UNIVERSITY OF CALIFORNIA, SAN DIEGO

Genetic and Anatomical Dissection of Sleep and Arousal in Drosophila melanogaster

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

in

Biomedical Sciences

by

James Edward Robinson

Committee in Charge:

Professor William Joiner, Chair
Professor Rolf Bodmer
Professor Joan Heller Brown
Professor Gabriel Haddad
Professor Jing Wang

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The dissertation of James Edward Robinson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015

DEDICATION

In recognition of the support, assistance, love, and patience given to me, this dissertation is dedicated to:

My parents, who have always fostered an environment of lifelong learning and curiosity. Thank you for providing me with the experiences that have shaped me into who I am today.

My siblings, who challenge and push each other toward betterment. Thank you for the fun we have had and will have.

My daughter, Edith, who despite the brevity of her time with us so far, has already shown me another fun-filled side of life. Thank you for the joy you bring and will continue to bring.

My wife, who tolerates and supports my ambitions and interests while keeping me focused on what is truly important. Thank you for your love and patience as we journey through life together.

EPIGRAPH

Always plan with the end result in mind… Be clear. Be specific.

Stephanie Goddard Davidson

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LIST OF ABBREVIATIONS

AD	Activating Domain
ADAR	Adenosine Deaminase Acting on RNA
AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AMPAR	AMPA Receptor
ANOVA	Analysis of Variance
AT50	Arousal Threshold for Half Maximal Response
au	Arbitrary Units
Cas9	CRISPR Associated Protein 9
CD8::GFP	Cluster of Differentiation 8 fused to Green Fluorescent Protein
CDS	Coding DNA Sequence
CGXXXX	Computed Gene Number XXXX
Cha	Choline Acetyltransferase
Clk	Clock
CNS	Central Nervous System
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats
CT	Circadian Time
Cyc	Cycle
DAMS	Drosophila Activity Monitoring System
DAM2	Drosophila Activity Monitoring System 2
DBD	DNA Binding Domain
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
DVGLUT	Drosophila Vesicular Glutamate Transporter
EC50	Effective Concentration for Half-Maximal Response
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
EJP	Excitatory Junctional Potential
EMG	Electromyogram
EtOH	Ethanol
G0	Generation 0
GAL4	Galactose pathway gene 4
GAL80	Galactose pathway gene 80
GFP	Green Fluorescent Protein
GluR1	Glutamate Receptor 1
GluR1B	Glutamate Receptor 1 B
GluRIIA	Glutamate Receptor 2 A
GluRIIB	Glutamate Receptor 2 B
GluRIIC	Glutamate Receptor 2 C
GluRIID	Glutamate Receptor 2 D
HA	Hemagglutinin
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HRP	Horse Radish Peroxidase

Hyp	Hypomorph
IBMX	3-isobutyl-1-methylxanthine
Kir	Inward Rectifying Potassium Channel
LNv	Lateral Ventral Neurons
MB	Mushroom Bodies
mEJP	Miniature Excitatory Junctional Potential
mGluRA	Metabotropic Glutamate Receptor A
nAChR	Nicotinic Acetylcholine Receptor
NF1	Neurofibromin Factor 1
NMDA	*N*-Methyl-D-Aspartate
NMDAR	NMDA Receptor
NMJ	Neuromuscular Junction
NR1	NMDA Receptor 1
NR2	NMDA Receptor 2
NTC	No Template Control
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Triton X-100
PCR	Polymerase Chain Reaction
PDF	Pigment Dispersing Factor
Per	Period
PER	Proboscis Extension Reflex
PFA	Paraformaldehyde
PKA	Protein Kinase A
ppk	Pick Pocket
qPCR	Quantitative PCR
REM	Rapid Eye Movements
RNA	Ribonucleic Acid
RNAi	RNA Interference
RP	Reserve Pool
RRP	Readily Releasable Pool
SD	Sleep Deprivation
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
Sh	Shaker
SHY	Synaptic Homeostasis Hypothesis
sss	SLEEPLESS
SWS	Slow Wave Sleep
Syn	Synapsin
Syt	Synaptotagmin
Tdc2	Tyrosine decarboxylase 2
TG	Thoracic Ganglia
TH	Tyrosine Hydroxylase
TrpA1	Transient Receptor Potential Cation Channel A1 Ortholog
UAS	Upstream Activating Sequence

VGLUT	Vesicular Glutamate Transporter
w	White
WT	Wild-Type
y	Yellow
ZT	Zeitgeber Time

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Work presented in Chapter 2 with minor modifications is submitted for publication to *Nature Communications* with coauthors Jeremy Paluch, Dion Dickman, and William Joiner. The dissertation author was the primary author of this work and performed all experiments except for those involving electrophysiology, which were performed by Jeremy Paluch.

Work presented in Chapter 3 will be submitted for publication upon completion of follow-up studies. The dissertation author was the primary author of this work in collaboration with Glen Seidner, Steve Roberts, and William Joiner.

Work presented in Chapter 4 with minor modifications is under review for publication in *Current Biology*. Coauthors Kurtresha Worden, Pavel Masek, and Alex Keene performed all learning and memory assays and analysis. Meilin Wu performed all of the molecular biology presented in this work. The

dissertation author was the co-primary author of this work with Glen Seidner and corresponding author William Joiner. The dissertation author performed all imaging experiments along with some of the sleep/deprivation assays, design, analysis and interpretation of the data.

Work presented in Chapter 5 will be submitted for publication upon completion of follow-up studies. The dissertation author was the primary author of this work in collaboration with Glen Seidner and William Joiner.

Work presented in Chapter 6 is modified from parts of “SLEEPLESS is a bi-functional regulator of excitability and cholinergic synaptic transmission,” which was published in *Current Biology* vol. 24, pages 621-629 in 2014 with primary author Meilin Wu and corresponding author William Joiner. The dissertation author performed experiments and analysis included in figures 6.2, 6.3, and 6.4.

VITA

Education:

2008 Bachelor of Science, Biology
Iowa State University
Magna Cum Laude

2015 Doctor of Philosophy, Biomedical Sciences
University of California, San Diego

Publications:

Ratliff EP, Mauntz RE, Kotzebue RW, Gonzalez A, Achal M, Barekat A, Finley KA, Sparhawk JM, **Robinson JE**, Herr DR, Harris GL, Joiner WJ, Finley KD. (2015). Aging and autophagic function influences the progressive decline of adult Drosophila behaviors. *Plos One*. 10, e0132768

Bailey KL, Wyatt TA, Wells SM, Klein EB, **Robinson JE**, Romberger DJ, Poole JA. (2014). Dimethylarginine Dimethylaminohydrolase (DDAH) Overexpression Attenuates Agricultural Organic Dust Extract-Induced Inflammation. *Journal of Env. Immun. and Toxicology.* 2, 72-78

Wu M, **Robinson JE**, Joiner WJ. (2014). SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission. *Current Biology*. 24, 621-629

Bailey KL, Bonasera SJ, Wilderdyke M, Hanisch BW, Pavlik JA, DeVasure J, **Robinson JE**, Sisson JH, Wyatt TA. (2014). Aging causes slowing in ciliary beat frequency, mediated by PKCε. *Am. J. Physiol. – Lung C.* 306, 584-589

Heinrichsen ET, Zhang H, **Robinson JE**, Ngo J, Diop S, Bodmer R, Joiner WJ, Metallo CM, Haddad GG. (2014). Metabolic and transcriptional response to a high-fat diet in *Drosophila melanogaster*. *Molecular Metabolism*. 3, 41-54

Bailey KL, **Robinson JE**, Sisson JH, Wyatt TA. (2011). Alcohol decreases RhoA activity through a nitric oxide (NO)/cyclic GMP (cGMP)/protein kinase G (PKG)-dependent pathway in airway epithelium. *Alcoholism: Clinical and Experimental Research*. 35, 1277-81

Bailey KL, Sisson JH, Romberger DJ, **Robinson JE**, Wyatt TA. (2010). Alcohol up-regulates TLR2 through a NO/cGMP dependent pathway. *Alcoholism: Clinical and Experimental Research.* 34, 51-56

Allen-Gipson DS, Jarrell JC, Bailey KL, **Robinson JE**, Kharbanda KK, Sisson JH, Wyatt TA. (2009). Ethanol blocks adenosine uptake via inhibiting the nucleoside transport system in bronchial epithelial cells. *Alcoholism: Clinical and Experimental Research*. 33, 791-798

ABSTRACT OF THE DISSERTATION

Genetic and Anatomical Dissection of Sleep and Arousal in Drosophila melanogaster

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James Edward Robinson

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Professor William Joiner, Chair

Sleep and arousal are among the major mysteries remaining in biology. Animals are rendered defenseless, unable to forage for food, and immobile during sleep and yet sleep is necessary since complete deprivation results in significant health deficits and eventually death. In humans, artificial lighting and societal pressures have reduced the total amount of time spent sleeping over the last century. Chapter 1 of this thesis provides background information on our current understandings of sleep, the role of sleep in disease and

Drosophila as a model organism for the study of sleep. Chapter 2 describes the RNA editing enzyme, ADAR, as a novel regulator of sleep and glutamatergic plasticity in *Drosophila* whereby ADAR acts to restrict the size of the synaptic vesicle reserve pool in glutamatergic sleep-promoting neurons. Chapter 3 details a novel arousal threshold measuring apparatus that expands our ability to measure and understand sleep and arousal in *Drosophila*. Chapter 4 presents a study in which we show multiple neurotransmitter systems promoting arousal yet differentially promote sleep homeostasis and the cognitive consequences of homeostatic recovery sleep. Chapter 5 describes work probing the role of the gene *Nf1* as a sleep-promoting factor and its localization. Finally, Chapter 6 presents an extension of studies performed on the previously described sleep-promoting gene *sss* and its bifunctional role in regulating neuronal excitability and cholinergic synaptic transmission.

Chapter 1: Introduction

1.1 Sleep as a historical question

The ubiquitous and prominent role that sleep plays in the life of every human being has prompted numerous theories about both the process of how sleep occurs and the role sleep plays in normal physiology. For many centuries, philosophers proposed theories about the role of sleep. Even within academic settings, sleep research was not formally conducted and the field lacked a sense of community and formalization. Many physiologists during these early periods of sleep theories postulated explanations for sleep involving animal spirits, cerebral vibrations, or changes in nervous fluid quantities or mobility [1-4]. In the late 18th century, Immanuel Kant's widely translated and published lectures marked an important transition from sleep being the subject matter of philosophers to a serious field of study for physiologists:

> By verbal definition, sleep is a condition in which a healthy person is unable to be aware of ideas through external senses. However, to find an explanation of this remains for the physiologists who, if they are able, may explain this exhaustion, which is also a gathering of strength for renewed external perception (whereby man considers himself a newborn in the world, and whereby probably a third of our life-span passes unaware and unregretted) [5].

For the better part of the 19th century, mounting evidence from Albrecht von Haller and David Hartley among others suggested a role for the circulation of blood in the regulation of sleep. This original theory stated that venous

congestion was responsible for increasing pressure on the brain and thus reducing flow of nervous fluid. Primarily, observations that included pressure placed on the brain through a fractured skull induces unconsciousness as well as abundant sleep in young children whose brains were thought to be growing within a restrictive skull were chief among the evidence supporting this theory.

In 1913, Henri Piéron published *Le problème physiologique du sommeil* [6] which examined sleep more rigorously from a physiological perspective and is considered the beginning of modern sleep research. As the 20th century progressed, crucial studies were performed detailing the homeostatic regulation of sleep [7], rapid eye movements (REM) during sleep [8], and the cyclical nature of sleep [9]. These seminal findings form the basis of all contemporary sleep research.

After describing the basis of fundamental sleep research, contemporary efforts have focused on the genetics of sleep regulation. Over the years, research has shown that sleep is a genetically regulated process. EEG measurements from monozygotic twins are more similar than dizygotic twins and monozygotic twins have more similar sleep onset times and amounts [10-13]. Further, family studies and dog models of sleep have identified individual genes which, when mutated, cause abnormal sleep [14-16].

1.2 Sleep in health and disease

Given the ubiquitous and conserved nature of sleep, it is unsurprising that sleep disruptions, genetically or otherwise, are detrimental to health. The

importance of sleep is exemplified by the fact that complete deprivation of sleep in rats leads to death in approximately the same amount of time as starvation [17]. Sleep-loss induced lethality has also been suggested in fatal familial insomnia [18].

To a lesser degree, sleep disruptions are involved in a variety of pathological conditions. Reduced sleep has been associated with depression, pain, hypertension, diabetes, and cardiovascular disease [19-24]. Given the broad range of processes that sleep influences, it can be safely assumed that many of the effects are secondary and not directly related to sleep. However, due to the wide range of disease states that can be influenced by lost sleep, it is easy to understand the underlying importance of sleep to health and society.

While sustained sleep deprivation leads to lethality and other chronic health issues, short-term sleep deficits result in cognitive deficits that can prove costly. Sleep deprivation as short as 17-19 hours results in significant increases in reaction time and reduces driving performance to that of or worse than individuals with blood alcohol contents of 0.05% or higher [25]. In fact, drowsy driving accounts for at least 2.5% of all fatal car accidents in the United States with some estimates as high as 15-33% [26].

From a cognitive perspective, sleep is intricately involved in learning and memory [27, 28]. In recent years, a theory of the function of sleep has arisen to incorporate the involvement of sleep in learning/memory and plasticity. The Synaptic Homeostasis Hypothesis (SHY) roots its foundation in non-Hebbian plasticity [29-31] and states that sleep promotes net synaptic

downscaling to offset net synaptic potentiation that accumulates during waking. This hypothesis, despite its criticisms [32], is sufficient to explain processes that are theorized to occur during sleep to consolidate learned information into memories. While our lab is agnostic regarding SHY, data presented in Chapter 2 of this thesis can be explained, in part, using SHY and thus feel it is worth addressing.

1.3 *Drosophila* as a model organism for sleep research

In order to facilitate the study of sleep, model organisms are a critical component to the field. By utilizing model organisms, genetic perturbations of sleep can occur in a rapid and directed fashion. To date, the primary genetic models for sleep research include mice, zebrafish, *Drosophila*, and *C. elegans* [33-37]. Each model organism has its own advantages and disadvantages. For example, mice are most closely related to humans and thus findings are more easily applicable to human health and disease. Also, mice are large relative to other organisms and can thus be fitted for such measurements as EEG and EMG [38], which provide the most information about sleep states and depths. On the other hand, the generation times of mice are often much slower than other genetic organisms and mice can be vastly more expensive to maintain than invertebrate models. Other organisms, such as *Drosophila*, offer advantages of rapid generation times, powerful genetic tools, low genetic redundancy, and low costs. However, *Drosophila* are not without their disadvantages. While reports have been published regarding

electrophysiological changes that occur during sleep in flies [39, 40], these techniques are technically challenging and difficult to interpret.

Our lab has chosen to focus on *Drosophila* as model organism to study sleep. It is our opinion that each model organism can provide unique perspectives on our understanding of sleep as a whole. We believe that the genetic contribution to sleep is underrepresented in the sleep field as a whole and that genetic regulators of sleep may yield insight into the biological function of sleep that may otherwise be difficult to glean from other models of sleep research.

The formal study of sleep in *Drosophila* is only beginning to leave its infancy since the field has only been around for approximately 15 years after the first descriptions of a behavior in flies that satisfies all of the fundamental features of sleep [35, 36]. Similar to mammals, sleep and arousal in *Drosophila* can be regulated through activation of dopaminergic or octopaminergic (homologous in function to noradrenergic neurons) [41-47]. Additionally, other neurotransmitter systems play a role in portions of arousal in flies [48-51]. For the most part, neurotransmitters that regulate sleep in mammals play similar roles in sleep regulation in *Drosophila* suggesting conserved regulation and logic for sleep as a behavior [52].

Drosophila exhibit robust circadian rhythms, which have been postulated in the two-process model of sleep regulation to govern the timing of sleep [53]. Indeed, altering activity of “clock” neurons in flies results in altered sleep timing and amounts [49-51, 54-56]. Many discoveries regarding

circadian rhythms were originally made in *Drosophila* such as the identification of *per* and *tim* [57, 58]. Through the identification of novel genes that regulate circadian rhythms, many of the core discoveries describing the molecular clock have been made using *Drosophila*. We postulate that if similarly definable molecular and genetic mechanisms for sleep regulation exist, we should be able to elucidate these pathways using the powerful genetics of *Drosophila*. To accomplish this task, we performed a screen for genes involved in sleep regulation. In order to perform this screen, we utilized the Gal4/UAS system [59] to couple the pan-neuronal driver *elav-Gal4* with various *UAS-RNAi* transgenes to knock down the expression of neuronally expressed genes specifically in the nervous system. From this screen, we identified two genes with significant deviation of total sleep from the mean of the population (data not shown). These genes, *Adar* and *Nf1,* are the focus of Chapter 2 and 5, respectively.

This work presented in this thesis aims to further our understanding of the genetic regulation of sleep (Chapters 2, 5, and 6), provide novel insights into experimental paradigms for the study of arousal (Chapter 3), and demonstrate privileged roles of independent arousal systems with differential effects on sleep homeostasis (Chapter 4).

1.4 References

1. Hammond, W.A. (1869). Sleep and its derangements, (Philadelphia: Lippincott).

2. Duval, M.M. (1895). Hypothèses sur la physiologie des centres nerveux: théorie histologique du sommeil.

3. Radestock, P. (1879). Schlaf und Traum, (Рипол Классик).

4. Pupin, C. (1896). Le neurone et les hypothèses histologiques sur son mode de fonctionnement: théorie histologique du sommeil, (G. Steinheil).

5. Kant, I., and Louden, R.B. (2006). Anthropology from a pragmatic point of view, (Cambridge, UK ; New York: Cambridge University Press).

6. Piéron, H. (1913). Le problème physiologique du sommeil, (Masson et Cie).

7. Lee, M.A., and Kleitman, N. (1923). Studies on the Physiology of Sleep. American Journal of Physiology--Legacy Content *67*, 141-152.

8. Aserinsky, E., and Kleitman, N. (1953). Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. Science *118*, 273-274.

9. Dement, W., and Kleitman, N. (1957). Cyclic variations in EEG during sleep and their relation to eye movements, body motility, and dreaming. Electroencephalogr Clin Neurophysiol *9*, 673-690.

10. Anokhin, A., Steinlein, O., Fischer, C., Mao, Y., Vogt, P., Schalt, E., and Vogel, F. (1992). A genetic study of the human low-voltage electroencephalogram. Hum Genet *90*, 99-112.

11. Ambrosius, U., Lietzenmaier, S., Wehrle, R., Wichniak, A., Kalus, S., Winkelmann, J., Bettecken, T., Holsboer, F., Yassouridis, A., and Friess, E. (2008). Heritability of sleep electroencephalogram. Biol Psychiatry *64*, 344-348.

12. De Gennaro, L., Marzano, C., Fratello, F., Moroni, F., Pellicciari, M.C., Ferlazzo, F., Costa, S., Couyoumdjian, A., Curcio, G., Sforza, E., Malafosse, A., Finelli, L.A., Pasqualetti, P., Ferrara, M., Bertini, M., and Rossini, P.M. (2008). The electroencephalographic fingerprint of sleep is genetically determined: a twin study. Ann Neurol *64*, 455-460.

13. Dauvilliers, Y., Maret, S., and Tafti, M. (2005). Genetics of normal and pathological sleep in humans. Sleep Med Rev *9*, 91-100.

14. Winkelmann, J., Schormair, B., Lichtner, P., Ripke, S., Xiong, L., Jalilzadeh, S., Fulda, S., Putz, B., Eckstein, G., Hauk, S., Trenkwalder, C., Zimprich, A., Stiasny-Kolster, K., Oertel, W., Bachmann, C.G.,

Paulus, W., Peglau, I., Eisensehr, I., Montplaisir, J., Turecki, G., Rouleau, G., Gieger, C., Illig, T., Wichmann, H.E., Holsboer, F., Muller-Myhsok, B., and Meitinger, T. (2007). Genome-wide association study of restless legs syndrome identifies common variants in three genomic regions. Nat Genet *39*, 1000-1006.

15. Hallmayer, J., Faraco, J., Lin, L., Hesselson, S., Winkelmann, J., Kawashima, M., Mayer, G., Plazzi, G., Nevsimalova, S., Bourgin, P., Hong, S.C., Honda, Y., Honda, M., Hogl, B., Longstreth, W.T., Jr., Montplaisir, J., Kemlink, D., Einen, M., Chen, J., Musone, S.L., Akana, M., Miyagawa, T., Duan, J., Desautels, A., Erhardt, C., Hesla, P.E., Poli, F., Frauscher, B., Jeong, J.H., Lee, S.P., Ton, T.G., Kvale, M., Kolesar, L., Dobrovolna, M., Nepom, G.T., Salomon, D., Wichmann, H.E., Rouleau, G.A., Gieger, C., Levinson, D.F., Gejman, P.V., Meitinger, T., Young, T., Peppard, P., Tokunaga, K., Kwok, P.Y., Risch, N., and Mignot, E. (2009). Narcolepsy is strongly associated with the T-cell receptor alpha locus. Nat Genet *41*, 708-711.

16. Lin, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., Qiu, X., de Jong, P.J., Nishino, S., and Mignot, E. (1999). The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell *98*, 365-376.

