantification of sleep deprivation (D1) and subsequent recovery sleep (D2) in (C). n ≥ 84 per group. E) Sleep profiles of genetic rescue with shRNA against transcript J and genetic control. F) Quantification of sleep deprivation (F1) and subsequent recovery sleep (F2) in (E). n ≥ 88 per group.

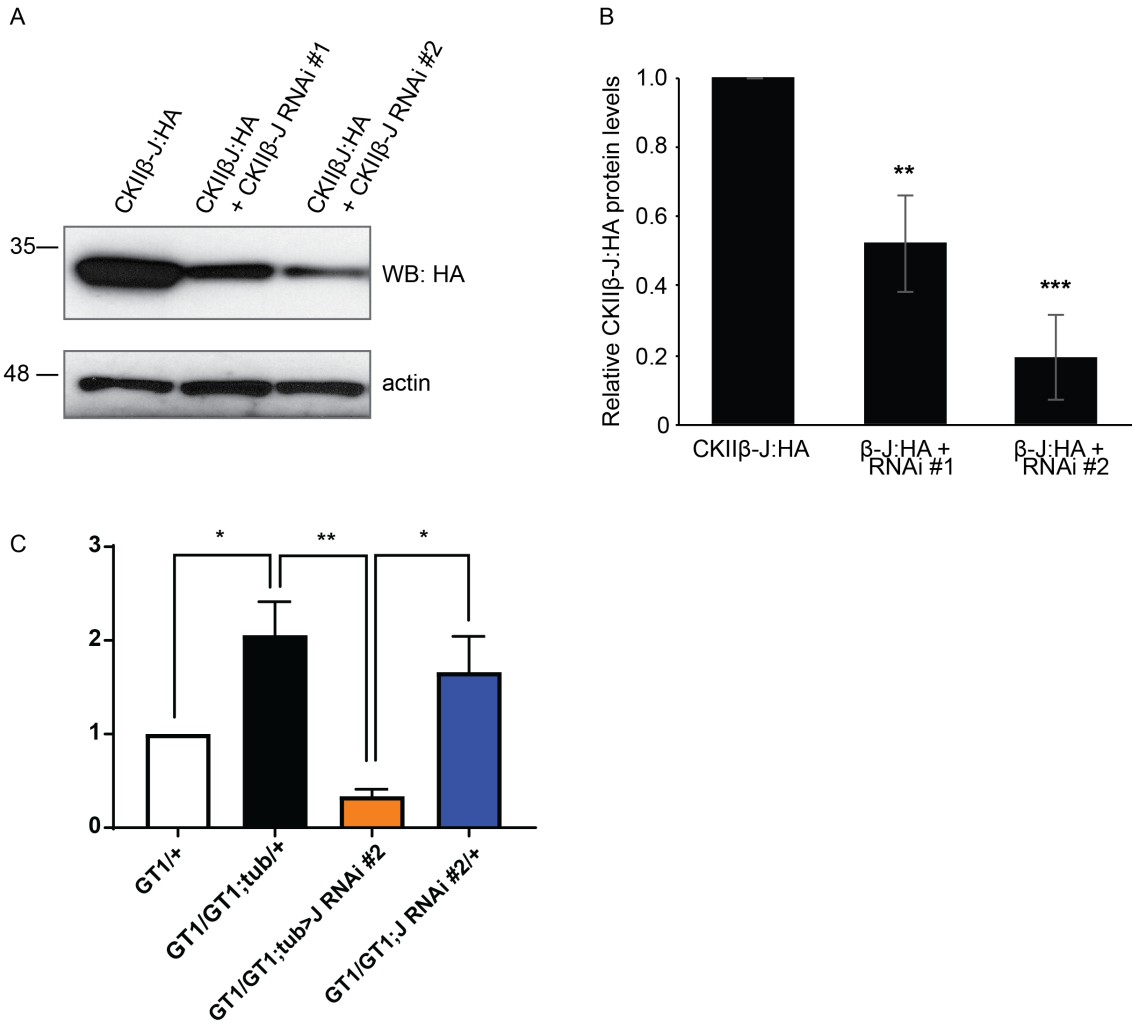


Figure 4.4 Validation of CKIIβ-J RNAi *in vitro* and *in vivo*. A) Representative western blot of CKIIβ-J:HA knockdown in transfected S2 cells. B) Quantification of knockdown in (A). C) Quantitative PCR demonstrates significant reduction in CKIIβ-J expression in heads of homozygous mutants relative to controls. n = 3 biological replicates of ≥ 20 animals per experiment.