17. Everson, C.A., Bergmann, B.M., and Rechtschaffen, A. (1989). Sleep deprivation in the rat: III. Total sleep deprivation. Sleep *12*, 13-21.

18. Goldfarb, L.G., Petersen, R.B., Tabaton, M., Brown, P., LeBlanc, A.C., Montagna, P., Cortelli, P., Julien, J., Vital, C., Pendelbury, W.W., and et al. (1992). Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism. Science *258*, 806-808.

19. Mullington, J.M., Haack, M., Toth, M., Serrador, J.M., and Meier-Ewert, H.K. (2009). Cardiovascular, inflammatory, and metabolic consequences of sleep deprivation. Prog Cardiovasc Dis *51*, 294-302.

20. Johnson, E.O., Roth, T., and Breslau, N. (2006). The association of insomnia with anxiety disorders and depression: exploration of the direction of risk. J Psychiatr Res *40*, 700-708.

21. Gangwisch, J.E., Heymsfield, S.B., Boden-Albala, B., Buijs, R.M., Kreier, F., Pickering, T.G., Rundle, A.G., Zammit, G.K., and Malaspina, D. (2006). Short sleep duration as a risk factor for hypertension: analyses of the first National Health and Nutrition Examination Survey. Hypertension *47*, 833-839.

22. Gangwisch, J.E., Heymsfield, S.B., Boden-Albala, B., Buijs, R.M., Kreier, F., Pickering, T.G., Rundle, A.G., Zammit, G.K., and Malaspina, D. (2007). Sleep duration as a risk factor for diabetes incidence in a large U.S. sample. Sleep *30*, 1667-1673.

23. Gottlieb, D.J., Punjabi, N.M., Newman, A.B., Resnick, H.E., Redline, S., Baldwin, C.M., and Nieto, F.J. (2005). Association of sleep time with diabetes mellitus and impaired glucose tolerance. Arch Intern Med *165*, 863-867.

24. Ayas, N.T., White, D.P., Manson, J.E., Stampfer, M.J., Speizer, F.E., Malhotra, A., and Hu, F.B. (2003). A prospective study of sleep duration and coronary heart disease in women. Arch Intern Med *163*, 205-209.

25. Williamson, A.M., and Feyer, A.M. (2000). Moderate sleep deprivation produces impairments in cognitive and motor performance equivalent to legally prescribed levels of alcohol intoxication. Occup Environ Med *57*, 649-655.

26. Centers for Disease, C., and Prevention (2013). Drowsy driving - 19 states and the District of Columbia, 2009-2010. MMWR Morb Mortal Wkly Rep *61*, 1033-1037.

27. Stickgold, R. (2005). Sleep-dependent memory consolidation. Nature *437*, 1272-1278.

28. Walker, M.P. (2009). The role of sleep in cognition and emotion. Ann N Y Acad Sci *1156*, 168-197.

29. Turrigiano, G.G. (2008). The self-tuning neuron: synaptic scaling of excitatory synapses. Cell *135*, 422-435.

30. Turrigiano, G. (2011). Too many cooks? Intrinsic and synaptic homeostatic mechanisms in cortical circuit refinement. Annu Rev Neurosci *34*, 89-103.

31. Burrone, J., and Murthy, V.N. (2003). Synaptic gain control and homeostasis. Curr Opin Neurobiol *13*, 560-567.

32. Frank, M.G. (2012). Erasing synapses in sleep: is it time to be SHY? Neural Plast *2012*, 264378.

33. Crocker, A., and Sehgal, A. (2010). Genetic analysis of sleep. Genes Dev *24*, 1220-1235.

34. Zhdanova, I.V., Wang, S.Y., Leclair, O.U., and Danilova, N.P. (2001). Melatonin promotes sleep-like state in zebrafish. Brain Res *903*, 263-268.

35. Hendricks, J.C., Finn, S.M., Panckeri, K.A., Chavkin, J., Williams, J.A., Sehgal, A., and Pack, A.I. (2000). Rest in Drosophila is a sleep-like state. Neuron *25*, 129-138.

36. Shaw, P.J., Cirelli, C., Greenspan, R.J., and Tononi, G. (2000). Correlates of sleep and waking in Drosophila melanogaster. Science *287*, 1834-1837.

37. Raizen, D.M., Zimmerman, J.E., Maycock, M.H., Ta, U.D., You, Y.J., Sundaram, M.V., and Pack, A.I. (2008). Lethargus is a Caenorhabditis elegans sleep-like state. Nature *451*, 569-572.

38. Franken, P., Malafosse, A., and Tafti, M. (1998). Genetic variation in EEG activity during sleep in inbred mice. Am J Physiol *275*, R1127-1137.

39. Nitz, D.A., van Swinderen, B., Tononi, G., and Greenspan, R.J. (2002). Electrophysiological correlates of rest and activity in Drosophila melanogaster. Curr Biol *12*, 1934-1940.

40. van Swinderen, B., Nitz, D.A., and Greenspan, R.J. (2004). Uncoupling of brain activity from movement defines arousal States in Drosophila. Curr Biol *14*, 81-87.

41. Andretic, R., van Swinderen, B., and Greenspan, R.J. (2005). Dopaminergic modulation of arousal in Drosophila. Curr Biol *15*, 1165-1175.

42. Van Swinderen, B., and Andretic, R. (2011). Dopamine in Drosophila: setting arousal thresholds in a miniature brain. Proc Biol Sci *278*, 906-913.

43. Liu, Q., Liu, S., Kodama, L., Driscoll, Maria R., and Wu, Mark N. (2012). Two Dopaminergic Neurons Signal to the Dorsal Fan-Shaped Body to Promote Wakefulness in Drosophila. Curr Biol *22*, 2114-2123.

44. Kume, K., Kume, S., Park, S.K., Hirsh, J., and Jackson, F.R. (2005). Dopamine is a regulator of arousal in the fruit fly. J Neurosci *25*, 7377-7384.

45. Ueno, T., Tomita, J., Tanimoto, H., Endo, K., Ito, K., Kume, S., and Kume, K. (2012). Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. Nat Neurosci *15*, 1516-1523.

46. Crocker, A., and Sehgal, A. (2008). Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. J Neurosci *28*, 9377-9385.

47. Crocker, A., Shahidullah, M., Levitan, I.B., and Sehgal, A. (2010). Identification of a neural circuit that underlies the effects of octopamine on sleep:wake behavior. Neuron *65*, 670-681.

48. Yuan, Q., Joiner, W.J., and Sehgal, A. (2006). A sleep-promoting role for the Drosophila serotonin receptor 1A. Curr Biol *16*, 1051-1062.

49. Parisky, K., Agosto, J., Pulver, S.R., Shang, Y., Kuklin, E., Hodge, J.J., Kang, K., Liu, X., Garrity, P.A., Rosbash, M., and Griffith, L. (2008). PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. Neuron *60*, 672-682.

50. Agosto, J., Choi, J., Parisky, K., Stilwell, G., Rosbash, M., and Griffith, L. (2008). Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in Drosophila. Nat Neurosci *11*, 354-359.

51. Li, Y., Zhou, Z., Zhang, X., Tong, H., Li, P., Zhang, Z.C., Jia, Z., Xie, W., and Han, J. (2013). Drosophila neuroligin 4 regulates sleep through modulating GABA transmission. J Neurosci *33*, 15545-15554.

52. Sehgal, A., and Mignot, E. (2011). Genetics of sleep and sleep disorders. Cell *146*, 194-207.

53. Borbely, A.A. (1982). A two process model of sleep regulation. Hum Neurobiol *1*, 195-204.

54. Chung, B., Kilman, V., Keath, J.R., Pitman, J., and Allada, R. (2009). The GABA(A) receptor RDL acts in peptidergic PDF neurons to promote sleep in Drosophila. Curr Biol *19*, 386-390.

55. Rogulja, D., and Young, M.W. (2012). Control of sleep by cyclin A and its regulator. Science *335*, 1617-1621.

56. Liu, S., Lamaze, A., Liu, Q., Tabuchi, M., Yang, Y., Fowler, M., Bharadwaj, R., Zhang, J., Bedont, J., Blackshaw, S., Lloyd, T.E., Montell, C., Sehgal, A., Koh, K., and Wu, M.N. (2014). WIDE AWAKE mediates the circadian timing of sleep onset. Neuron *82*, 151-166.

57. Vosshall, L.B., Price, J.L., Sehgal, A., Saez, L., and Young, M.W. (1994). Block in nuclear localization of period protein by a second clock mutation, timeless. Science *263*, 1606-1609.

58. Sehgal, A., Price, J.L., Man, B., and Young, M.W. (1994). Loss of circadian behavioral rhythms and per RNA oscillations in the Drosophila mutant timeless. Science *263*, 1603-1606.

59. Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Chapter 2: ADAR-mediated RNA editing suppresses sleep by acting as a brake on glutamatergic synaptic plasticity

2.1 Summary

It has been postulated that synaptic potentiation during waking is offset by a homeostatic reduction in net synaptic strength during sleep. However, molecular mechanisms to support such a process are lacking. Here we demonstrate that deficiencies in the RNA editing gene *Adar* increase sleep due to synaptic dysfunction in glutamatergic neurons in *Drosophila*. Specifically, the vesicular glutamate transporter is upregulated, leading to over-activation of NMDA receptors, and the reserve pool of glutamatergic synaptic vesicles is selectively expanded in *Adar* mutants. Collectively these changes lead to sustained neurotransmitter release under conditions that would otherwise result in synaptic depression. We propose that a shift in the balance from synaptic depression toward synaptic potentiation in sleep-promoting neurons underlies the increased sleep pressure of *Adar*-deficient animals. Our findings provide a plausible molecular mechanism linking sleep and synaptic plasticity.

2.2 Introduction

Chronically unfulfilled sleep need contributes to numerous medical problems including depression, pain, hypertension, diabetes, and cardiovascular disease [1-6]. On a shorter timescale, even 1-2 nights of poor

sleep result in attention deficits that can prove costly, or even deadly, in situations in which reaction time is critical [7]. Remarkably, despite decades of intense study, the mechanisms that control sleep need and the cellular functions that they fulfill are largely unknown. In recent years much attention has focused on the hypothesis that sleep need arises from an experience-dependent increase in net synaptic strength during waking [8]. According to the same hypothesis, sleep homeostatically reverses this increase to maintain average synaptic strength within an optimal dynamic range for synaptic plasticity. Although much experimental data support this hypothesis, criticisms persist, and detailed mechanistic support for the proposed phenomenon is lacking [9].

We set out to identify novel sleep-regulating genes in the fruit fly, *Drosophila melanogaster,* due to the genetic tractability of this organism as well as the striking parallels between sleep/wake behavior in flies and mammals, which suggest that core functions of sleep are evolutionarily conserved. This notion has been reinforced in recent years by identification of sleep-regulating genes and signaling mechanisms in flies and mice that are believed to share similar functions, including cAMP signaling, voltage-gated K^+ channels, and dopamine among others [10]. Notably lacking, however, have been molecular discoveries relating sleep to defined forms of synaptic plasticity.

We hypothesized that if sleep is indeed functioning to maintain overall synaptic strength within a physiological range, then identifiable genes should

exist that reflect the reciprocal relationship between both processes. For example, if net potentiation during waking truly drives the need to sleep, then genetic lesions resulting in increased synaptic strength should cause an increase in sleep, whereas genetic lesions resulting in decreased synaptic strength should cause a decrease in sleep. Finally, we reasoned that the resulting dysregulation should shed light on the mechanistic relation between synaptic plasticity and sleep, for which very little information is currently known.

To test these ideas we performed a genetic screen in *Drosophila* and found that the RNA editing gene *Adar* is required for stabilization of the waking state. Consistent with a related role in synaptic plasticity, *Adar* suppresses sleep-promoting glutamatergic signaling through postsynaptic AMPA and NMDA receptors by reducing the reserve pool of synaptic vesicles available for release during sustained trains of neuronal activity. We therefore conclude that *Adar* suppresses sleep in *Drosophila* by negatively regulating short-term potentiation.

2.3 Results

2.3.1 ADAR is required for wake maintenance

We systematically screened for neuronal genes that control sleep need in *Drosophila melanogaster* and tested to what extent their mechanisms of action involve synaptic plasticity. Our approach involved coupling the GAL4/UAS system [11] to RNA interference (RNAi)-dependent knockdown of genetic targets specifically in the nervous system and then assaying for effects

on daily sleep. After retesting promising lines, we found that efficient knockdown of the conserved RNA-editing gene *Adar* (*Adenosine deaminase acting on RNA*) led to hypersomnolence in both male and female animals (*elav>Adar* RNAi; Figure 2.1a-c; Figure 2.2a,b), an effect that was recapitulated with a hypomorphic *Adar* allele ($Adar^{hyp}$) [12] that expresses just 20% of normal ADAR protein (Figure 2.3a,b). Consistent with a deficit in sleep/wake control rather than in locomotion, *Adar*-deficient animals were at least as active during waking as controls (Figure 2.1d; Figure 2.2c) and had inactive periods that could be fully overcome by mechanical agitation (Figure 2.1e). To determine which component of sleep/wake control is regulated by *Adar*, we analyzed the durations of both sleep and wake bouts and found that depletion of *Adar* selectively affected the latter, leading to destabilization of the waking state (Figure 2.1f,g; Figure 2.2d,e).

Two possible explanations for the increase in sleep caused by reduction in *Adar* are altered sleep homeostasis and increased sleep pressure. To discriminate between these possibilities we sleep-deprived *Adar* hypomorphs and control flies during the final 4 hours of night and measured homeostatic recovery sleep the next morning. We found that both groups of animals recovered ~1.5 hours of lost sleep and then returned to baseline levels in the following days (Figure 2.3c-g). We interpret these data to indicate that depletion of *Adar* does not affect sleep homeostasis. We therefore suggest that *Adar* is required for normal sleep pressure, and that in *Adar*

mutants this process is decoupled from compensatory homeostatic mechanisms that would otherwise fix total daily sleep at normal levels.

2.3.2 ADAR suppresses glutamatergic signaling

Previous studies in *Drosophila* have suggested that ADAR protein is expressed broadly throughout the brain [12]. To map where *Adar* functions to modulate sleep behavior, we initially performed an anatomical screen in which we coupled UAS-*Adar* RNAi to a variety of well-characterized GAL4 drivers that express in populations of circadian clock neurons, established sleep-regulating regions of the brain, and in neurons distinguishable from one another by their distinct neurotransmitter systems (Table 2.1). We also screened an additional collection of ~500 randomly selected GAL4 lines derived from cloned putative enhancer fragments [13]. Out of both collections, the GAL4 driver 40B03 was most effective at recapitulating the increase in sleep observed with pan-neuronal knockdown of *Adar* (Figure 2.4).

As controls to confirm that knockdown of *Adar* by 40B03-*Gal4* led to increased sleep, rather than a physical disability or generalized defect in CNS function, we performed a series of additional experiments. In the first, we measured the responsiveness of knockdown animals to an arousal stimulus of fixed intensity. Consistent with the rapidly reversible nature of sleep, we found that the tendency of 40B03>*Adar* RNAi flies to remain immobile could be fully overcome by mechanical stimulation (Figure 2.5a). In a second experiment, we fed flies the caffeine analog, IBMX, and found that it was able to efficiently

maintain waking in 40B03>*Adar* RNAi animals (Figure 2.5b). In a third experiment, we tested climbing ability and found that 40B03>*Adar* RNAi flies showed no performance deficits relative to controls (Figure 2.5c). In a fourth experiment, we tested whether increased sleep in 40B03>*Adar* RNAi animals occurs through effects on selected neuronal circuits or through a general depression of neuronal function. We reasoned that widespread effects should sensitize animals to other general CNS depressants. To test this possibility we measured the amount of time it took for flies to stop responding to a repeated mechanical stimulus in the presence of volatilized ethanol. We found that 40B03>*Adar* RNAi flies had normal sensitivity and development of tolerance to ethanol (Figure 2.5d), suggesting that *Adar* selectively modulates neural circuitry involved in sleep regulation rather than acting as a global gain control of brain activity.

We also tested the role of 40B03 neurons in regulating sleep by increasing their electrical excitability with the bacterial sodium channel, NaChBac [14]. We found that 40B03>*NaChBac* flies had increased sleep compared to controls (Figure 2.6a), similar to what we observed in 40B03>*Adar* RNAi animals. Our data thus suggest that 40B03 neurons promote sleep and that *Adar* suppresses output of these neurons.

To continue to address where *Adar* functions to regulate sleep, we then coupled 40B03-*Gal4* to *UAS-CD8::GFP* (40B03>*CD8GFP*) and examined whole mounts of dissected brains by confocal microscopy (Figure 2.6b). Although 40B03>*CD8GFP* expression was restricted to a subset of neurons in

the fly brain, we were unable to correlate specific cell types with sleep function. However, the increased sleep we observed upon pan-neuronal knockdown of *Adar* suggested a nervous system requirement for this molecule, and indeed we also found that the neuronal suppressor of GAL4 activity, *elav-Gal80*, blocked the ability of 40B03>*Adar* RNAi to increase sleep. In contrast, the cholinergic-specific suppressor of GAL4 activity, *cha-Gal80*, had no effect on sleep in 40B03>*Adar* RNAi animals (Figure 2.6c), suggesting a non-cholinergic role for *Adar* in sleep regulation. Thus, despite the fact that RNA editing alters the identities of thousands of transcripts [15, 16], our ability to map the effect of this process to non-cholinergic neurons suggested that specific cellular mechanisms might mediate *Adar*'s effects on sleep.

2.3.3 Elevated DVGLUT and NMDA receptor activity are required for increased sleep in *Adar* mutants

Since *Adar* is known to alter synaptic transmission by unresolved mechanisms at the neuromuscular junction (NMJ) [17], which is glutamatergic in flies, we hypothesized that *Adar*'s effects on sleep might be mediated by alterations in glutamatergic signaling in the central brain. To test this hypothesis, we compared sleep in *Adar* null mutants ($Adar^{P}$) [18] alone and in the presence of transgenic *Adar* expressed exclusively in glutamatergic neurons ($Adar^{P}$;OK371>*Adar*). As expected, $Adar^{P}$ mutants exhibited an increase in sleep, and this phenotype was abolished in $Adar^{P}$;OK371>*Adar*

animals (Figure 2.6d). Thus, *Adar* expression in glutamatergic neurons is sufficient to restore normal sleep to *Adar*-deficient animals.

Together with evidence that synaptic vesicles accumulate at the NMJ in *Adar* mutants [17], these data prompted us to measure protein levels of the *Drosophila* vesicular glutamate transporter (DVGLUT) in fly heads. Consistent with a defect in synaptic signaling in central glutamatergic neurons, we found a striking increase in DVGLUT protein in flies depleted of *Adar* (Figure 2.7a). To determine whether this increase was responsible for the increase in sleep we observed in *Adar*-deficient animals, we paired *Adar* hypomorphs with a single copy of either a strong hypomorphic or a null *dvglut* allele [19] and found that this compensation for elevated DVGLUT expression led to normal sleep (Figure 2.7b). Thus, elevated DVGLUT is required for the increased sleep in *Adar*-deficient animals. Interestingly, even more severe reductions in *dvglut* expression led to significantly less sleep than in controls (Figure 2.8), suggesting that levels of glutamatergic signaling must be tightly maintained within a narrow range to avoid excesses and shortfalls in daily sleep.

To determine the identities of postsynaptic mediators of increased glutamatergic signaling in *Adar*-deficient animals, we knocked down various glutamate receptor transcripts in the fly genome while simultaneously reducing *Adar* expression (Figure 2.9). Interestingly, knockdown of either of two NMDA-type glutamate receptors, *NR1 or NR2*, was sufficient to partially or completely restore normal sleep to animals depleted of *Adar*. Similar results were obtained with two independent *NR2* RNAi lines (Figure 2.7c). Since activation

of NMDA receptors is known to require simultaneous synaptic release of glutamate and depolarizing current through AMPA-type glutamate receptors, AMPA receptor signaling would also be expected to be involved in *Adar*-dependent increases in sleep. Consistent with this expectation, we observed restoration of normal sleep to *Adar*-deficient animals upon knockdown of the AMPA receptor transcript *GluRI* (Figure 2.9). Thus, *Adar* is required to reduce signaling through excitatory glutamate receptors in the brain.

2.3.4 Expansion of the reserve pool of synaptic vesicles is required for increased sleep in *Adar* mutants

Enhanced excitatory glutamate signaling through AMPA/NMDA receptors could be achieved through several distinct mechanisms related to elevated DVGLUT expression. First, each synaptic vesicle could contain more DVGLUT, thus increasing the amount of glutamate packaged into each vesicle, which would result in an increase in quantal size [20]. To test this possibility, we recorded spontaneous miniature excitatory junctional potentials (mEJPs) at the well-characterized larval NMJ. In this preparation, a null allele of *Adar* has been reported to exhibit increased mEJP amplitude [17]. However, these changes were not detectable with more moderate reductions in *Adar* that are still able to increase sleep (Figure 2.10a-c; Figure 2.3a,b). Second, more vesicles could be released per action potential that arrives at presynaptic terminals. However, we did not measure a difference in quantal content in *Adar* hypomorphs relative to controls, as reflected in the amplitude of evoked

EJPs at the NMJs of both groups of animals (Figure 2.10d-f). These data strongly suggest that reductions in *Adar* that are sufficient to increase sleep do not alter the amount of glutamate in each synaptic vesicle or the number of vesicles released per action potential during baseline synaptic transmission.

These measurements also permitted us to clearly distinguish changes in synaptic vesicle size and number from potential expansions or contractions of different vesicle pools that could alternatively underlie constitutive potentiation of glutamatergic synapses. Specifically, we hypothesized that the increased sleep observed in *Adar*-depleted flies was mediated by an increase in availability of glutamatergic vesicles during sustained neuronal activity. In support of this hypothesis, a previous study reported that *Adar* mutants accumulate synaptic vesicles and vesicle-related proteins at the NMJ [17]. We also tested our hypothesis by stimulating axons at the NMJ for 10 minutes at 15 Hz to deplete synaptic vesicle pools while measuring the quantal content per stimulus (Figure 2.11a). As described by others, we found that change in quantal content followed two temporally distinct phases: an initial period involving rapid decay followed by a subsequent period of slower, more sustained decay. Such changes have been attributed to fast and slow depletion of what are often referred to as the readily releasable pool (RRP) and reserve pool (RP) of synaptic vesicles, respectively [21]. Intriguingly, in *Adar* hypomorphs with increased sleep we found that the fast phase decayed more quickly (Figure 2.11b) and the slow phase decayed more slowly than in controls (Figure 2.11c). These data are consistent with both an expanded RP

in *Adar* mutants and chronically potentiated glutamatergic signaling, which could be a signal to sleep.

To determine whether a larger RP size was responsible for the increase in sleep we observed in *Adar* mutants, we focused on the possible genetic interaction between *Adar* and *Synapsin* (*Syn*). *Syn* encodes a synaptic vesicle protein that is thought to act as a barrier to transitions from the RP to the RRP [22]. A reduction in the amount of Syn should thus lower the barrier to this transition. By this logic, if increased sleep in *Adar* mutants results from an expanded glutamatergic RP, then reducing the levels of Syn should compensate for this effect. To test this hypothesis we coupled a hypomorphic mutation in *Adar* that increased sleep with a heterozygous null mutation in *Syn* (Syn^{97}) [23] and measured rates of depletion of the RP and RRP during high frequency presynaptic stimulation of the NMJ. Consistent with our hypothesis, the rate of depletion of the RP was restored nearly to control levels in $Adar^{hyp}$;;Syn^{97}/+ double mutants without altering the rate of depletion of the RRP (Figure 2.11b,c). We then reasoned that if an expanded glutamatergic RP indeed increases sleep need, then restoring the size of the RP to normal by reducing levels of *Syn* should also restore normal levels of sleep to *Adar* mutants. To test this hypothesis, we measured total daily sleep in animals containing $Adar^{hyp}$ alone or in combination with the heterozygous Syn^{97} allele. Consistent with our hypothesis, sleep was fully restored to control levels in $Adar^{hyp}$;;Syn^{97}/+ mutants (Figure 2.11d). Together, these data strongly suggest that *Adar* normally restricts the size of the RP, thus reducing

glutamatergic synaptic potentiation via downstream NMDAR signaling, which in turn limits sleep pressure. In the absence of *Adar,* this brake on synaptic potentiation appears to be released in sleep-promoting neurons, thus leading to increased sleep pressure.

2.4 Discussion

By describing a role for *Adar* in suppressing sleep, we have discovered a mechanistic link between sleep and synaptic plasticity. Such a link has been hypothesized for many years but has lacked molecular details to support it [8, 9]. To arrive at this link, we initially identified *Adar* as a sleep-regulating gene through a genetic screen, mapped the hypersomnolence of *Adar* mutants to sleep-promoting glutamatergic neurons, showed that this phenotype was associated with an increase in DVGLUT, and found that it could be compensated by genetic reductions in *dvglut*, *NR1, NR2,* and *GluRI*. Collectively, these data suggest that *Adar* normally suppresses glutamatergic signaling to reduce sleep.

Based on our results, this signaling pathway appears to be at least as important as other arousal systems in regulating sleep. For example, previous reports have shown that overall dopamine, octopamine, and acetylcholine promote waking in flies [24-29], whereas serotonin predominantly promotes sleep [30]. Similar to these neurotransmitters, we found that availability of glutamate correlates with arousal state, wherein elevated glutamate promotes sleep and reduced glutamate leads to increased waking. Thus, it is likely that

glutamatergic signaling is tightly regulated to maintain proper levels and timing of sleep – a process in which we have now implicated *Adar*.

In elucidating the pathway through which *Adar* acts to regulate sleep, we found two points at which synaptic plasticity might be implicated. The first is based on our observation that the sleep-promoting effects of *Adar* mutants require AMPA and NMDA receptors. NMDA receptors are frequently involved in synaptic plasticity [31-33], making them prime candidates for potentiated synaptic responses that have been proposed to accumulate during waking and in turn drive compensatory sleep need. In fact, a recent report has described *NR1* as a novel sleep-promoting gene in *Drosophila* [34]. Here we have confirmed that NMDA receptors promote sleep, and we have demonstrated that they are required for the sleep phenotype of *Adar* mutants. Thus, it is likely that a major role of wild-type *Adar* is to act as a presynaptic brake on NMDA-dependent postsynaptic potentiation.

We were able to more thoroughly investigate the second mechanism by which *Adar* appears to regulate glutamatergic synaptic plasticity to influence sleep. In this case, we found that basal synaptic transmission in glutamatergic neurons was not altered by depletion of *Adar*. That is, *Adar* hypomorphs had normal glutamate loading into synaptic vesicles, postsynaptic responses to spontaneously released glutamate, and number of glutamatergic vesicles released per evoked junctional potential. However, upon sustained stimulation, *Adar* mutants exhibited decreased synaptic depression that could be compensated by reducing *Syn*, a gene that limits depletion of the RP[22].

Notably, reducing *Syn* was also sufficient to reduce sleep in *Adar* mutants to control levels. Our data thus support the hypothesis that *Adar* mutants have an expanded synaptic RP, which reduces depression of sleep-promoting glutamatergic neurons to increase intrusions of sleep into the waking state.

Expansion of the RP of synaptic vesicles, like those we have observed in *Adar* mutants, has been shown to substantially increase the amount of information transmitted per burst of action potentials due to decreased short-term depression [35]. In essence, this phenomenon shifts the balance of depression and potentiation toward the latter. We suggest that in glutamatergic sleep-promoting neurons, this shift translates to an increased probability of sleep onset from spike trains that would normally be sub-threshold for the behavior. It will be interesting to determine if similar mechanisms link sleep to potentiating responses involved in other forms of behavioral plasticity.

Our studies may also direct research about RNA editing toward a system that can be studied and dissected at the molecular level. The link between RNA editing and glutamatergic plasticity that we have described may be particularly fruitful to explore in other contexts as well. Hints of such a relationship have been described previously. For example, early observations of the role of RNA editing in the brain showed that ADAR functions postsynaptically to limit Ca^{2+} permeability and channel conductance of GluR2-containing receptors [36, 37]. Furthermore, editing of GluR2, GluR3, and GluR4 at the R/G position has been shown to alter receptor gating kinetics,

resulting in more rapid desensitization and recovery from desensitization[38]. Together with evidence that RNA editing is reduced in glutamate excitotoxic diseases such as ALS [39-41], we suggest that a major function of RNA editing in the nervous system is to limit glutamatergic signaling. Since we have shown that ADAR acts as a presynaptic glutamatergic brake in flies, it will be interesting to determine whether this function is conserved in mammalian nervous systems and whether ADAR is also able to achieve the same effect through postsynaptic regulatory mechanisms.

2.5 Methods

2.5.1 Fly stocks

Flies were grown at room temperature (20-22°C) on standard cornmeal media with yeast. Unless otherwise indicated, all animals were outcrossed a minimum of 5 times into a w^{1118} *iso31* genetic background. $Adar^{HA}$ and $Adar^{hyp}$ were obtained from Dr. Robert Reenan. $Adar^{P}$ and *UAS-Adar* were obtained from Dr. Gabriel Haddad. $dvglut^{1}$ and $Df(2L)dvglut^{2}$ were obtained from Dr. Aaron DiAntonio. The *Adar* RNAi line, *v7764*, was obtained from the Vienna Drosophila Resource Center and was used in the presence of *UAS-Dicer* in all experiments. *UAS-CD8::GFP*, *UAS-Dicer* (second and third chromosome insertions), *elav-Gal4*, $dvglut^{MI02805}$, *OK371-Gal4*, Syn^{97}, *NR1* RNAi (HMS02200), *NR2* #1 RNAi (HMS02012), *NR2* #2 RNAi (HMS02176), *GluRI* RNAi (HMS02155), *GluRIB* RNAi (JF02752), *GluRIIA* RNAi (JF03145), *GluRIIB* RNAi (JF03145), *GluRIIC* RNAi (JF01854), *GluRIID* RNAi (JF02035),

GluRIIE RNAi (JF01962), *Clumsy* RNAi (JF02987), *CG3822* RNAi (JF01873), *CG5621* RNAi (JF01840), *CG11155* RNAi (JF03425), *mGluRA* RNAi (JF01958) [42, 43], 40B03-*Gal4* and all other *Gal4* lines[13] were obtained from the Bloomington Stock Center. Male flies were used for all behavioral assays unless otherwise indicated.

2.5.2 Sleep and locomotor measurements

Individual 3-7 day old flies were placed in 5 mm x 65 mm Pyrex tubes containing a mixture of 2% agarose and 5% sucrose at one end as a food source. Animals were entrained for 2 days in 12 hr:12 hr light:dark conditions at 25°C, and infrared beam breaks were recorded in 1 min bins for the next 2-4 days using the Drosophila Activity Monitoring System (Trikinetics). Sleep analysis was performed as previously described using custom MATLAB (Mathworks) software [28]. For IBMX treatment, 1 day of baseline sleep was recorded on standard food before animals were moved at ZT0 into new glass tubes containing food supplemented with 0.1mg/mL IBMX (Sigma). Sleep was calculated for the second day on drug.

2.5.3 Sleep deprivation and rebound

Flies in DAM2 *Drosophila* activity monitors (Trikinetics) were sleep deprived by periodic shaking in a VX-2500 multi-tube vortexer (VWR) for 2 sec/min from ZT20-24. Rebound sleep was measured as immediate post-deprivation minus pre-deprivation sleep at ZT0-6.

2.5.4 Sleep reversibility measurements

Flies were loaded into DAM2 activity monitors and placed on a custom built platform that oscillated along a line perpendicular to the axis of locomotion. Oscillation speed and timing were controlled using an Arduino Uno-powered motor. Arousal stimuli consisted of 10 sec oscillations every minute for 5 minutes at ZT06. Sleep was considered to be reversible for any fly that moved within 5 minutes of the final stimulation. Dead flies were also included as negative controls to rule out spurious detection of activity due to involuntary motion across the infrared beam.

2.5.5 Climbing assays

3-7 day old flies were transferred at ZT2 to 2 empty vials (25 mm x 95 mm) connected to each other vertically at their open ends. After 10 min acclimation, flies were tapped to the bottom of the lower vial. Thirty seconds later a divider was placed between the vials and the percentage of flies that successfully climbed into the upper vial was measured. Means for each genotype were recorded after 3 trials.

2.5.6 Ethanol sensitivity and tolerance

3-7 day old flies were transferred at ZT2 to empty vials and tested every minute for loss-of-righting reflex during exposure to ethanol as previously described [44]. The time for 50% of the animals to remain stationary (ST50)

was measured. Continued exposure to ethanol was maintained for approximately twice this time before transferring animals to a fresh vial. After 4 hrs recovery, the same animals were transferred back to ethanol-containing vials, and ST50 was measured again to determine ethanol tolerance.

2.5.7 Immunohistochemistry

Immunohistochemistry was performed as described previously [28]. Briefly, 3-7 day-old brains were dissected in ice-cold PBS, fixed in 4% paraformaldehyde, and blocked in PBST (PBS and 0.3% Triton X-100) with 5% normal donkey serum (Jackson Laboratory) prior to staining. Brains were then incubated with 1:1,000 rabbit anti-GFP (Invitrogen) and 1:50 mouse anti-nc82 (Developmental Studies Hybridoma Bank) antibodies overnight at 4°C and washed five times in PBST. Brains were then incubated with 1:1000 Alexa 568 anti-rabbit (Life Technologies) and 1:1,000 Alexa 633 anti-mouse (Life Technologies) antibodies for 4 hours at room temperature prior to washing five times in PBST and coverslip mounting in Vectashield (Vector Labs). Images were taken at 40x magnification on a Leica SP5 confocal microscope at 0.5 µm intervals and reassembled for display as maximum projections using Fiji [45].

2.5.8 Western blot analysis

15-20 brains or heads were dissected from 3-7 day old flies and lysed in sample buffer (20mM HEPES, pH 7.5, 100mM KCl, 10mM EDTA, 50mM

NaF, 0.1% Triton X-100, 10% glycerol, 1mM DTT, 1X Roche Complete Protease Inhibitors). Lysates were cleared of particulate debris by centrifugation at 5,000g for 5 minutes at 4°C prior to protein quantification. Lysates were resolved on 10-well 4-12% NuPAGE SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were probed using 1:500 mouse anti-HA (Covance), 1:10,000 rabbit anti-DVGLUT [46], and 1:10,000 mouse anti-actin primary antibodies (EMD Millipore) followed by 1:5,000 anti-mouse, 1:5,000 anti-rabbit and 1:10,000 anti-mouse HRP antibodies, respectively (VWR). Visualization of bound secondary antibodies was achieved using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

2.5.9 Electrophysiology

All dissections and recordings were performed in modified HL3 saline [47] containing (in mM): 70 NaCl, 5 KCl, 10 MgCl2, 10 $NaHCO_3$, 115 Sucrose, 5 Trehelose, 5 HEPES, and 0.4 $CaCl_2$ (unless otherwise specified), pH 7.2. Neuromuscular junction sharp electrode (electrode resistance between 10-35 MΩ) recordings were performed on muscles 6 and 7 of abdominal segments A2 and A3 in wandering 3rd instar larvae. Larvae were cultured in standard molasses medium, raised at 25C, and dissected; the guts, trachea, and ventral nerve cord were removed from the larval body walls with the motor nerve carefully cut, and the preparation was rinsed several times with HL3 saline. Recordings were performed on an Olympus BX51 WI microscope using a

40x/0.80 water-dipping objective. Recordings were acquired using an Axoclamp 900A amplifier, Digidata 1440A acquisition system and pClamp 10.5 software (Molecular Devices). Electrophysiological sweeps were digitized at 10kHz, and filtered at 1kHz. Data were analyzed using Clampfit (Molecular devices), MiniAnalysis (Synaptosoft), and Excel (Microsoft).

Miniature excitatory junctional potentials (mEJPs) were recorded in the absence of any stimulation, and cut motor axons were stimulated at ~5 nA for 3 msec to elicit excitatory junctional potentials (EJPs). To fine tune stimulus intensity, an ISO-Flex stimulus isolator was used (A.M.P.I.). Intensity was adjusted for each cell, set high enough to consistently elicit full responses in both axons innervating the muscle segment. Average mEJP amplitude, EJP amplitude, and quantal content were calculated for each genotype with corrections for nonlinear summation[48]. Muscle input resistance (R_{in}) and resting membrane potential (V_{rest}) were monitored during each experiment. Recordings were rejected if the V_{rest} was more depolarized than -60mV, if the R_{in} was less than 5MΩ, or if either measurement deviated by more than 10% during the experiment. For synaptic vesicle rundown experiments, postsynaptic responses for each preparation were fit to a single exponential curve (readily-releasable pool) or averaged into 30-second bins and fit from 60 sec to 9 min by linear regression (reserve pool).

2.5.10 Data analysis and statistics

Replicates (n values) represent the number of biological replicates for each experimental condition. Bar graphs depict the mean ± SEM, except for box and whisker plots, which depict the median (line), 25^{th} to 75^{th} percentiles (box) and minimum/maximum values (whiskers). Unless otherwise indicated, datasets that approximate a normal Gaussian distribution were analyzed with unpaired Student's t-test followed by Welch's correction for comparisons between two groups. For experiments of single factor design, we analyzed data using one-way ANOVA followed by the Tukey-Kramer test for multiple comparisons or Sidak's multiple comparisons test for select comparisons. Two-way ANOVA followed by Sidak's multiple comparisons test was used to analyze experiments of two-factor design. For datasets from non-Gaussian distributions, comparisons were performed using Kruskal-Wallis test followed by Dunn's multiple comparisons test. All statistical tests were two-sided and performed using Prism 6.0f for Mac OS X (GraphPad Software).

2.6 Acknowledgements

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2.7 Figures

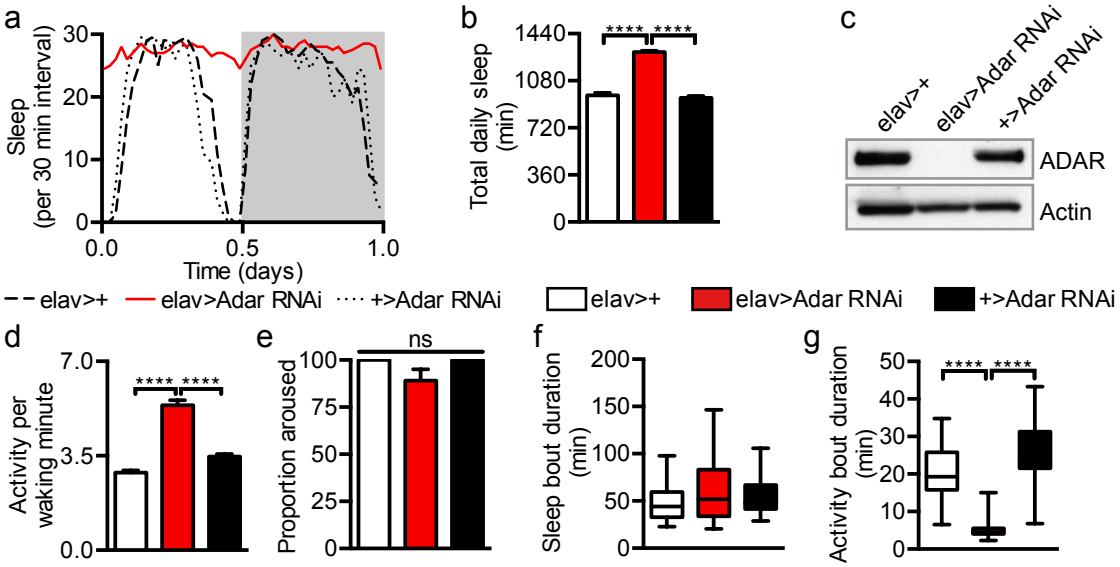

Figure 2.1 *Adar* stabilizes the waking state to suppress sleep. (A) Representative sleep profiles of male elav>*Adar* RNAi and controls. **(B)** Quantification of sleep in (A). Pan-neuronal knockdown of *Adar* increases sleep in elav>*RNAi* animals relative to controls. **(C)** Representative western blot of fly brains indicates efficient knockdown of ADAR expression in elav>*Adar* RNAi flies. **(D)** Waking activity is not reduced in elav>*Adar* RNAi animals. **(E)** Sleep in elav>*Adar* RNAi animals is acutely reversible by mechanical perturbation. **(F)** Sleep maintenance is unaffected in elav>*Adar* RNAi animals. **(G)** The wake state is destabilized in elav>*Adar* RNAi animals relative to controls. For each panel: *elav*>+ (n=39); elav>*Adar* RNAi (n=54); +>*Adar* RNAi (n=39). *, **, ***, **** indicate $p < 0.05$, 0.01, 0.001 and 0.0001, respectively, for all figures.

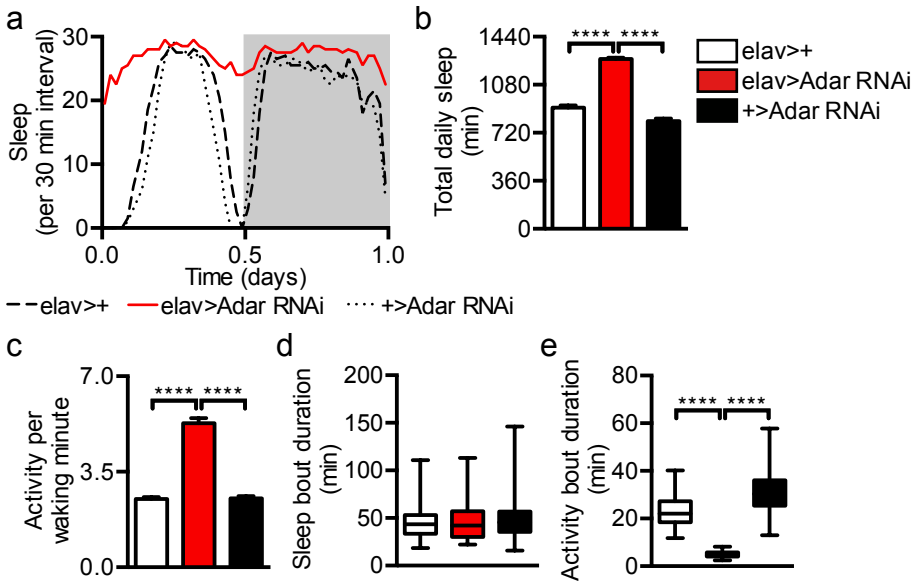

Figure 2.2 Reducing *Adar* expression in female flies increases sleep. (a) Representative sleep profiles of female elav>*Adar* RNAi and controls. **(b)** Quantification of sleep in (a). Pan-neuronal knockdown of *Adar* increases sleep in elav>*RNAi* animals relative to controls. **(c)** Waking activity is not reduced in elav>*Adar* RNAi females. **(d)** Sleep maintenance is unaffected in elav>*Adar* RNAi females. **(e)** The wake state is destabilized in elav>*Adar* RNAi females relative to controls. For all panels elav>*Adar* RNAi (n=37); *elav*>+ (n=63); +>*Adar* RNAi (n=40).