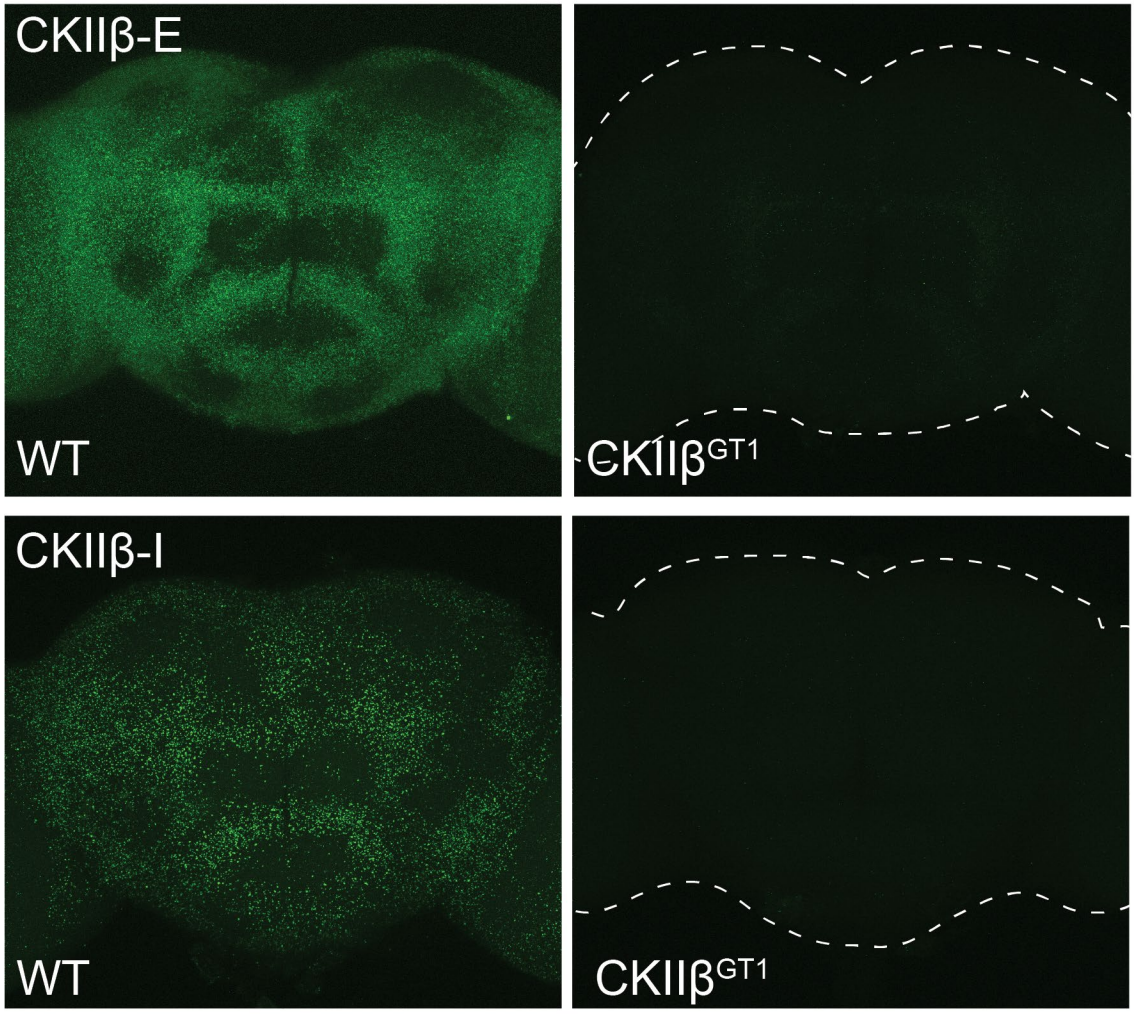


Figure 4.5 CKIIβ transcripts E and I express broadly throughout the brain. Representative images of CKIIβ-E and -I transcript expression in WT and GT1 mutant brains. RNAscope reveals broad expression of CKIIβ-E and -I transcripts in WT brains and that expression is nearly undetectable in GT1 mutants.