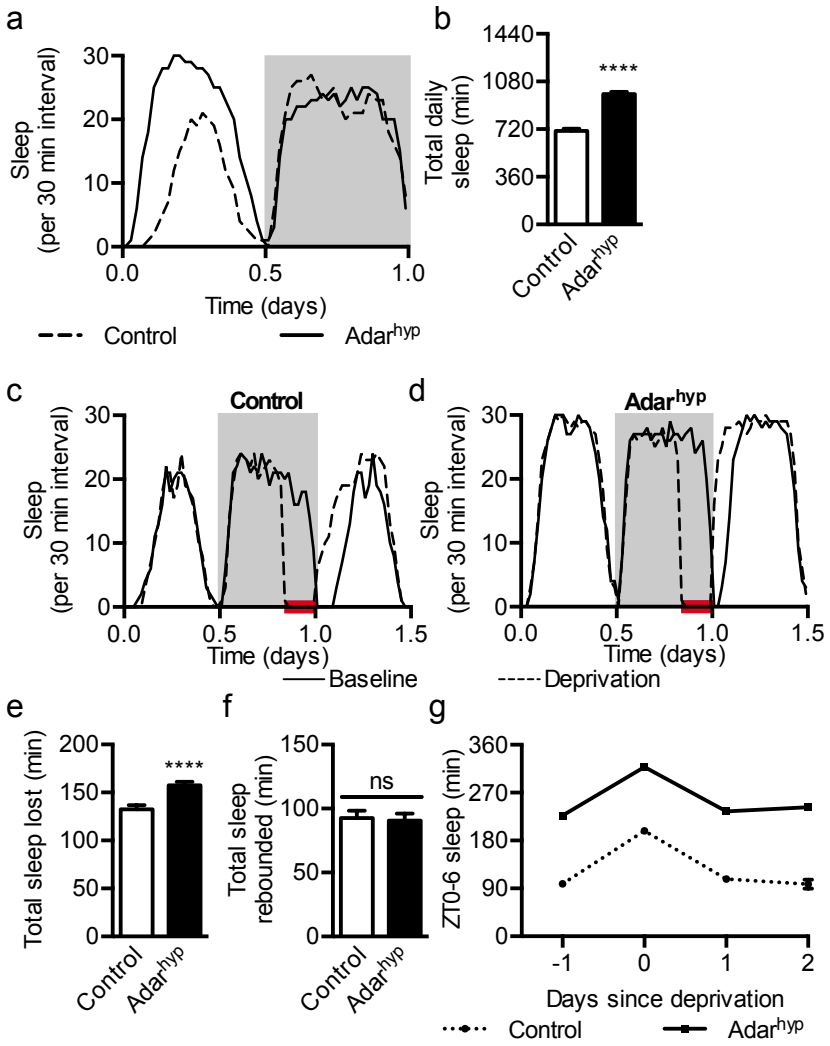

Figure 2.3 Reducing *Adar* levels does not alter sleep homeostasis. (a and **b)** Representative sleep profiles (a) and total daily sleep (b) for *Adar*^{hyp} and sibling control animals [females; *Adar*^{hyp} (n=93); control (n=95)]. (**c** and **d**) Representative sleep profiles during baseline, deprivation (red bar; ZT20-24) and recovery periods for sibling control (c) and *Adar*^{hyp} animals (d). (**e** and **f**) Quantification of sleep lost during deprivation period (e) and recovered the next morning from ZT0-6 (f). **(g)** Total sleep each day from ZT0-6. Sleep returns to baseline after one day of recovery. [In C-G: *Adar*^{hyp} (n=90); control (n=95)]. Females were used for all sleep homeostasis experiments.

Table 2.1 Sleep effects of GAL4>*Adar* RNAi combinations using established, well-characterized drivers. Total number of animals tested (n), total daily sleep, standard deviation, and standard error of the mean (SEM) are listed for the various GAL4 lines used to knock down *Adar*.

Genotype	n	Total daily sleep mean (min)	Std. deviation	SEM
+>Adar RNAi	32	906	99	17.5
elav>+	31	944	92	16.5
elav>Adar RNAi	28	1299	61	11.6
c522>+	31	1022	103	18.4
c522>Adar RNAi	32	1000	102	18.1
104y>+	31	898	102	18.4
104y>Adar RNAi	32	789	120	21.2
cha>+	32	971	118	20.8
cha>Adar RNAi	31	805	115	20.7
sss>+	32	874	67	11.9
sss>Adar RNAi	32	893	107	19.0
30y>+	31	859	102	18.4
30y>Adar RNAi	32	852	116	20.6
TPH>+	32	932	79	13.9
TPH>Adar RNAi	32	954	108	19.0
201y>+	24	1061	78	15.9
201y>Adar RNAi	20	1046	97	21.8
Tdc2>+	32	983	98	17.3
Tdc2>Adar RNAi	31	918	107	19.3
TH>+	32	1005	88	15.6
TH>Adar RNAi	32	962	98	17.3
OK371>+	30	939	139	25.5
OK371>Adar RNAi	32	814	130	23.0
GAD>+	32	1028	81	14.2
GAD>Adar RNAi	32	958	81	14.3
Ddc>+	32	977	90	15.8
Ddc>Adar RNAi	29	874	94	17.5
MB247>+	25	865	157	31.4
MB247>Adar RNAi	32	742	138	24.4
tim>+	32	974	72	12.6
tim>Adar RNAi	25	925	116	23.2
clk-4.1m>+	32	980	84	14.8
clk-4.1m>Adar RNAi	28	876	112	21.2
cry>+	15	963	90	23.2
cry>Adar RNAi	11	962	103	30.9
24B>+	29	890	97	18.1
24B>Adar RNAi	29	778	125	23.1
OK107>+	15	922	106	27.2
OK107>Adar RNAi	16	810	81	20.3
npf>+	16	932	89	22.2
npf>Adar RNAi	16	770	123	30.6
pdf>+	15	841	111	28.7
pdf>Adar RNAi	36	824	184	30.6
vGlut>+	16	828	67	16.8
vGlut>Adar RNAi	16	806	79	19.7
trh>+	16	750	122	30.4
trh>Adar RNAi	16	836	126	31.5
ple>+	16	914	74	18.5
ple>Adar RNAi	16	897	97	24.3

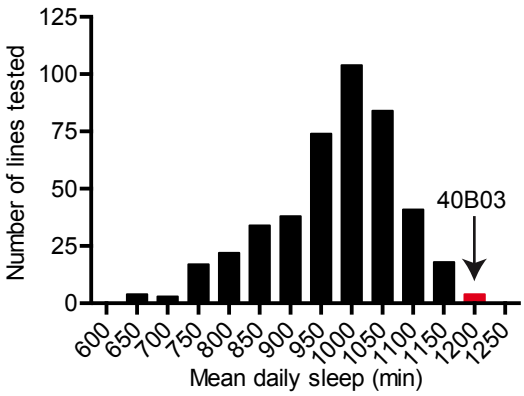

Figure 2.4 Enhancer-GAL4 screen for *Adar*-mediated sleep-regulating neurons. Daily sleep was calculated for each Gal4>*Adar* RNAi combination and binned accordingly. The bin containing 40B03-*Gal4* (arrow) is indicated in red.

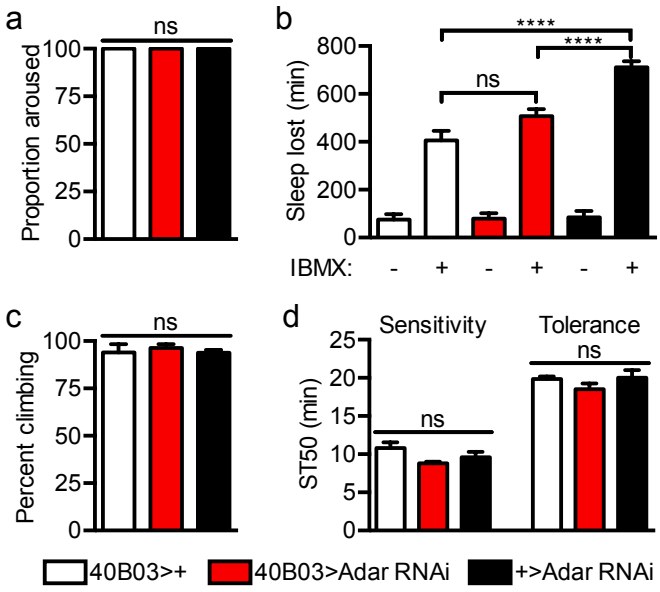

Figure 2.5 Behavioral changes in 40B03>*Adar* RNAi animals are attributable to increased sleep. (a) Behavioral immobility in 40B03>*Adar* RNAi animals was acutely reversible by mechanical perturbation [n=3 trials for each genotype; 8 animals were used for each trial]. **(b)** Behavioral immobility in 40B03>*Adar* RNAi animals was suppressible by the caffeine analog, IBMX [n=31 animals for each genotype]. **(c)** Like controls, 40B03>*Adar* RNAi animals climbed 9.5 cm in 30 sec [n=3 trials of 10 flies for each genotype]. **(d)** 40B03>*Adar* RNAi and control flies exhibited equivalent sensitivity and tolerance to ethanol [n=3 trials for each genotype; 10 animals were used for each trial].

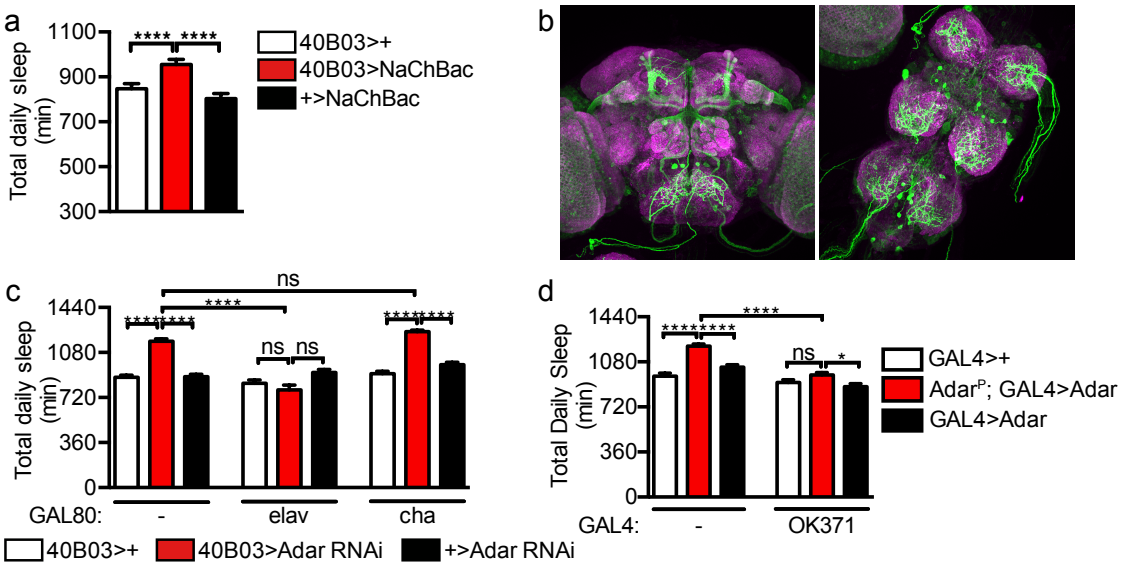

Figure 2.6 *Adar* suppresses output of glutamatergic sleep-promoting neurons. (**a**) Total daily sleep is increased in flies expressing the depolarizing NaChBac channel under control of 40B03-*Gal4* [40B03>+ (n=24); 40B03>*NaChBac* (n=30); +>*NaChBac* (n=31)]. **(b)** Representative expression pattern of 40B03>CD8GFP (green) and of the neuropil marker, nc82 (magenta) in central brain and thoracic ganglion. **(c)** Increased sleep in 40B03>*Adar* RNAi animals is suppressed by pan-neuronal (*elav*) but not cholinergic (*cha*) expression of GAL80 [n=23-48 for each genotype]. **(d)** Restoration of *Adar* expression in glutamatergic neurons (OK371-*Gal4*) restores sleep to control levels in *Adar* null mutants ($Adar^{P}$) [n=23-32 for each genotype].

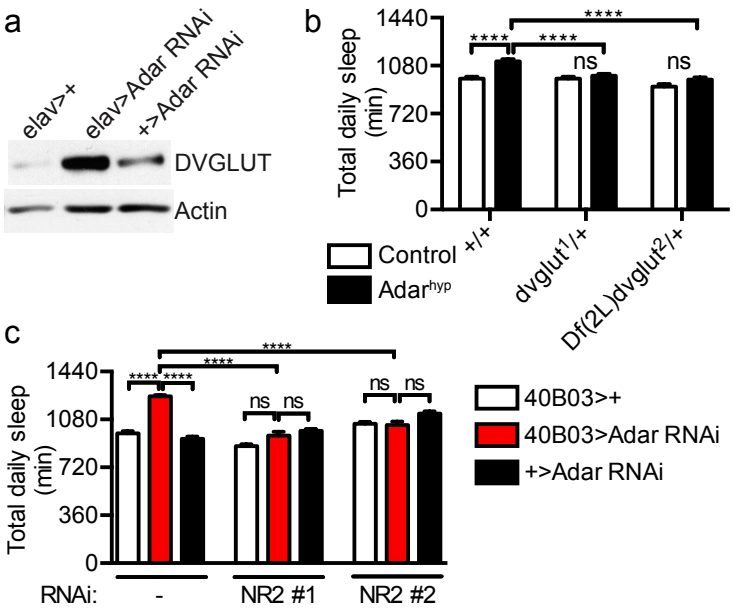

Figure 2.7 *Adar* suppresses sleep by reducing DVGLUT expression and signaling through NMDA receptors. (a) Representative western blot showing elevated expression of DVGLUT in heads of elav>*Adar* RNAi flies relative to controls. Actin is a loading control. **(b)** Heterozygous reduction in *dvglut* reduces sleep to control levels in *Adar*^{hyp} mutants [n=46-48 for each genotype]. **(c)** Knockdown of *NR2* restores sleep to control levels in 40B03>*Adar* RNAi animals [n=18-62 for each genotype].

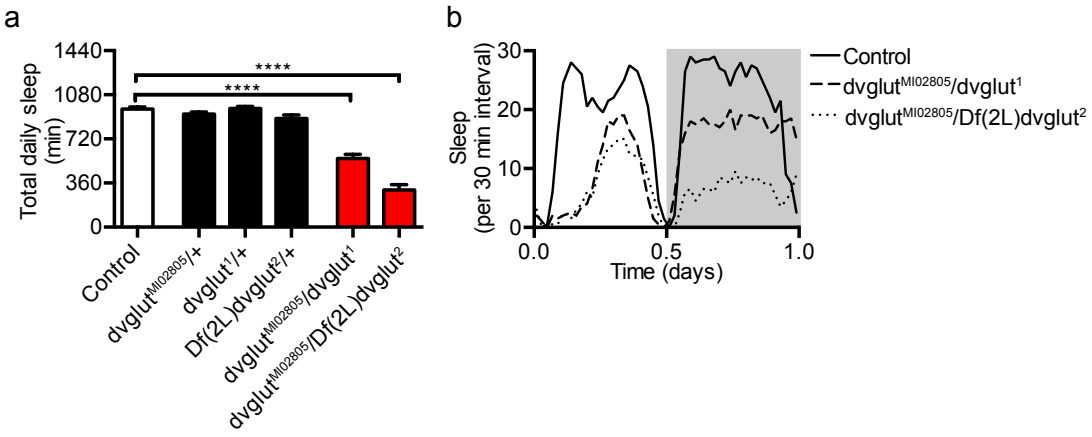

Figure 2.8 Severe reduction in *dvglut* suppresses sleep. (a and **b)** Total daily sleep (a) and representative sleep profiles (b) of heteroallelic combinations of *dvglut* mutants and controls [n=22-31 for each genotype].

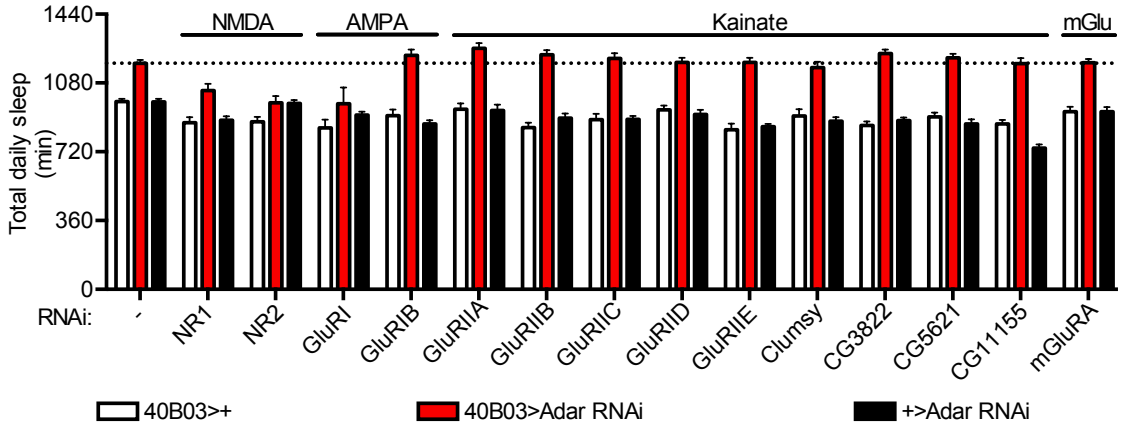

Figure 2.9 Knockdown of NMDA and AMPA receptors restores sleep to *Adar*-deficient animals. Total daily sleep measured in control, 40B03>*Adar* RNAi, and 40B03>*Adar* RNAi in combination with RNAi's against ionotropic (NMDA, AMPA, kainate) and metabotropic (mGlu) glutamate receptors [n=7-31 for all genotypes].

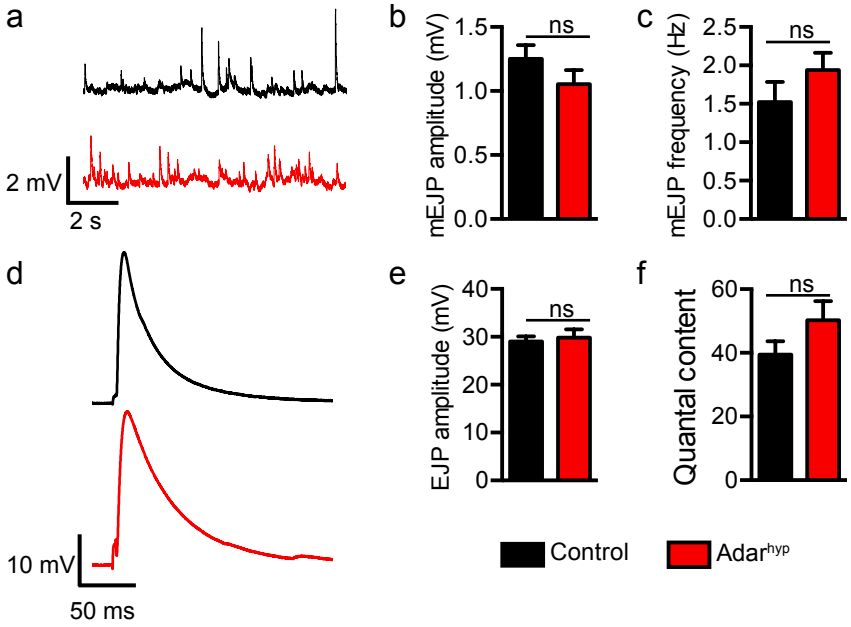

Figure 2.10 *Adar* hypomorphs have normal spontaneous and evoked synaptic transmission at the NMJ. (a) Representative recordings of mEJPs in control (black) and *Adar*^{hyp} animals (red). **(b** and **c)** Average mEJP amplitudes (B) and frequencies (c) do not differ significantly between control and *Adar*^{hyp} animals. **(d)** Representative recordings of evoked EJPs in control (black) and *Adar*^{hyp} animals (red). **(e and f)** Average evoked EJP amplitude (e) and quantal content per stimulus (f) do not differ significantly between control and *Adar*^{hyp} animals [n=10 for control and n=11 for *Adar*^{hyp} in all panels].

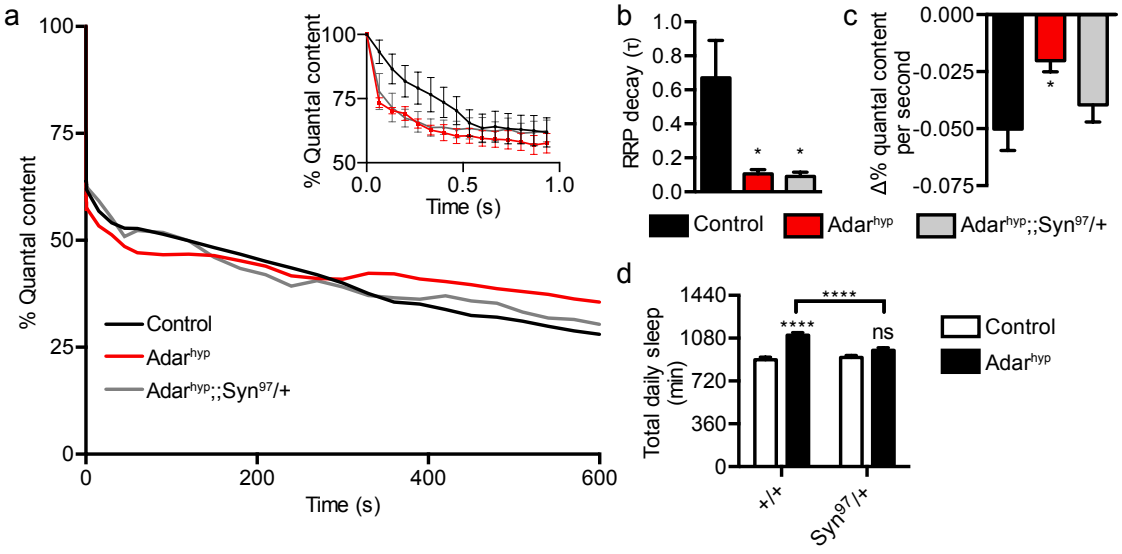

Figure 2.11 Reversing the expansion of the reserve vesicle pool restores normal sleep to *Adar* mutants. (a) High frequency (15 Hz) stimulation at the NMJ causes a faster rate of depletion of the RRP (inset) and a slower rate of depletion of the RP (main figure) in *Adar*^{hyp} mutants relative to controls. Heterozygous loss of *Synapsin* only rescues changes in the reserve pool [control (n=8); *Adar*^{hyp} (n=6); *Adar*^{hyp};;*Syn*^{97}/+ (n=6)]. **(b)** Quantification of the decay rate of the RRP in (a). Tau values are based on an exponential fit to the change in quantal content over time, ending at 1.0 sec [control (n=8); *Adar*^{hyp} (n=6); *Adar*^{hyp};;*Syn*^{97}/+ (n=6)]. **(c)** Quantification of the decay rate of the reserve pool. Values are based on linear regression fit of changes in quantal content over time, beginning at 60 sec [control (n=8); *Adar*^{hyp} (n=6); *Adar*^{hyp};;*Syn*^{97}/+ (n=6)]. **(d)** Heterozygous loss of *Synapsin* restores normal sleep to *Adar* mutants (black bars) without altering sleep in controls (white bars) [n=45-48 for each genotype].

2.8 References

1. Mullington, J.M., Haack, M., Toth, M., Serrador, J.M., and Meier-Ewert, H.K. (2009). Cardiovascular, inflammatory, and metabolic consequences of sleep deprivation. Prog Cardiovasc Dis *51*, 294-302.

2. Johnson, E.O., Roth, T., and Breslau, N. (2006). The association of insomnia with anxiety disorders and depression: exploration of the direction of risk. J Psychiatr Res *40*, 700-708.

3. Gangwisch, J.E., Heymsfield, S.B., Boden-Albala, B., Buijs, R.M., Kreier, F., Pickering, T.G., Rundle, A.G., Zammit, G.K., and Malaspina, D. (2007). Sleep duration as a risk factor for diabetes incidence in a large U.S. sample. Sleep *30*, 1667-1673.

4. Gangwisch, J.E., Heymsfield, S.B., Boden-Albala, B., Buijs, R.M., Kreier, F., Pickering, T.G., Rundle, A.G., Zammit, G.K., and Malaspina, D. (2006). Short sleep duration as a risk factor for hypertension: analyses of the first National Health and Nutrition Examination Survey. Hypertension *47*, 833-839.

5. Gottlieb, D.J., Punjabi, N.M., Newman, A.B., Resnick, H.E., Redline, S., Baldwin, C.M., and Nieto, F.J. (2005). Association of sleep time with diabetes mellitus and impaired glucose tolerance. Arch Intern Med *165*, 863-867.

6. Ayas, N.T., White, D.P., Manson, J.E., Stampfer, M.J., Speizer, F.E., Malhotra, A., and Hu, F.B. (2003). A prospective study of sleep duration and coronary heart disease in women. Arch Intern Med *163*, 205-209.

7. McCoy, J.G., and Strecker, R.E. (2011). The cognitive cost of sleep lost. Neurobiol Learn Mem *96*, 564-582.

8. Tononi, G., and Cirelli, C. (2014). Sleep and the Price of Plasticity: From Synaptic and Cellular Homeostasis to Memory Consolidation and Integration. Neuron *81*, 12-34.

9. Frank, M.G. (2012). Erasing synapses in sleep: is it time to be SHY? Neural Plast *2012*, 264378.

10. Sehgal, A., and Mignot, E. (2011). Genetics of sleep and sleep disorders. Cell *146*, 194-207.

11. Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

12. Jepson, J.E., Savva, Y.A., Yokose, C., Sugden, A.U., Sahin, A., and Reenan, R.A. (2011). Engineered alterations in RNA editing modulate complex behavior in Drosophila: regulatory diversity of adenosine deaminase acting on RNA (ADAR) targets. The Journal of biological chemistry *286*, 8325-8337.

13. Jenett, A., Rubin, G.M., Ngo, T.T., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B.D., Cavallaro, A., Hall, D., Jeter, J., Iyer, N., Fetter, D., Hausenfluck, J.H., Peng, H., Trautman, E.T., Svirskas, R.R., Myers, E.W., Iwinski, Z.R., Aso, Y., DePasquale, G.M., Enos, A., Hulamm, P., Lam, S.C., Li, H.H., Laverty, T.R., Long, F., Qu, L., Murphy, S.D., Rokicki, K., Safford, T., Shaw, K., Simpson, J.H., Sowell, A., Tae, S., Yu, Y., and Zugates, C.T. (2012). A GAL4-driver line resource for Drosophila neurobiology. Cell Rep *2*, 991-1001.

14. Nitabach, M.N. (2006). Electrical Hyperexcitation of Lateral Ventral Pacemaker Neurons Desynchronizes Downstream Circadian Oscillators in the Fly Circadian Circuit and Induces Multiple Behavioral Periods. J Neurosci *26*, 479-489.

15. St Laurent, G., Tackett, M.R., Nechkin, S., Shtokalo, D., Antonets, D., Savva, Y.A., Maloney, R., Kapranov, P., Lawrence, C.E., and Reenan, R.A. (2013). Genome-wide analysis of A-to-I RNA editing by single-molecule sequencing in Drosophila. Nat Struct Mol Biol *20*, 1333-1339.

16. Graveley, B.R., Brooks, A.N., Carlson, J.W., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren, M.J., Boley, N., Booth, B.W., Brown, J.B., Cherbas, L., Davis, C.A., Dobin, A., Li, R., Lin, W., Malone, J.H., Mattiuzzo, N.R., Miller, D., Sturgill, D., Tuch, B.B., Zaleski, C., Zhang, D., Blanchette, M., Dudoit, S., Eads, B., Green, R.E., Hammonds, A., Jiang, L., Kapranov, P., Langton, L., Perrimon, N., Sandler, J.E., Wan, K.H., Willingham, A., Zhang, Y., Zou, Y., Andrews, J., Bickel, P.J., Brenner, S.E., Brent, M.R., Cherbas, P., Gingeras, T.R., Hoskins, R.A., Kaufman, T.C., Oliver, B., and Celniker, S.E. (2011). The developmental transcriptome of Drosophila melanogaster. Nature *471*, 473-479.

17. Maldonado, C., Alicea, D., Gonzalez, M., Bykhovskaia, M., and Marie, B. (2012). Adar is essential for optimal presynaptic function. Molecular and Cellular Neuroscience *52*, 173-180.

18. Ma, E., Gu, X.Q., Wu, X., Xu, T., and Haddad, G.G. (2001). Mutation in pre-mRNA adenosine deaminase markedly attenuates neuronal tolerance to O2 deprivation in Drosophila melanogaster. J Clin Invest *107*, 685-693.

19. Daniels, R.W., Collins, C.A., Chen, K., Gelfand, M.V., Featherstone, D.E., and Diantonio, A. (2006). A single vesicular glutamate transporter is sufficient to fill a synaptic vesicle. Neuron *49*, 11-16.

20. Daniels, R.W., Collins, C.A., Gelfand, M.V., Dant, J., Brooks, E.S., Krantz, D.E., and DiAntonio, A. (2004). Increased expression of the Drosophila vesicular glutamate transporter leads to excess glutamate release and a compensatory decrease in quantal content. J Neurosci *24*, 10466-10474.

21. Kuromi, H., and Kidokoro, Y. (1998). Two distinct pools of synaptic vesicles in single presynaptic boutons in a temperature-sensitive Drosophila mutant, shibire. Neuron *20*, 917-925.

22. Akbergenova, Y., and Bykhovskaia, M. (2007). Synapsin maintains the reserve vesicle pool and spatial segregation of the recycling pool in Drosophila presynaptic boutons. Brain Res *1178*, 52-64.

23. Godenschwege, T.A., Reisch, D., Diegelmann, S., Eberle, K., Funk, N., Heisenberg, M., Hoppe, V., Hoppe, J., Klagges, B.R., Martin, J.R., Nikitina, E.A., Putz, G., Reifegerste, R., Reisch, N., Rister, J., Schaupp, M., Scholz, H., Schwarzel, M., Werner, U., Zars, T.D., Buchner, S., and Buchner, E. (2004). Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. Eur J Neurosci *20*, 611-622.

24. Kume, K., Kume, S., Park, S.K., Hirsh, J., and Jackson, F.R. (2005). Dopamine is a regulator of arousal in the fruit fly. J Neurosci *25*, 7377-7384.

25. Andretic, R., van Swinderen, B., and Greenspan, R.J. (2005). Dopaminergic modulation of arousal in Drosophila. Curr Biol *15*, 1165-1175.

26. Crocker, A., and Sehgal, A. (2008). Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. J Neurosci *28*, 9377-9385.

27. Crocker, A., Shahidullah, M., Levitan, I.B., and Sehgal, A. (2010). Identification of a neural circuit that underlies the effects of octopamine on sleep:wake behavior. Neuron *65*, 670-681.

28. Wu, M., Robinson, J.E., and Joiner, W.J. (2014). SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission. Curr Biol *24*, 621-629.

29. Shi, M., Yue, Z., Kuryatov, A., Lindstrom, J.M., and Sehgal, A. (2014). Identification of Redeye, a new sleep-regulating protein whose expression is modulated by sleep amount. Elife *3*, e01473.

30. Yuan, Q., Joiner, W.J., and Sehgal, A. (2006). A sleep-promoting role for the Drosophila serotonin receptor 1A. Curr Biol *16*, 1051-1062.

31. Tsien, J.Z., Huerta, P.T., and Tonegawa, S. (1996). The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell *87*, 1327-1338.

32. Liu, L., Wong, T.P., Pozza, M.F., Lingenhoehl, K., Wang, Y., Sheng, M., Auberson, Y.P., and Wang, Y.T. (2004). Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science *304*, 1021-1024.

33. Zhao, M.G., Toyoda, H., Lee, Y.S., Wu, L.J., Ko, S.W., Zhang, X.H., Jia, Y., Shum, F., Xu, H., Li, B.M., Kaang, B.K., and Zhuo, M. (2005). Roles of NMDA NR2B subtype receptor in prefrontal long-term potentiation and contextual fear memory. Neuron *47*, 859-872.

34. Tomita, J., Ueno, T., Mitsuyoshi, M., Kume, S., and Kume, K. (2015). The NMDA Receptor Promotes Sleep in the Fruit Fly, Drosophila melanogaster. PLoS One *10*, e0128101.

35. Rotman, Z., Deng, P.-Y., and Klyachko, V.A. (2011). Short-Term Plasticity Optimizes Synaptic Information Transmission. J Neurosci *31*, 14800-14809.

36. Burnashev, N., Monyer, H., Seeburg, P.H., and Sakmann, B. (1992). Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. Neuron *8*, 189-198.

37. Swanson, G.T., Kamboj, S.K., and Cull-Candy, S.G. (1997). Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. J Neurosci *17*, 58-69.

38. Lomeli, H., Mosbacher, J., Melcher, T., Hoger, T., Geiger, J.R., Kuner, T., Monyer, H., Higuchi, M., Bach, A., and Seeburg, P.H. (1994). Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. Science *266*, 1709-1713.

39. Kawahara, Y., Ito, K., Sun, H., Aizawa, H., Kanazawa, I., and Kwak, S. (2004). Glutamate receptors: RNA editing and death of motor neurons. Nature *427*, 801.

40. Hideyama, T., Yamashita, T., Suzuki, T., Tsuji, S., Higuchi, M., Seeburg, P.H., Takahashi, R., Misawa, H., and Kwak, S. (2010). Induced loss of ADAR2 engenders slow death of motor neurons from Q/R site-unedited GluR2. J Neurosci *30*, 11917-11925.

41. Yamashita, T., Chai, H.L., Teramoto, S., Tsuji, S., Shimazaki, K., Muramatsu, S., and Kwak, S. (2013). Rescue of amyotrophic lateral sclerosis phenotype in a mouse model by intravenous AAV9-ADAR2 delivery to motor neurons. EMBO Mol Med *5*, 1710-1719.

42. Ni, J.-Q., Liu, L.-P., Binari, R., Hardy, R., Shim, H.-S., Cavallaro, A., Booker, M., Pfeiffer, B.D., Markstein, M., Wang, H., Villalta, C., Laverty, T.R., Perkins, L.A., and Perrimon, N. (2009). A Drosophila Resource of Transgenic RNAi Lines for Neurogenetics. Genetics *182*, 1089-1100.

43. Ni, J.Q., Zhou, R., Czech, B., Liu, L.P., Holderbaum, L., Yang-Zhou, D., Shim, H.S., Tao, R., Handler, D., Karpowicz, P., Binari, R., Booker, M., Brennecke, J., Perkins, L.A., Hannon, G.J., and Perrimon, N. (2011). A genome-scale shRNA resource for transgenic RNAi in Drosophila. Nat Methods *8*, 405-407.

44. Maples, T., and Rothenfluh, A. (2011). A simple way to measure ethanol sensitivity in flies. J Vis Exp.

45. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods *9*, 676-682.

46. Daniels, R.W., Collins, C.A., Gelfand, M.V., Dant, J., Brooks, E.S., Krantz, D.E., and DiAntonio, A. (2004). Increased expression of the Drosophila vesicular glutamate transporter leads to excess glutamate release and a compensatory decrease in quantal content. J Neurosci *24*, 10466-10474.

47. Dickman, D.K., and Davis, G.W. (2009). The schizophrenia susceptibility gene dysbindin controls synaptic homeostasis. Science *326*, 1127-1130.

48. Martin, A.R. (1955). A further study of the statistical composition on the end-plate potential. J Physiol *130*, 114-122.

Chapter 3: Development of a novel method for quantitative measurements of arousal thresholds in *Drosophila*

3.1 Summary

Drosophila have become a widely used genetic model for sleep research, however, given their small size, it is difficult to assess various features of sleep such as arousal threshold. While there have been previous studies utilizing *ad hoc* measurements of arousal threshold, we set out to develop and validate a semi-high throughput assay to measure arousal thresholds in *Drosophila*. We found that arousal threshold measurements can be fit with four-parameter sigmoidal dose-response curves that can then be used to determine a best-fit intensity at which a half-maximal arousal response is attained. We validated our assay to determine reproducibility and to optimize a protocol that does not introduce added complexity to the analysis. Finally, we found that arousal thresholds for animals with or without a functional circadian clock do not change throughout the entire day. Our novel arousal threshold assay will provide an added layer of depth to sleep studies in *Drosophila* in the future.

3.2 Introduction

Sleep is a fundamental process that is conserved across all organisms with a central nervous system [1]. The ubiquitous nature of sleep suggests an important role for the behavior in maintaining normal physiology for survival

despite the disadvantages posed by the periods of immobility and lost consciousness. While much is known about sleep and its electrophysiological correlates, very little is known about the function of sleep. To address this gap in knowledge, numerous genetic models of sleep have arisen ranging from *C. elegans* to mammals [2]. In order to properly study sleep as a genetically tractable behavior, we must first define the behavior in a way that is applicable to all organisms.

After years of study, it is generally accepted that sleep has at least 5 fundamental features: 1) Sleep occurs as periods of inactivity or immobility. 2) Sleeping animals have an elevated arousal threshold. 3) It is homeostatically regulated. 4) Sleep is rapidly reversible. 5) Sleep is identifiable by changes in electrical activity of the brain.

Most of these fundamental features of sleep are easier to study in some genetic models than others. For example, it is much easier to measure altered brain activity in mice than it is in *Drosophila*. For this reason, model systems such as *Drosophila* require additional experimental measures to verify changes in locomotor activity are indeed changes in sleep rather than hyper- or hypoactivity.

In order to maximize the throughput of screening for novel genes involved in the regulation of sleep and arousal, our lab focuses on the study of sleep in *Drosophila melanogaster*. Previous studies have empirically determined that quiescence in flies of at least 5 minutes correlates very well with the fundamental features of sleep. Animals that have been quiescent for

at least 5 minutes have a stereotypical posture, increased arousal threshold, and a net decrease in neuronal activity thus strongly conforming to the definition of sleep [3-6]. Additionally, quiescence in flies, like mammals, is under both circadian and homeostatic regulation thus controlling the timing and amount of sleep, respectively [7, 8].

A primary challenge in studying sleep behaviors in *Drosophila* is the lack of practical assays to measure neuronal activity in a behaving fly. While such experiments have been performed [5], the experiments are technically challenging and difficult to interpret. In order to expand the repertoire of assays available to probe sleep behaviors in flies, we focused on developing a reliable quantitative assay for measuring arousal thresholds in flies. While previous works have been published utilizing various methods [9-11], many of these methods are prone to user-error, inter-run variability, and low sensitivity.

In this study, we developed an apparatus that can accommodate up to 8 DAM2 monitors for simultaneous measurements of up to 256 animals. By oscillating the platform at programmable frequencies at specified times of day or night, we found that we could quantitatively assess arousal thresholds in *Drosophila*. Using this method, we optimized the parameters for arousal measurements and found that the measurements are robust and reproducible. Finally, we found that arousal levels do not vary throughout the day.

3.3 Results

3.3.1 An apparatus for measuring arousal threshold

To accommodate a sufficient number of activity monitors to accurately and reliably assess arousal thresholds, we built a platform with slots to hold up to 8 DAM2 activity monitors. This platform was connected to a rotating arm and stepper motor such that the platform could oscillate perpendicular to the axis of locomotion of the *Drosophila* (Figure 3.1a). By programmatically setting the timing and intensity of oscillations, we hypothesized that we would be able to arouse increasing proportions of animals with increasing intensities. After empirically determining a range of stimulus intensities that resulted in arousal values that fall on the "linear" region of the curve (Figure 3.1b), we found that we could minimize baseline arousal levels by maintaining 0.1 Hz oscillations starting one hour before the first stimulus and continuing throughout the experiment for up to 30 minutes after the final stimulus (data not shown). Based on the expected shape of the resulting data, we hypothesized that the arousal data could be well fit with a 4-paramater dose-response sigmoidal curve (Figure 3.1c). Using the best-fit values from this curve, we should then be able to determine a half maximal intensity for arousal – a value we refer to as the Arousal Threshold for 50% of animals (AT50).

3.3.2 Daytime and nighttime arousal are highly similar

Due to the profound effects of light on the circadian clock and behavioral rhythms [12, 13], we first wanted to determine if arousal thresholds were different between daytime and nighttime sleep. In order to address this question, we performed arousal measurements on both male and female w^{1118}

flies between ZT5-7 and ZT17-19 representing daytime and nighttime, respectively. To our surprise, we did not observe any differences in arousal curves or AT50 values for daytime compared to nighttime (Figure 3.2). These data suggest that daytime sleep and nighttime sleep are equivalent behavioral states in *Drosophila* – a conclusion that has not been thoroughly examined before. Additionally, we found that despite differences in total daily sleep between male and female flies, we did not observe significant differences in arousal thresholds between the two sexes.

3.3.3 Arousal threshold measurements are reproducible across days

Drosophila, like many organisms with a central nervous system, exhibit a high degree of behavioral plasticity [14, 15]. In order to determine if flies acclimate to repeated stimuli across multiple days, we measured arousal in flies on three consecutive days. Additionally, to determine whether the order of stimulus intensities influenced subsequent arousal measurements, we ran the arousal measurement protocol in reverse on the second day of stimulations. We found that across three consecutive days of measurements, flies did not acclimate to stimulation but instead, displayed a high degree of reproducibility (Figure 3.3). Further, the order of stimuli (low to high vs high to low) had no effect on arousal measurements. Together, these data suggest that our arousal measurement method is robust, reproducible and not subject to acclimation.