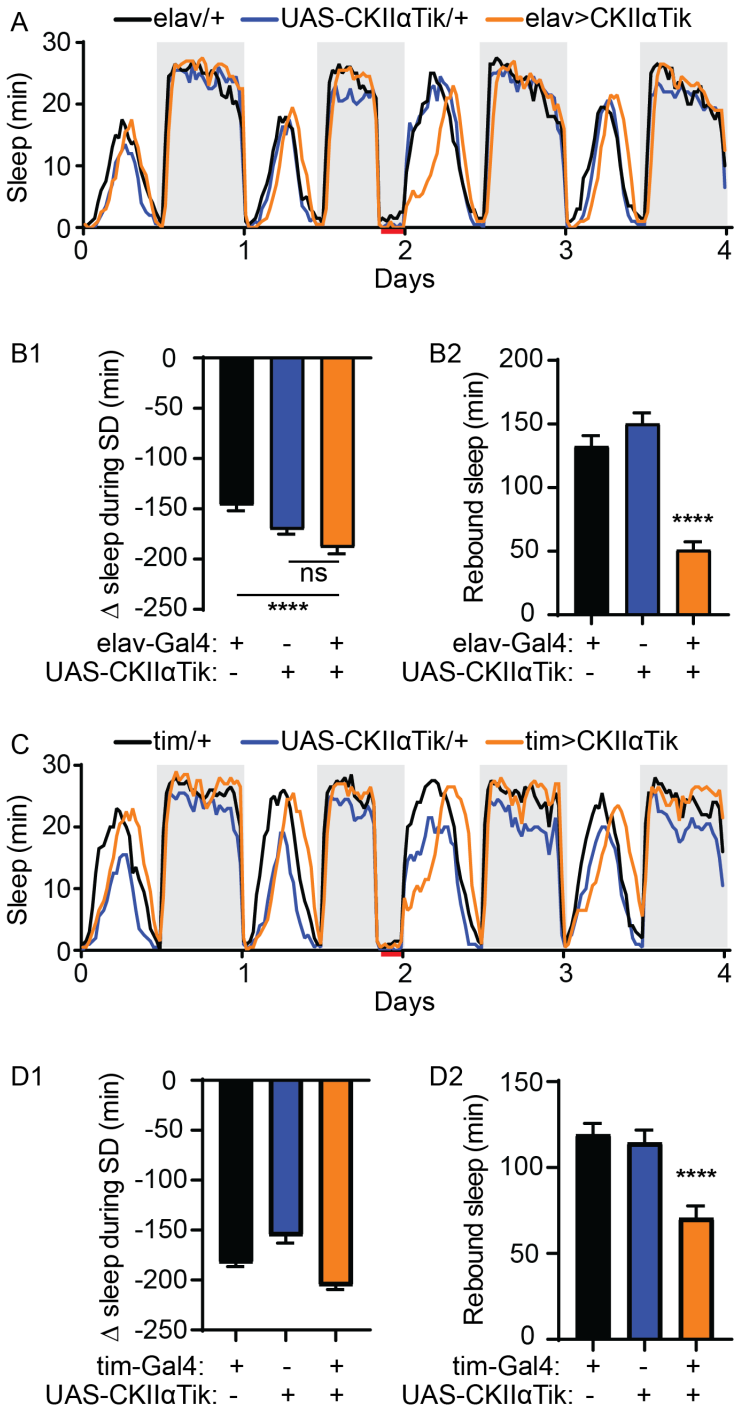


Figure 4.6 CKIIα is required for sleep homeostasis. A) Sleep profiles of mechanically sleep deprived elav-Gal4>CKIIαTik females and genetic controls shows that overexpression of dominant negative CKIIα in the CNS phenocopies CKIIβ^{GT1} phenotype. B) Quantification of sleep deprivation (B1) and subsequent recovery sleep (B2) in (A). n ≥ 60 per group. C) Sleep profiles of mechanically sleep deprived tim-Gal4>CKIIαTik females and genetic controls shows that sleep homeostasis phenotype maps to clock neurons. D) Quantification of sleep deprivation (D1) and subsequent recovery sleep (D2) in (C). n ≥ 60 per group.