3.3.4 Interpulse interval length does not influence arousal

A recent study suggested that repeated stimulation of flies at an interval of 30 minutes results in disruptions that influence subsequent stimulations [9]. This study found that interstimulus intervals of 1 hour or more did not influence subsequent arousal when repeated for 72 hours. We hypothesized that since our arousal protocol only requires 2 to 2.5 hours, that our 30-minute interpulse interval time would not alter arousal if performed at 1-hour intervals over the same time period. To test this hypothesis, we tested flies on subsequent days wherein flies were measured using the 5-stimulus 30-minute interval protocol on the first day and a 3 stimulus 1 hour interval on the second day. We found that for each protocol, almost identical proportions of flies were aroused at given stimulus intensities (Figure 3.4). These data support our hypothesis that our 30-minute interpulse interval protocol is sufficient to generate arousal curves without needing to extend the interval periods to 1 hour or more.

3.3.5 Arousal threshold across time of day does not change

Given the lack of change in arousal threshold between daytime and nighttime sleep, we hypothesized that sleep in *Drosophila* is a behavioral state with arousal characteristics that are independent of the circadian clock. It has long been postulated that the circadian clock sets the timing of activity and arousal; however, only activity has been measured due to the ease of the measurements. We decided to test our hypothesis that arousal threshold for sleeping flies does not change across the time of day. To test this hypothesis,

we measured arousal threshold at 6 different times of day: early day, mid-day, late day, early night, mid-night, and late night. Additionally, we simultaneously performed these measurements in animals lacking a circadian clock (*Per*^{0} animals). Despite difficulties measuring arousal at time points closest to light-dark transitions due to low numbers of sleeping animals (data not shown), we found that arousal thresholds as measured by AT50, do not vary significantly across time of day (Figure 3.5). These data suggest that the sleep state in *Drosophila* does not change throughout time of day.

3.4 Discussion

By developing a reliable assay to measure the arousal threshold of *Drosophila*, we have opened up new avenues for sleep research to probe the underlying genetics and neuroanatomy of arousal. While assays measuring arousal threshold have been previously reported [9-11], we have developed the first quantitative, semi-high throughput, reliable assay to assess arousal thresholds. In developing this assay, we have shown that arousal data can be fit with a four-parameter sigmoidal dose-response curve that can then be used to find the best-fit intensity at which half maximal arousal occurs. Additionally, we found that our assay was highly reproducible across several days and that the interval between stimuli had no effect on arousal levels as had been previously suggested for much longer stimulation periods [9]. Finally, we found that arousal thresholds are not influenced by loss of the molecular circadian clock and that these thresholds do not change across time of day.

Based on these results, we conclude that arousal in flies is an assayable behavior that can be assessed in populations of animals. Rather than correlating increased total sleep with depth of sleep, this assay will allow for a more direct measurement of depth of sleep and sleep need. While arousal threshold does not give the same type of information as would be possible with an EEG, it does increase the breadth of possible experiments and information relative to measurements of total sleep alone.

We feel that one of the more interesting aspects of this study revolves around our data not indicating a difference in arousal between daytime versus nighttime or across time of day. Conventional wisdom would suggest that due to the effects of light on arousal [12, 13], we should expect to observe increased arousal during daytime in *Drosophila*. However, the fact that we were unable to detect such a difference suggests that arousal mechanisms are independent of light and the circadian clock. One rational for how this may work is that sleep bouts may be a pre-set behavioral state with a physiological set of instructions much like observed with the various discriminable stages of sleep in mammals. Unlike in mammals and several previous works in invertebrates [16-18], we observed no difference in arousal threshold at any of the time points throughout the day. One potential explanation for this discrepancy may come from the fact that we did not analyze our data in such a way to identify various sleep stages as was previously done in honey bee foragers [19] and thus may have missed small differences in specific stages of sleep across the 24 hour day.

The real power of using our arousal threshold measurements will come in studies directed at determining changes in depth of sleep as assessed by arousal threshold. We envision utilizing this assay for studies on sleep homeostasis and rebound sleep.

3.5 Methods

3.5.1 Fly stocks

Flies were grown at room temperature (20-22°C) on standard cornmeal media with yeast. All fly lines used unless otherwise indicated were w^{1118} *iso31* or outcrossed a minimum of 5 times into a w^{1118} *iso31* genetic background.

3.5.2 Sleep and locomotor measurements

Individual 3-7 day old flies were placed in 5 mm x 65 mm Pyrex tubes containing a mixture of 2% agarose and 5% sucrose at one end as a food source. Animals were entrained for a minimum of 2 days in 12 hr:12 hr light:dark conditions at 25°C, and infrared beam breaks were recorded in 1 min bins for the next 2 days prior to arousal measurements using the DAM2 Drosophila Activity Monitoring System (Trikinetics). Sleep analysis was performed as previously described using custom MATLAB (Mathworks) software [20].

3.5.3 Arousal threshold stimulations

Flies were loaded into up to 8 DAM2 activity monitors and placed on a custom built platform that oscillates along a line perpendicular to the axis of *Drosophila* locomotion. Oscillation speed and timing were controlled through custom MATLAB software communicating with an Arduino Uno-powered stepper motor. Arousal stimulus protocols, unless otherwise indicated, consisted of a 60 min pre-pulse oscillation speed of 0.1 Hz followed by arousal stimuli of 1 or 2 oscillations at indicated speeds spaced every 30 minutes for a total of 5 or 6 stimulations separated by continuous oscillations at 0.1 Hz. To confirm stimulus intensities, the oscillating platform was equipped with an accelerometer that reported values back to the MATLAB program at an interval of once every 4 steps where one full rotation equals 2,000 steps. Each round of arousal stimulations were performed 1-3 times and averaged for each batch of flies.

3.5.4 Arousal threshold analysis

Arousal was determined in three steps. First, only animals that were asleep at the time of the stimulus were considered for arousal. Sleeping animals were defined as those with no activity in the 5 or 10 minutes immediately before the stimulation depending on the experiment. Second, animals that had at least one activity count during the one-minute bin encompassing the stimulation through 5 minutes after were considered to have been aroused. Third, the total number of animals aroused divided by the total arousable number of animals (animals asleep during the 5 or 10 minutes

prior to stimulation) was calculated to determine the proportion of animals aroused.

The stimulation intensity for which half maximal arousal was achieved above baseline (referred to here as AT50) was determined by fitting the average proportion of animals aroused at each intensity to a sigmoidal 4-parameter dose-response curve with the Top and Bottom parameters constrained to be between 0 and 1. The best-fit value for EC50 was then used for the AT50 of the experiment. Error bars represent the 95% confidence interval for the AT50.

3.5.5 Statistics

Replicates (n values) represent the number of biological replicates for each experimental condition. Unless otherwise indicated, bar graphs depict the mean ± SEM. Unless otherwise indicated, datasets that approximate a normal Gaussian distribution were analyzed with unpaired Student's t-test followed by Welch's correction for comparisons between two groups. For experiments of single factor design, we analyzed data using one-way ANOVA followed by the Tukey-Kramer test for multiple comparisons or Sidak's multiple comparisons test for select comparisons. For comparisons between best-fit values, the extra-sum-of-squares F test was used with a p-value of less than 0.05 considered significant. For all figures, *, **, ***, and **** represent $p < 0.05$, 0.01, 0.001, and 0.0001.

3.6 Acknowledgements

Work presented in Chapter 3 will be submitted for publication upon completion of follow-up studies. The dissertation author was the primary author of this work in collaboration with Glen Seidner, Steve Roberts, and William Joiner.

3.7 Figures

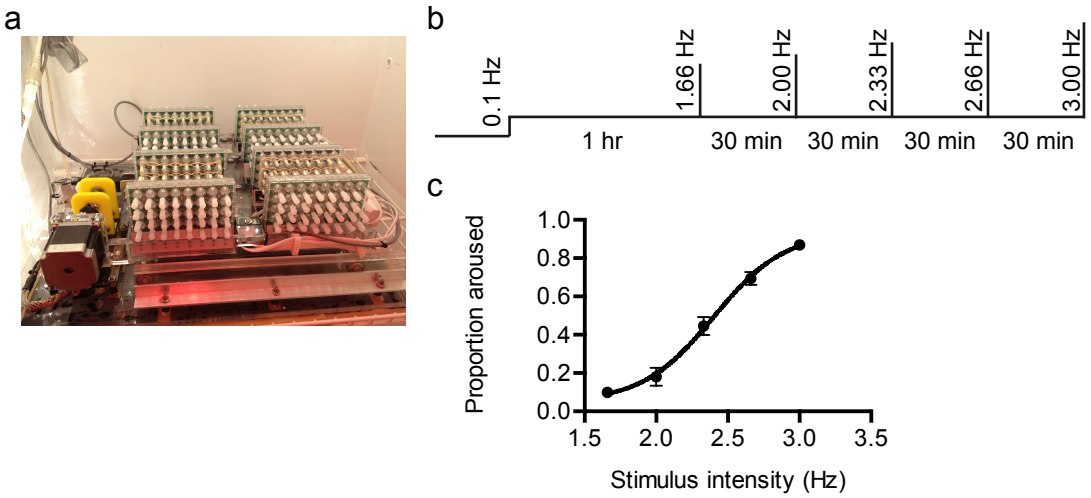

Figure 3.1 A novel apparatus for arousal threshold measurements. (a) Photograph of the MAELSTRM apparatus. The platform oscillates at programmable frequencies along a 1-dimensional axis perpendicular to the axis of *Drosophila* locomotion. **(b)** Schematic of the standard arousal determination protocol. One hour prior to the first stimulus, the platform begins oscillating at a baseline 0.1 Hz followed by individual stimuli at the indicated frequencies spaced 30 minutes apart. **(c)** A representative arousal trace indicating the stimulus intensity (X-axis) and proportion of animals aroused (Y-axis). The data are then fit with a 4-parameter dose response sigmoidal curve.

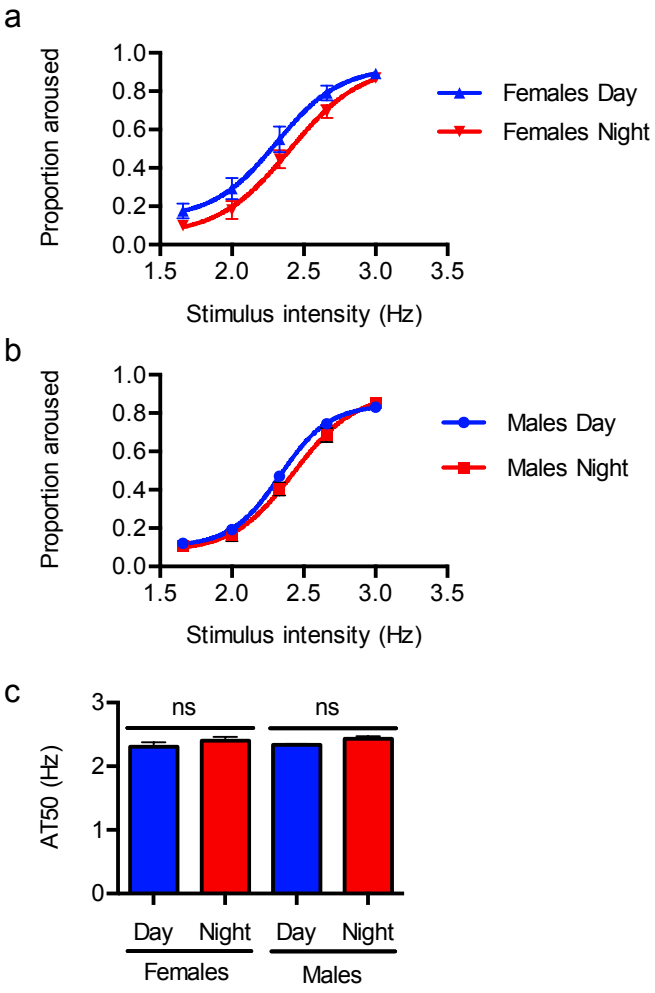

Figure 3.2 Baseline arousal of female and male flies does not differ between nighttime and daytime sleep. (a and **b)** Arousal threshold curves for (a) female and (b) male w^{1118} flies during the daytime siesta (ZT5-7; blue) and night (ZT17-19; red) [n=3].

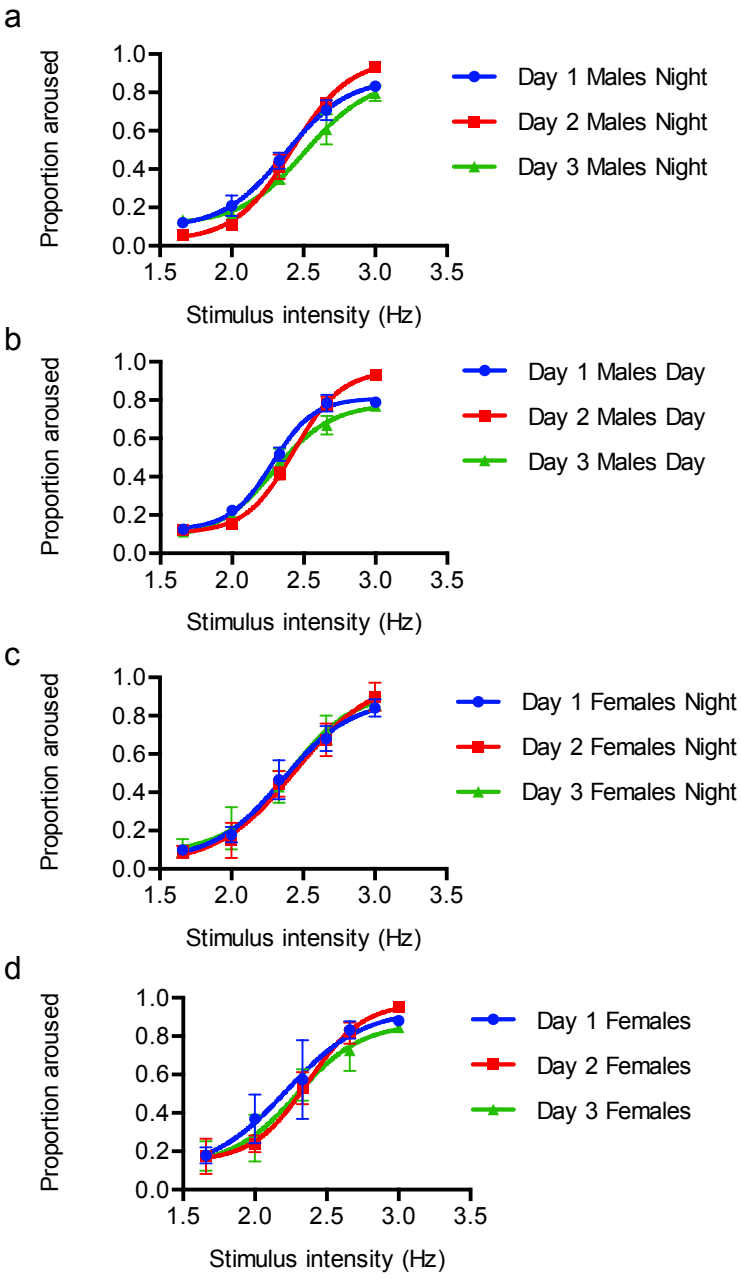

Figure 3.3 Arousal curves are highly similar across technical replicates. **(a** and **b)** Arousal curves for male w^{1118} flies between ZT5-7 (daytime siesta; a) and ZT17-19 (nighttime; b). **(c** and **d)** Arousal curves for female w^{1118} flies between ZT5-7 (daytime siesta; c) and ZT17-19 (nighttime; d). Notice the similarity in arousal curves from the first (blue), second (red), and third (green) day of stimulus trials [n=3].

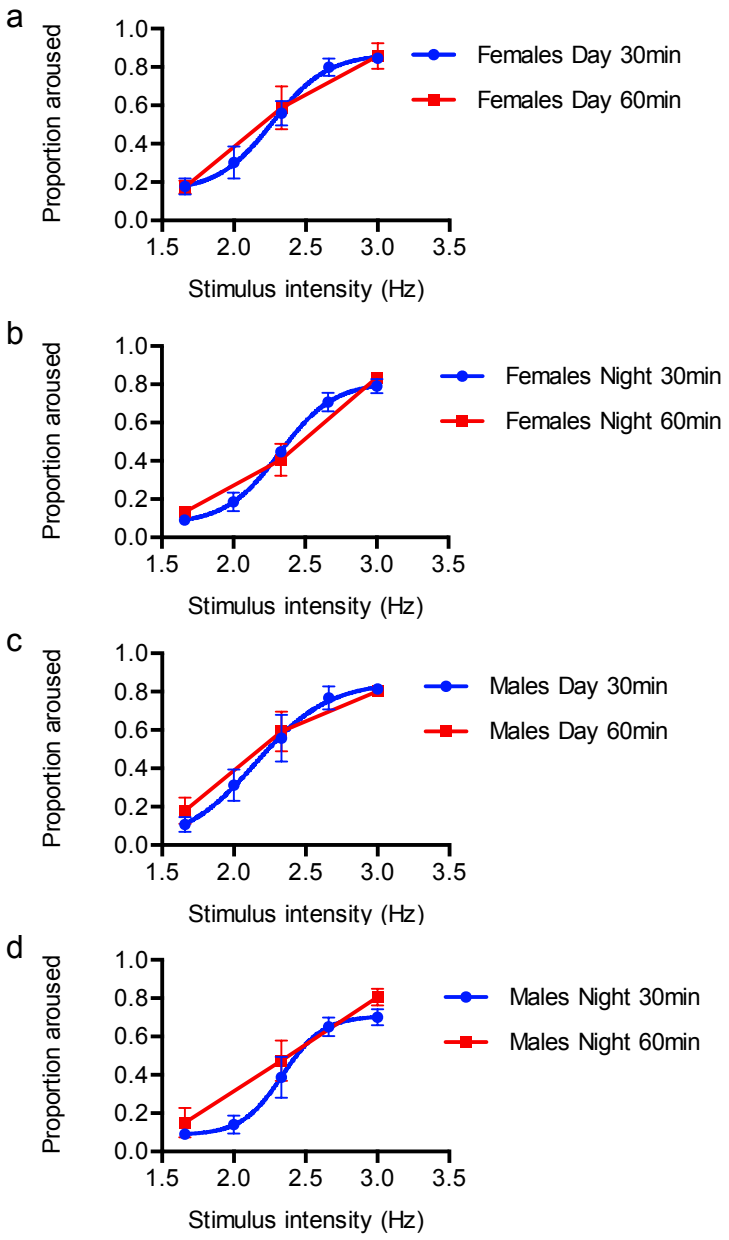

Figure 3.4 Arousal levels are not influenced by 30- vs 60- minute inter-pulse intervals. (a-d) Arousal threshold curves for females during day (a) and night (b) and males during day (c) and night (d). No difference was observed between 30- and 60-minute inter-pulse intervals at each stimulus intensity [n=3].

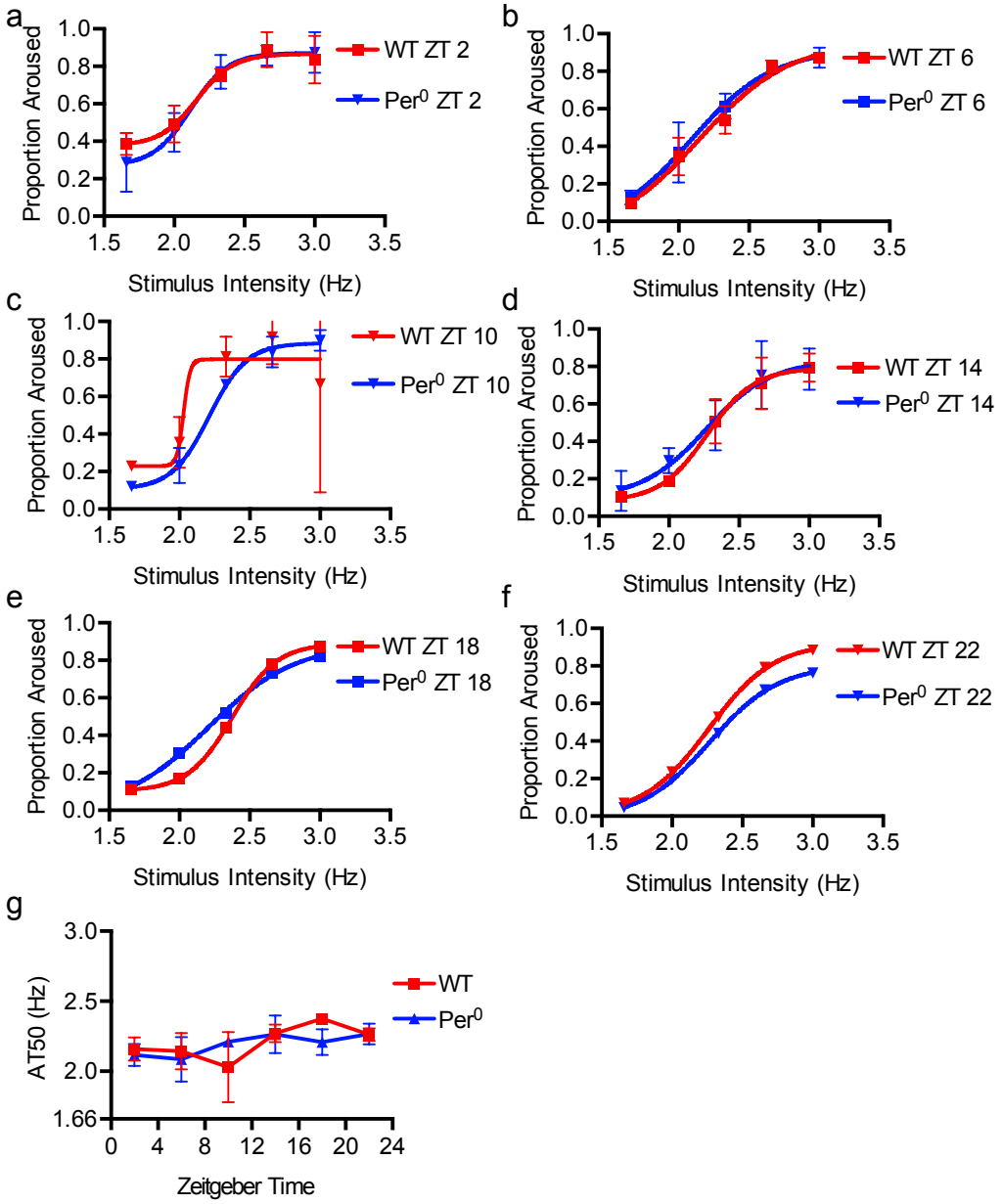

Figure 3.5 Arousal levels do not change across ZT time and are not under control of the clock. (a-f) Arousal curves at ZT 2 (a), ZT 6 (b), ZT 10 (c), ZT 14 (d), ZT 18 (e), and ZT 22 (f) for both w^{1118} (WT; red) and Per^{0} (blue) animals. **(g)** Summary graph for the AT50 values of WT and Per^{0} animals at each indicated time of day [n≥3 for each time point].