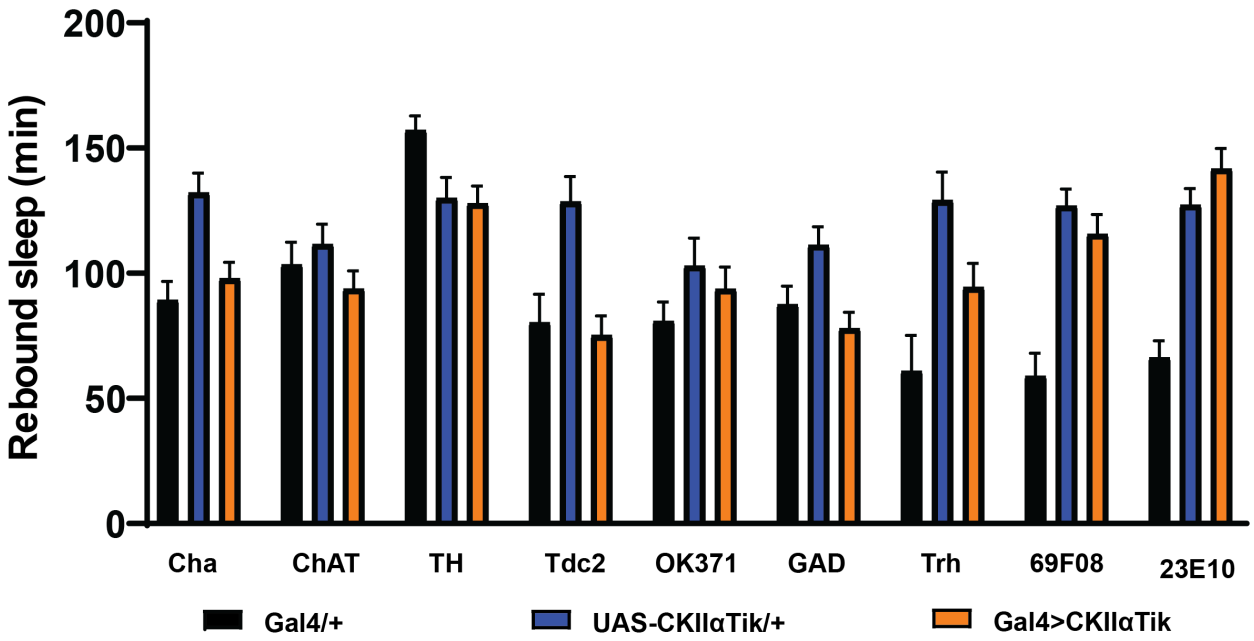


Figure 4.7 CKII sleep homeostasis phenotype does not map to neurons that express common neurotransmitters or sleep promoting brain regions. Total recovery sleep following mechanical sleep deprivation measured in indicated Gal4>Tik combinations and genetic controls. n ≥ 50 per group.