3.8 References

1. Allada, R., and Siegel, J.M. (2008). Unearthing the phylogenetic roots of sleep. Curr Biol *18*, R670-R679.

2. Crocker, A., and Sehgal, A. (2010). Genetic analysis of sleep. Genes Dev *24*, 1220-1235.

3. Shaw, P.J., Cirelli, C., Greenspan, R.J., and Tononi, G. (2000). Correlates of sleep and waking in Drosophila melanogaster. Science *287*, 1834-1837.

4. Hendricks, J.C., Finn, S.M., Panckeri, K.A., Chavkin, J., Williams, J.A., Sehgal, A., and Pack, A.I. (2000). Rest in Drosophila is a sleep-like state. Neuron *25*, 129-138.

5. Nitz, D.A., van Swinderen, B., Tononi, G., and Greenspan, R.J. (2002). Electrophysiological correlates of rest and activity in Drosophila melanogaster. Curr Biol *12*, 1934-1940.

6. van Swinderen, B., and Greenspan, R.J. (2003). Salience modulates 20-30 Hz brain activity in Drosophila. Nat Neurosci *6*, 579-586.

7. Huber, R., Hill, S.L., Holladay, C., Biesiadecki, M., Tononi, G., and Cirelli, C. (2004). Sleep homeostasis in Drosophila melanogaster. Sleep *27*, 628-639.

8. Keene, A.C., Duboue, E.R., McDonald, D.M., Dus, M., Suh, G.S., Waddell, S., and Blau, J. (2010). Clock and cycle limit starvation-induced sleep loss in Drosophila. Curr Biol *20*, 1209-1215.

9. van Alphen, B., Yap, M.H., Kirszenblat, L., Kottler, B., and van Swinderen, B. (2013). A dynamic deep sleep stage in Drosophila. J Neurosci *33*, 6917-6927.

10. Crocker, A., and Sehgal, A. (2008). Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. J Neurosci *28*, 9377-9385.

11. Kayser, M.S., Yue, Z., and Sehgal, A. (2014). A critical period of sleep for development of courtship circuitry and behavior in Drosophila. Science *344*, 269-274.

12. Zeng, H., Qian, Z., Myers, M.P., and Rosbash, M. (1996). A light-entrainment mechanism for the Drosophila circadian clock. Nature *380*, 129-135.

13. Hunter-Ensor, M., Ousley, A., and Sehgal, A. (1996). Regulation of the Drosophila protein timeless suggests a mechanism for resetting the circadian clock by light. Cell *84*, 677-685.

14. Spatz, H.C., Emanns, A., and Reichert, H. (1974). Associative learning of Drosophila melanogaster. Nature *248*, 359-361.

15. Quinn, W.G., Harris, W.A., and Benzer, S. (1974). Conditioned behavior in Drosophila melanogaster. Proc Natl Acad Sci U S A *71*, 708-712.

16. Neckelmann, D., and Ursin, R. (1993). Sleep stages and EEG power spectrum in relation to acoustical stimulus arousal threshold in the rat. Sleep *16*, 467-477.

17. Sauer, S., Kinkelin, M., Herrmann, E., and Kaiser, W. (2003). The dynamics of sleep-like behaviour in honey bees. J Comp Physiol A Neuroethol Sens Neural Behav Physiol *189*, 599-607.

18. Tobler, I.I., and Neuner-Jehle, M. (1992). 24-h variation of vigilance in the cockroach Blaberus giganteus. J Sleep Res *1*, 231-239.

19. Eban-Rothschild, A.D., and Bloch, G. (2008). Differences in the sleep architecture of forager and young honeybees (Apis mellifera). J Exp Biol *211*, 2408-2416.

20. Wu, M., Robinson, J.E., and Joiner, W.J. (2014). SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission. Curr Biol *24*, 621-629.

Chapter 4: Identification of neurons with a privileged role in sleep homeostasis in *Drosophila melanogaster*

4.1 Summary

Sleep is thought to be controlled by two main processes: a circadian clock that primarily regulates sleep timing and a homeostatic mechanism that detects and responds to sleep need. While an abundance of experimental evidence suggests sleep need increases with time spent awake, the contributions of various arousal systems have not been assessed independently of each other to determine if certain neural circuits, rather than waking *per se*, selectively contribute to sleep homeostasis. Using the fruit fly, *Drosophila melanogaster*, we found that sustained thermogenetic activation of three independent neurotransmitter systems promoted nighttime wakefulness. However, only sleep deprivation resulting from activation of cholinergic neurons was sufficient to elicit subsequent homeostatic recovery sleep, as assessed by multiple behavioral criteria. In contrast, sleep deprivation resulting from activation of octopaminergic neurons suppressed homeostatic recovery sleep, indicating that wakefulness can be dissociated from accrual of sleep need. Neurons involved in promoting sleep homeostasis were found to connect to the central brain and motor control regions of the thoracic ganglion. Blocking activity of these neurons suppressed recovery sleep but did not alter baseline sleep, further differentiating between neural control of sleep homeostasis and daily fluctuations in the sleep/wake cycle. Selective

activation of wake-promoting neurons without engaging the sleep homeostat impaired subsequent short-term memory, thus providing evidence that the neural circuitry regulating sleep homeostasis is important for behavioral plasticity. Together, our data suggest a simple neural circuit model involving distinct populations of wake-promoting neurons, only some of which are involved in homeostatic control of sleep. Our findings suggest that proper cognition requires function of neural circuits that promote sleep homeostasis.

4.2 Introduction

A growing body of literature indicates that sleep is critical for restorative properties involved in cognition, metabolic regulation, and cardiovascular and immune function [1-13]. Despite the established roles for sleep in these processes, the molecular and neuroanatomical underpinnings of sleep regulation are poorly understood. Recent progress on these fronts has largely come from identification of molecules and neurons that are permissive for activation or suppression of different arousal circuits [14, 15]. But mechanisms underlying sleep homeostasis, i.e. the dynamic process of detecting and responding to sleep need, have remained far more elusive.

A particularly intriguing question is how sleep need is detected and communicated across the brain to enable distinct sleep and wake states. For example, several studies have demonstrated that slow-wave activity, which is often used as an indicator of mammalian sleep need, manifests first in regions of the brain that are most intensely activated during prior waking tasks [16-22].

These studies suggest that sleep need exists at a cellular level in many brain regions and is driven by local activity. However, EEG and intracortical recordings demonstrate that electrophysiological correlates of slow-wave activity occur simultaneously in neurons throughout the brain [23-25]. Thus, sleep may require coordinated regulation by circuits that detect and communicate sleep need to the rest of the brain. In other words, sleep as a behavioral state could emerge from the summed activities of various populations of locally synchronized neurons, or it could be driven by dedicated homeostatic circuitry that exerts global control of brain arousal.

The latter hypothesis would certainly be strengthened by identification of distinct populations of neurons that drive waking: that is, those whose activities cause subsequent homeostatic recovery sleep and those that do not. Although it has generally been assumed that prior waking by any means drives subsequent sleep need [26], this hypothesis has never been formally tested by comparing the effects of activating different arousal systems on sleep homeostasis. Until recently it has been technically impossible to conduct such experiments. However, with the advent of optogenetics and thermogenetics [27], and with the exquisite control available for dissection of functional neural circuits in genetically tractable model organisms such as flies and worms, technical hurdles to such experiments have been lowered substantially.

We directly tested the contributions of distinct arousal-regulating neurons to sleep homeostasis by expressing transgenic temperature-sensitive

TrpA1 channels in flies. Using a mild temperature pulse we activated these channels at night to depolarize neurons and deprive animals of sleep, and we measured subsequent recovery sleep the next morning. Our results indicate that different populations of known wake-promoting neurons have varied effects ranging from suppression to facilitation of sleep homeostasis. Wake-promoting neurons that drive sleep homeostasis appear to be rare, and at least some of these project to the brain and to the vicinity of motor control circuitry in the thoracic ganglion. We also show that neural circuits involved in sleep homeostasis promote restoration of short-term memory following sleep deprivation. Taken together, these findings indicate that sleep homeostasis and baseline sleep can be regulated by distinct neural circuits that differentially impact cognition.

4.3 Results

4.3.1 Homeostatic recovery sleep can be uncoupled from prior waking time

The standard assay for assessing sleep homeostasis in flies involves mechanically perturbing animals to prevent them from sleeping at night and then measuring subsequent recovery sleep, or rebound, the following morning [28, 29]. To better understand this process, we examined several metrics for recovery sleep following mechanical stimulation. We found that recovery sleep has several key features commonly observed in vertebrates [30]. First, it exceeds the amount of sleep that unperturbed controls would normally obtain

(Figure 4.1A-C). Second, it is transitory, typically subsiding within the first 6-12 hrs after sleep deprivation ends (Figure 4.1A). Third, it is associated with increased intensity, or depth of sleep, which we measured as an increase in arousal threshold. We assessed this feature by creating an apparatus that oscillates flies along a one-dimensional axis at programmable frequencies. We confirmed that arousal threshold is elevated during recovery sleep following mechanical sleep deprivation (Figure 4.1D; Figure 4.3A). At the highest intensity of stimulation, sleep during this period was fully and acutely reversible, illustrating a fourth key feature of recovery sleep that distinguishes it from seizure, coma or locomotor deficiency. Finally, recovery sleep is associated with decreased sleep latency (Figure 4.1A,E).

Although this technique is regularly used to study sleep homeostasis, recovery sleep resulting from mechanical deprivation is highly variable, thus complicating comparisons of different groups of animals. We reasoned that direct activation of wake-promoting neurons might overcome this problem by bypassing sensory transduction processes that convert environmental signals into arousal cues. Such a technical improvement might also allow us to assess the roles of different arousal systems in sleep homeostasis. To test this hypothesis we first expressed transgenic TrpA1 channels in cholinergic neurons, which we previously implicated in waking [31]. Indeed, a 6 hr heat pulse of animals expressing TrpA1 in cholinergic neurons (cha>TrpA1) led to a nearly complete suppression of sleep and a robust increase in recovery sleep

the next day (Figure 4.2A,B,E,F). Longer heat pulses led to paralysis and were not studied further.

Notably, thermogenetically induced recovery sleep conformed to the criteria established earlier for sleep homeostasis. First, during the 6 hrs immediately following sleep deprivation, recovery sleep exceeded sleep in controls lacking TrpA1, demonstrating that activation of cholinergic neurons rather than heat exposure *per se* was required for the effect (Figure 4.2A,F). Second, recovery sleep largely subsided within 12 hrs after sleep deprivation ended (Figure 4.2B). Third, recovery sleep was associated with increased sleep intensity, as measured by increased arousal threshold (Figure 4.2G; Figure 4.3B). Fourth, recovery sleep was acutely and fully reversible (Figure 4.3B). Fifth, it was accompanied by decreased sleep latency (Figure 4.2B,H). Recovery sleep was also not an artifact of phase-shifting the circadian clock because cha>TrpA1 and cha>+ animals that remained in constant darkness following the heat pulse showed the same timing of sleep/wake cycling (Figure 4.4). Thus, activation of cholinergic neurons is sufficient to sleep deprive animals and induce subsequent homeostatic recovery sleep.

We then asked whether the sleep homeostat is engaged by waking *per se* or by engagement of specific arousal circuits normally associated with waking. The former idea suggests that any means of prolonging waking should lead to homeostatic recovery sleep, whereas the latter suggests that it may be possible to uncouple waking from sleep need. To distinguish between these two possibilities we thermogenetically activated dopaminergic and

octopaminergic wake-promoting neurons and assessed the resulting effects on recovery sleep. To activate dopaminergic neurons we expressed TrpA1 channels under control of the tyrosine hydroxylase driver (TH>TrpA1). As expected from previous studies [32, 33], activation of these neurons with a heat pulse of up to 12 hrs was well-tolerated by animals and led to nearly complete loss of sleep (Figure 4.2C,E). Surprisingly, however, this effect elicited less recovery sleep than either sleep deprivation by mechanical means or by shorter activation of cha>TrpA1 (Figure 4.1C; Figure 4.2B,C,F). Changes in arousal threshold and sleep latency following thermogenetic sleep deprivation were also either indistinguishable or only weakly changed in sleep deprived TH>TrpA1 animals compared to undeprived TH>+ controls (Figure 4.2C,G,H; Figure 4.3C).

Even more striking was the lack of sleep homeostasis following activation of TrpA1 channels in octopaminergic neurons (Tdc2>TrpA1), which are known to promote waking [34, 35]. In this case a 12 hr heat pulse effectively kept animals awake, but no subsequent recovery sleep was observed (Figure 4.2D-F). Consistent with this observation, arousal threshold and sleep latency following activation of Tdc2>TrpA1 were unchanged (Figure 4.2D,G,H; Figure 4.3D). In summary, whereas activation of neurons harboring any of the three neurotransmitter systems we tested was sufficient to deprive animals of sleep, only cholinergic neurons contributed to a recovery process that quantitatively and qualitatively fulfilled all criteria for sleep homeostasis.

4.3.2 Select arousal-promoting circuits suppress sleep homeostasis

Our unexpected results suggest that sleep need can be separated from prior waking time, which ostensibly supports the hypothesis that specific neural circuits participate in sleep homeostasis. However, it is still possible that sleep need arises from waking in general but is actively suppressed by certain circuits [34, 35]. To test this hypothesis, we mechanically sleep-deprived flies for 12 hrs at night while simultaneously activating TrpA1 channels in either dopaminergic neurons (TH>TrpA1) or octopaminergic neurons (Tdc2>TrpA1). Control animals lacking TrpA1 were subjected to the same procedure (TH>+ or Tdc2>+). Using this “shake and bake” protocol, we found that apparent recovery sleep in TH>TrpA1 animals was not reduced compared to TH>+ controls (Figure 4.5A,C). Thus, activation of dopaminergic neurons does not suppress sleep homeostasis. In contrast, recovery sleep was much lower in Tdc2>TrpA1 animals compared to Tdc2>+ controls, despite similar sleep deprivation in both groups (Figure 4.5B,C). These results support the hypothesis that activation of octopaminergic, but not dopaminergic neurons suppresses sleep homeostasis.

If sleep homeostasis resulted from extended waking as a behavioral state, rather than from activation of specific wake-promoting neural circuits, then sleep deprivation should always elicit recovery sleep, providing the latter was not actively inhibited. However, the failure of wake-promoting dopaminergic neurons to inhibit subsequent recovery sleep suggests that sleep homeostasis does not result by default during waking. Instead this

finding suggests that sleep need may be driven by specific wake-promoting neural circuits. To search for these, we first performed an anatomical screen for populations of neurons that are capable of activating waking when animals would normally sleep. To accomplish this task we performed two experiments. In the first we determined the effects of a 6 hr nighttime heat pulse on a subset of Gal4 drivers in the absence of transgenic TrpA1. The sleep changes we observed for these Gal4>+ control animals during and after pulses allowed us to establish boundaries beyond which heat could not account for behavior of Gal4>TrpA1 animals in the next experiment. In this second experiment, we screened for Gal4>TrpA1 combinations whose sleep behavior during and after heat pulses fell outside the established control range. As we observed with Gal4>+ controls, many Gal4>TrpA1 groups exhibited a mild loss of sleep during the heat pulse and no recovery sleep afterward (Figure 4.5D). However, we found that ~10% of Gal4>TrpA1 combinations exhibited sleep loss outside the control range, indicating that TrpA1 activation in these Gal4 lines promoted arousal. Within this group, 89% of Gal4>TrpA1 combinations failed to exhibit significant homeostatic recovery sleep (Figure 4.5D, left of red line and below blue line). This phenotype was confirmed for four Gal4>TrpA1 lines (Figure 4.5E; Figure 4.6). Thus, most groups of wake-promoting neurons do not elicit sleep homeostasis under our experimental conditions, again indicating that sleep homeostasis can be dissociated from prior waking in the majority of cases.

4.3.3 Select groups of cholinergic neurons promote sleep homeostasis

Only 11% of the Gal4>TrpA1 combinations from our screen that enabled robust sleep deprivation also exhibited significant subsequent recovery sleep (Figure 4.5C, left of red line and above blue line). The relative infrequency of recovery sleep following sleep deprivation suggested that neurons labeled by select Gal4 drivers might have privileged roles in promoting sleep homeostasis. To address this question and to determine how functionally redundant such neurons might be, we first retested Gal4>TrpA1 combinations with strong phenotypes and found that one in particular, 24C10, triggered consistent sleep homeostasis (Figure 4.7A,C). We also tested a variety of Gal4 drivers derived from enhancer trap screens and previously described promoters, and we found another, ppk, that exhibited a similarly robust phenotype (Figure 4.7B,C). This phenotype conforms to our definition of recovery sleep in both cases since it satisfied all the criteria established above for sleep homeostasis, including overshoot of baseline sleep, rapid decay, increased arousal threshold, rapid reversibility, and decreased sleep latency (Figure 4.7A-C; Figure 4.8A-D).

Since we previously established that homeostatic recovery sleep could be elicited most effectively by prior activation of cholinergic neurons (Figure 4.2B-H), we then asked whether excluding TrpA1 from these neurons could block 24C10- and ppk-driven recovery sleep. To address this question we crossed our two Gal4>TrpA1 combinations to animals bearing the Gal4 suppressor, Gal80, expressed in cholinergic neurons (cha-Gal80). We found

that the 24C10 and ppk drivers could no longer deprive animals of sleep during a heat pulse or elicit subsequent recovery sleep (Figure 4.7D). Thus, 24C10 and ppk neurons that contribute to sleep homeostasis appear to be cholinergic.

We then asked whether 24C10 and ppk drivers express in a functionally unique or redundant subset of cholinergic neurons that are capable of triggering recovery sleep. To address this question we combined cha>TrpA1, which normally elicits recovery sleep, with 24C10-Gal80 vs ppk-Gal80. 24C10-Gal80 had no effect, perhaps because it was too weak to fully suppress cha-Gal4 (data not shown). However, 3 or 4 copies of ppk-Gal80 significantly reduced recovery sleep in cha>TrpA1 animals (Figure 4.7E,F). These results suggest that a significant proportion of cholinergic neurons that contribute to sleep homeostasis are ppk neurons.

4.3.4 Intersecting expression patterns of two promoters identifies a restricted set of neurons that promote sleep homeostasis

The similar sleep behaviors of 24C10>TrpA1 and ppk>TrpA1 as well as the ability of cha-Gal80 to suppress these behaviors suggests that neurons that promote sleep homeostasis might be shared between the two drivers. To test this hypothesis we cloned the 24C10 and ppk enhancer fragments and coupled them to the two halves of split Gal4 (Figure 4.9A) [36]. After pairing transgenic TrpA1 with the intersectional expression of 24C10 and ppk (split>TrpA1), we found that neurons in common with both drivers were

sufficient to elicit sleep deprivation and subsequent recovery sleep (Figure 4.9B,C). We then asked whether the magnitude of the homeostatic response is sensitive to the duration with which split Gal4 neurons are activated. Indeed, recovery sleep increased up to ~3.5 hrs with up to ~2 hrs prior waking time elicited by heat pulsing of split>TrpA1 animals. The homeostatic mechanism appears to have saturated beyond this point (Figure 4.9D). Nonetheless, recovery sleep was ~50% higher and sleep latency was ~70% lower in split>TrpA1 animals following thermogenetic sleep deprivation compared to animals that had been mechanically sleep deprived even though the latter were kept awake for up to six times longer (Figure 4.9D,E; Figure 4.1B,C,E). Thus, thermogenetic activation of select neuronal circuits appears to amplify the homeostatic response relative to traditional behavioral means of transducing arousal stimuli to the brain. We suggest that either thermogenetic manipulation is more efficient at activating behaviorally relevant neural circuits or that currents methods of mechanical sleep deprivation have not been optimized for maximum behavioral responsiveness.