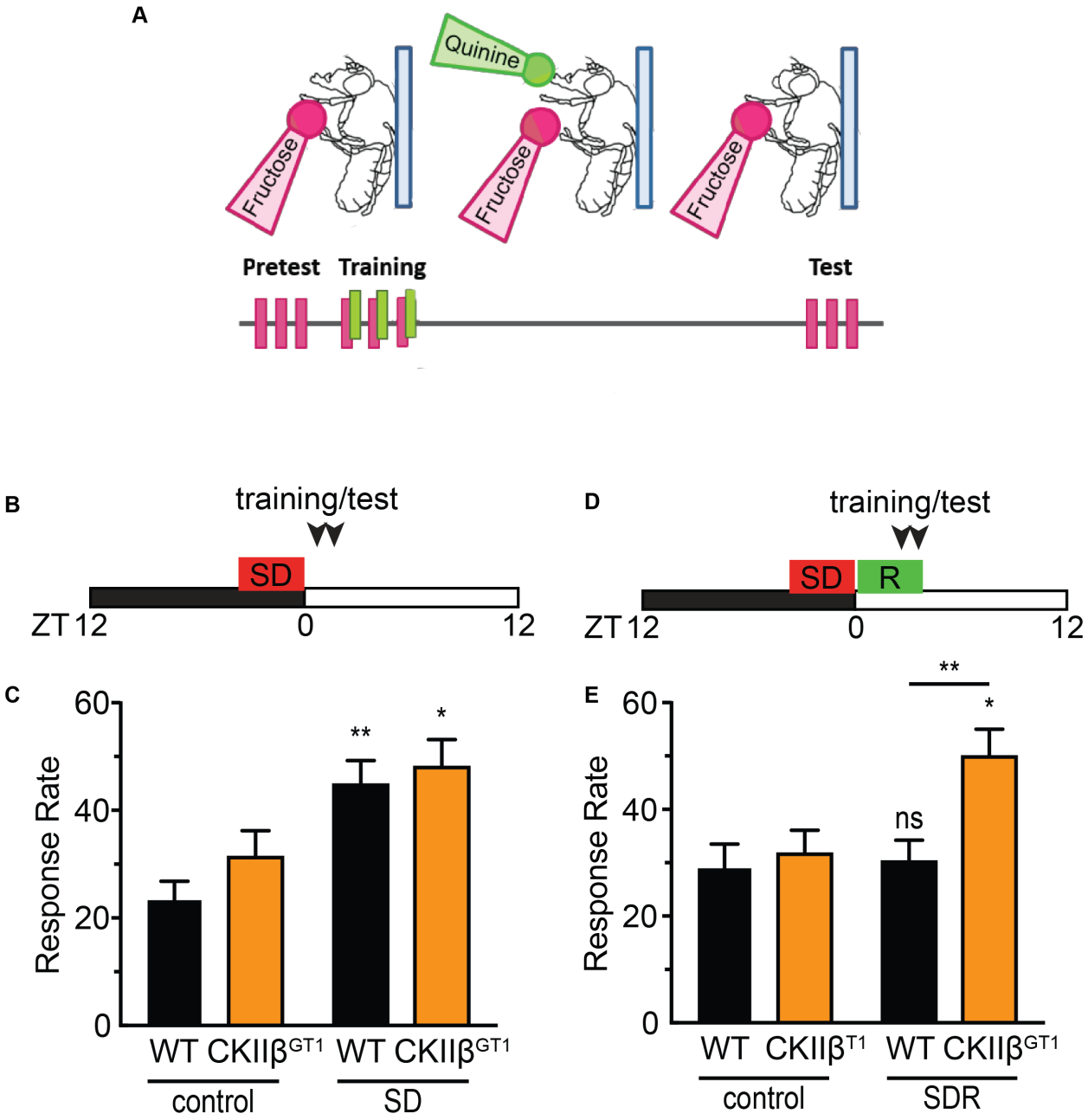


Figure 4.8 CKII is required for sleep-dependent memory formation. A) Protocol for aversive taste memory assay. Animals are trained with three presentations of fructose and quinine solutions leading to a reduction in the proboscis extension reflex (PER) during testing when fructose is presented alone. B) Protocol for testing in C. Animals were sleep deprived form ZT 20 – 24, glued to a platform, then trained and immediately tested at ZT 2. C) WT and GT1 mutant both have impaired memory formation after sleep deprivation compared to undisturbed controls. D) Protocol for testing in E. Animals were sleep deprived as in B but allowed to rest until ZT 4 before training and testing. E) Only sleep deprived GT1 mutants that fail to recover sleep have impaired memory formation.

Table 4.1 Sleep homeostasis phenotypes of CKIIβ transposon mutant genetic complementation experiments. Genotypes, total number of animals tested (n), mean of sleep lost during deprivation and rebound sleep following deprivation with standard deviation for each.

Genotype	n	Mean Sleep Deprivation (min)	Standard Dev.	Mean Rebound Sleep (min)	Standard Dev.
iso31	95	-171.2	46.68	124.9	61.79
CKIIβ GT1	93	-164.3	68.14	15.27	44.99
CKIIβ GT1/+	64	-175.2	41.31	129	81.09
CKIIβ TP2	31	-211.8	19.65	166.9	66.7
CKIIβ TP3	52	-130.8	80.23	82.48	57.9
CKIIβ TP3/+	64	-173.8	35.21	188.9	69.47
CKIIβ GT1/TP3	63	-132.5	40.51	89.84	52.68
CKIIβ TP3/Df	62	-105.1	82.11	78.03	65.1
CKIIβ Df/+	61	-104.1	75.22	92.61	64.91

Table 4.2 Primers used for quantitative PCR.

Name	5'->3'
CkIIbeta Ex3 qR1	AGATCATGTCCAACGCTTGC
CkIIbeta Ex5-6 qF	TCAGTTTGTTCCAAGGCTATATG
CKIIbeta-RB Ex7 qF4	CGTTGCGGATTGTTTGTG
CKIIbeta-RB Ex7 qR3	GTTCTCCTAATTACGCAAATATTC
CKIIbeta-RE Ex7 qF3	CCAGACCTGTTCCCAAATTC
CKIIbeta-RE Ex7 qR2	TTGATGTTGGTCCTCCTGTC
CkIIbeta-R-DFG Ex1 qF1	ACAGTGTGTGCTTCTTCTTGC
CkIIbeta-RH Ex1 qF1	CAGCGGGAAAAATACAGAATAGC
CKIIbeta-RI Ex7 qR3	GTATCGTCGTTGCTGAATGG
CKIIbeta-RI Ex7 qR4	GAGTGCAATGTAGTCCGCTC
CkIIbeta-RJ Ex6-7 qR1	CCGTTGTAAACGCTCGTA
CKIIbeta-RK Ex6-7 qR1	GCTTCGGGCGCTCGTAGT
CKIIbeta-RK Ex7 qR2	CTTGCCGGCCACTACTTT

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