Next we asked whether ppk, 24C10 and our split Gal4 drivers label neurons throughout the brain, as might be predicted by diffuse and independent homeostatic sensors of sleep need, or whether these drivers label a restricted set of neurons, as might be expected for neural circuitry dedicated to sleep homeostasis. To determine the expression patterns of our drivers we coupled 24C10-Gal4, ppk-Gal4 and split-Gal4 to UAS-CD8::GFP (Gal4>GFP) and examined the nervous systems of dissected animals by

confocal microscopy. We found that the 24C10 driver expressed broadly, whereas the ppk driver expressed in more restricted regions of the brain and thoracic ganglion (Figure 4.10A,B).

The region of anatomical overlap between these drivers was narrow, and as a result split>GFP expression was quite limited. In this case processes appeared to terminate in the gnathal ganglion, whose various functions have been poorly defined, and in regions of the thoracic ganglion (Figure 4.10C). To confirm that these processes were indeed axonal and forming synapses with downstream neurons, we targeted a reporter to synaptic vesicles and visualized neuronal polarity by coupling UAS-Syt::smGFP::HA [37] to split Gal4. As expected, large puncta indicative of presynaptic terminals were observed in the gnathal and thoracic ganglia (Figure 4.10D). We thus suggest that these neurons represent inputs into motor control or modulatory circuits in the thoracic ganglion and central brain.

4.3.5 Split Gal4-labeled neurons are distinct from neural circuitry that controls baseline sleep

Next we asked what role, if any, split Gal4-labeled neurons play in regulating normal daily fluctuations in sleep. To address this question we reduced the activity of these neurons by expressing the potassium channel, Kir2.1, under the control of split Gal4. In these animals, hyperpolarization of labeled neurons led to sleep profiles and total daily levels of sleep that were indistinguishable from controls lacking Kir.2.1 (Figure 4.11A,C). Activity of split

Gal4-labeled neurons was clearly suppressed because thermogenetic stimulation of split>TrpA1 did not elicit sleep deprivation or recovery sleep in the presence of Kir2.1 (Figure 4.11A,B). Thus, although activity of split Gal4-labeled neurons is sufficient for waking, it is not required for normal daily sleep. These findings indicate that neural circuitry that engages sleep homeostasis is at least partly distinct from brain circuitry that controls normal daily fluctuations in the sleep/wake cycle. Collectively, our data also suggest that split Gal4-labeled neurons are conditional drivers of waking that have privileged roles as upstream activators of sleep homeostasis.

4.3.6 Sleep homeostasis is important for short-term memory

The commonality of sleep throughout the animal kingdom and the performance deficits that often result from sleep loss have led to the widespread view that sleep is required for optimal brain function [38]. As in mammals, sleep facilitates learning and memory and even shares underlying neural circuitry with these processes in flies [39-43]. Therefore, we asked whether suppression of sleep by functionally distinct circuits has correspondingly distinct consequences on learning and memory. To address this question we utilized an aversive-taste memory assay where flies learn to suppress their proboscis extension reflex (PER) in response to the simultaneous pairing of appetitive fructose to the tarsi and noxious quinine at their proboscis [44] (Figure 4.12A). This assay provides robust induction of short-term memory in individual flies. Importantly, the formation of aversive

taste memory is dependent upon the mushroom bodies and dopamine neurons, two neuronal populations associated with sleep [45, 46].

We first determined whether thermogenetic sleep deprivation impairs aversive taste memory. split>TrpA1, Tdc2>TrpA1 flies and associated controls were trained and tested immediately following a 4 hr heat pulse at the end of the night (Figure 4.12B). This protocol resulted in reduced short-term memory formation in both split>TrpA1 and Tdc2>TrpA1 flies compared to non-sleep deprived controls harboring Gal4 or UAS-TrpA1 alone (Figure 4.12B). This effect was not due to reduced PER since the responses of split>TrpA1 and Tdc2>TrpA1 flies to fructose were comparable to controls immediately following the heat pulse (Figure 4.12D). Thus, sleep deprivation reduced aversive taste memory.

To examine the effect of sleep homeostasis on memory, we heat pulsed animals over the same 4 hr period, but this time we allowed them to recover for 3 hrs at room temperature prior to training and testing. Under these conditions we found that memory was comparably high in split>TrpA1 flies and heat-pulsed controls but not in Tdc2>TrpA1 animals (Figure 4.12C), suggesting that the recovery period allows for the formation of short-term memories previously impaired by sleep deprivation. Importantly, under both training protocols, neuronal activity was not manipulated during the memory assay. Therefore loss of memory was due to prior activation of arousal-promoting neurons resulting in sleep loss, rather than an acute effect of those neurons on memory formation. Since significant recovery sleep occurred in

split>TrpA1 but not in Tdc2>TrpA1 flies over this period (Figure 4.2D,F; Figure 4.9B,C), our results strongly suggest that neural circuits that control sleep homeostasis are important for memory.

4.4 Discussion

One of the most surprising findings from these studies is that homeostatic recovery sleep can be suppressed by activation of select wake-promoting neural circuits. This observation seems counterintuitive considering the evolutionary conservation of sleep, which has historically been interpreted to mean that sleep confers important survival advantages that make it indispensable [47]. However, temporary suppression of sleep need could actually be advantageous by enhancing survival in dire conditions in which sleepiness could be acutely fatal, such as starvation or predation. Such a short-term advantage could in theory outweigh any long-term disadvantage associated with sleep loss. A related rationale has been put forward to explain why newborn cetaceans and their mothers temporarily suppress sleep[48], as well as why cetaceans, pinnipeds and certain birds sometimes exhibit only unihemispheric sleep [49-51].

Still, it is surprising that prolonged waking without triggering a homeostatic response was so frequently observed following activation of different arousal-promoting neurons in the brain. This observation is difficult to reconcile with the tenet that waking as a behavioral state drives subsequent sleep need [26]. This tenet is based on a relationship that has been observed

for many years. However, this relationship is correlative rather than causative in that waking is associated with activation of many arousal systems simultaneously. As such, the contribution of waking to sleep need historically has not been distinguished from the potentially varied contributions of the underlying arousal systems that cause waking. In contrast, by activating individual neurotransmitter systems that are each capable of inducing waking, we have shown that cholinergic neurons seem to contribute disproportionately to homeostatic compensation for increased sleep need. Hints of differences in arousal have also been noted in rodents that were sleep-deprived for similar durations but by different methods. For example, sleep deprivation by gentle handling is more effective than sleep deprivation by continual cage change at reducing subsequent sleep latency in mice [52]. Thus, the nature of the neural circuitry involved in prior waking may determine subsequent arousal states in both flies and mammals.

Our work may also help address whether sleep need is globally or locally sensed. Our finding that a subset of wake-promoting neurons has a privileged role in stimulating homeostatic recovery sleep suggests that specific neural circuit(s) with cholinergic inputs may serve as a surrogate sensor of global sleep need that provides significant advantages for memory retention. Although sleep homeostasis could still arise from waking *per se* in the absence of activated circuits that inhibit sleep need, the failure of dopaminergic wake-promoting neurons to elicit or inhibit full-blown sleep homeostasis suggests that this process requires waking to be driven by

particular neural circuits. In future experiments it will be interesting to determine under what conditions, and when during the sleep/wake cycle, different types of arousal circuits are activated. It will also be interesting to determine if the neurons we identified as drivers of sleep homeostasis are functionally unique. Certainly we suspect that they are uncommon since our unbiased anatomical screen revealed very few candidates that contribute to this process, and exclusion of TrpA1 from the relatively sparse ppk neurons blocked recovery sleep following thermogenetic activation of cholinergic neurons.

Taking those caveats into account, the most parsimonious interpretation of our results is that neural circuits that promote sleep homeostasis are a subset of neurons that control baseline sleep, as reflected in the following model. We suggest that at least two classes of functionally distinct wake-promoting (W) neurons exist: those that trigger subsequent sleep homeostasis, and those that inhibit it. From the presence of the former we can infer the presence of sleep-promoting (S) neurons as well (Figure 4.13A). We propose that privileged W neurons inhibit S neurons and thus suppress sleep either directly (not shown) or indirectly through induction of waking. Similarly, we propose that S neurons inhibit waking (Figure 4.13A). Thus, like the flip-flop model of sleep/wake stability in mammals [14], mutual inhibition by W and S neurons in flies would stabilize waking and sleep states, respectively. We further hypothesize that sustained inhibition of S neurons causes compensatory upregulation of S neuron activity (Figure 4.13B). For the

duration of sleep deprivation under our conditions, this upregulation is insufficient for S neurons to overcome the wake-promoting influence of W neurons. However, when stimulation of W neurons is withdrawn at the end of a period of sustained sleep deprivation, upregulated S neurons now have an exaggerated influence and are able to induce recovery sleep when animals would normally be awake (Figure 4.13C). Our model thus attributes behavioral homeostasis to underlying synaptic homeostasis. We note that this model does not differ substantially from some established models of pharmacodynamic tolerance, in which synaptic pathways compensate for perturbations in activity caused by a drug [53]. In our model waking functions like the drug.

Our model does not depict additional classes of W neurons that have no effect on sleep homeostasis. We also cannot rule out the possible existence of sub-classes of W neurons, such as those that might respond to different environmental conditions or behavioral states. However, our simple model is sufficient to explain the function of split Gal4-labeled neurons, which appear to represent unusual inputs into a homeostatic circuit regulating sleep need. Interestingly, a recent study has established that activity of sleep-promoting dorsal fan-shaped bodies is upregulated following sleep deprivation [54]. It is possible that these structures represent the S neurons in our model. Thus, our model provides a context with which to interpret previous and future perturbations to sleep homeostasis. Our thermogenetic approach to studying

sleep homeostasis also provides a means to uncover previously unknown mechanisms that govern sleep need.

4.5 Methods

4.5.1 Fly stocks and transgenic fly lines

Drosophila lines used in this study were provided as follows: *TH*-Gal4 and UAS-*Kir2.1* (Mark Wu); *ppk*-Gal4 and *ppk*-Gal80 (Yuh Nung Jan); *cha*-Gal80 (Toshihiro Kitamoto); pJFRC51-*3XUAS-IVS-Syt::smGFP::HA* in su(Hw)attP1 (Gerry Rubin); and UAS-*TrpA1* (Paul Garrity). Other lines used in this study were obtained from the Bloomington Stock Center (*cha*-Gal4 [6793], *Tdc2*-Gal4 [9313], *Trh*-Gal4 [38388], OK371-Gal4 [26160], 24C10-Gal4 [49075], 11H07-Gal4 [45016], 44F01-Gal4 [45313], 60D04-Gal4 [45356], and 86C06-Gal4 [45379]).

Additional transgenic fly lines were generated by targeted insertion using PhiC31 integration after injection (Rainbow Transgenics), including: *ppk*-DBD into VK00027, 24C10-AD into attP2, and 24C10-Gal80 into attP2. At least 4 independent insertions were obtained per transgene and were outcrossed into the w^{1118} iso31 isogenic background at least two generations.

4.5.2 Behavioral assays

Sleep measurements were performed as previously described[31]. Briefly, one- to five-day old female flies were loaded into glass tubes containing 5% sucrose and 2% agarose. Animals were entrained for 2 days on

a 12 hr:12 hr light:dark cycle before sleep/wake patterns were measured using the *Drosophila* Activity Monitoring System (DAMS; Trikinetics). Thermogenetic sleep deprivation was performed using a baseline temperature of 22C for a minimum of one day of baseline sleep followed by up to a 12 hr nighttime heat pulse at either 27C or 29C. Following the heat pulse, the incubator temperature was reduced back to the baseline 22C for a minimum of two days for recovery sleep measurements. Mechanical sleep deprivation was achieved by loading flies in DAM2 activity monitors (Trikinetics) into a VX-2500 multi-tube vortexer (VWR) fitted with a custom base. Flies were vortexed at the lowest intensity setting for 2sec/min. Sleep deprivation was calculated as the total amount of sleep during the deprivation period minus sleep during the same period of the previous day. Likewise, recovery sleep was calculated over the 6 hrs immediately after deprivation (ZT0-6) minus the same period during the previous day.

Arousal threshold measurements were performed using a custom built apparatus that oscillates on a one-dimensional axis controlled by an Arduino Uno (Arduino). Briefly, immediately after the end of sleep deprivation, activity monitors were set to perpetually oscillate at a low frequency (0.1 Hz). Beginning at ZT2, the platform was then rotated twice every 30 min at increasing intensities up to 4 Hz. The percentage of flies aroused at each intensity was calculated as the percent of flies moving immediately after the stimulus that were immobile for at least 5 min prior to the stimulus. The arousal threshold for 50% (AT50) of the population was computed by fitting the

percent awakened at each intensity to a non-linear four-parameter dose-response curve where the best-fit EC50 value was used as the AT50. Curves were plotted as the best-fit curve ± 95% confidence interval.

Aversive taste memory assays were performed as previously described [44-46] with the following modifications. One-week-old mated females were entrained on a 12 hr:12 hr light:dark cycle at 22° C prior to all experiments. All animals were then heat pulsed to 29° C from ZT20-24 and returned to 22° C. Animals were then removed from the incubator at ZT24 or 3 hrs later at ZT3 and assayed for taste memory. For this assay, flies were satiated with water before and during the experiment. Flies that did not initially satiate within 5 min were excluded from conditioning. We used a 1 ml syringe (Tuberculine, Becton Dickinson & Comp) for tastant presentation. We used purified water, 100 mM fructose, 100 mM and 1000 mM sucrose, or 10 mM quinine solutions (all Sigma). Each fly was given 100 mM fructose on its tarsi three times with 10 sec inter-trial intervals and the number of full proboscis extensions was recorded. Only flies that responded every time were retained for further analysis. For taste suppression experiments, flies were given 100 mM fructose on tarsi followed by a droplet of 10 mM quinine placed on the extended proboscis. Flies were allowed to drink the latter for up to 2 sec or until they retracted their proboscis. At the end of each experiment, flies were given 100 mM and 1000 mM sucrose to check retained ability to undergo PER, and non-responders were excluded.

4.5.3 Molecular biology

For *ppk*-DBD, the *ppk* promoter sequence was subcloned from pCasper-*ppk*-hs43-*EGFP*[55] into the entry vector pENTR1A (Life Technologies) using the EcoRI and XhoI sites of each plasmid. The *ppk* promoter sequence was then inserted into pBPZpGAL4DBDUw[56] using Gateway recombination (Life Technologies). The 24C10-AD plasmid was generated by obtaining the 24C10 enhancer fragment sequence via plasmid rescue from the P(GMR24C10-GAL4)attP2 fly line. The enhancer fragment was PCR amplified using 24C10-Kpn-F (5'-AAGGTACCGCTCTGGTGTTCTGTTGGGCTGATA-3') and 24C10-XhoR1 (5'-AACTCGAGGTAAGTTTGGGGCATCCCATCGAGA-3') primers and cloned into pCR2.1 TOPO (Life Technologies). The 24C10 sequence was then subcloned into a modified mENTRY vector (Life Technologies) using the KpnI and EcoRI sites. The 24C10 sequence was then recombined into the pBPp65ADZpUw and pBPGAL80Uw-4 plasmids via Gateway recombination.

4.5.4 Immunohistochemistry

Immunostaining of whole-mount brains and thoracic ganglia was performed as previously described[31]. Briefly, 3- to 5-day old female brains were dissected in cold PBS and fixed in 4% PFA for 20 min. After brief washes in PBST, brains were blocked in 5% normal donkey serum (Jackson Laboratory) in PBST. Brains were incubated with 1:1,000 rabbit anti-GFP (Life Technologies), or 1:1,000 rabbit anti-HA (Rockland) and 1:50 mouse anti-nc82

(Developmental Studies Hybridoma Bank). After brief washes in PBST, brains were incubated in 1:1,000 Alexa 488 anti-rabbit and 1:1,000 Alexa 568 anti-mouse (Life Technologies) prior to a final set of washes. Brains were mounted in Vectashield (Vector Labs) before imaging at 40x magnification on a Leica SP5 confocal microscope at 0.5 to 0.75 μm intervals and reassembled for maximum projection using Fiji.

4.5.5 Statistics

Bar graphs are presented as mean ± SEM unless otherwise stated. Comparisons between two groups were performed with unpaired Student's t-test. For comparisons between multiple groups, data were analyzed using one-way ANOVA followed by Tukey-Kramer test for multiple comparisons. P values for AT50s were computed by pairwise comparison using the extra sum-of-squares F test of the best fits for each group. All statistical tests were two-sided and performed using Prism 6.0f for Mac OS X (GraphPad Software).

4.6 Acknowledgements

Work presented in Chapter 4 with minor modifications is under review for publication in *Current Biology*. Coauthors Kurtresha Worden, Pavel Masek, and Alex Keene performed all learning and memory assays and analysis. Meilin Wu performed all of the molecular biology presented in this work. The dissertation author was the co-primary author of this work with Glen Seidner and corresponding author William Joiner. The dissertation author performed all

imaging experiments along with some of the sleep/deprivation assays, design, analysis and interpretation of the data.

4.7 Figures

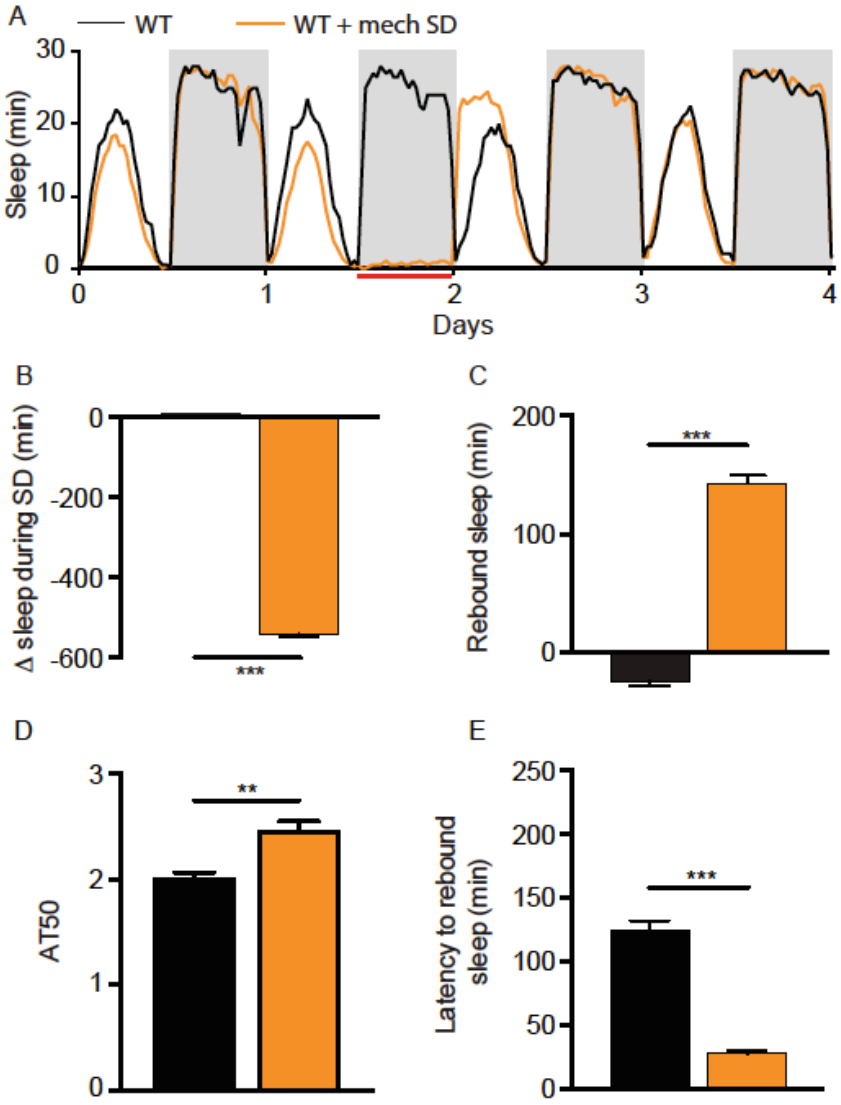

Figure 4.1 Key features of homeostatic recovery sleep following mechanical sleep deprivation. (**A**) Mechanical perturbation for 12 hrs at night (red bar) effectively sleep deprived wild-type flies and elicited subsequent rebound sleep the next morning. WT, wild-type controls (black); WT+mech SD, wild-type sleep deprived (orange). (**B**, **C**) Sleep lost during nighttime shaking (**B**) and recovered the next morning (**C**). (**D**) Arousal threshold at which 50% of animals could be awoken (AT50) was elevated during the rebound period (see also Figure 4.3). (**E**) Latency to sleep was reduced following mechanical sleep deprivation. N ≥ 122 for each condition. * p< .05, ** p< .01, *** p < 0.001 by one-way ANOVA with Bonferroni post-test. Error bars indicate s.e.m.

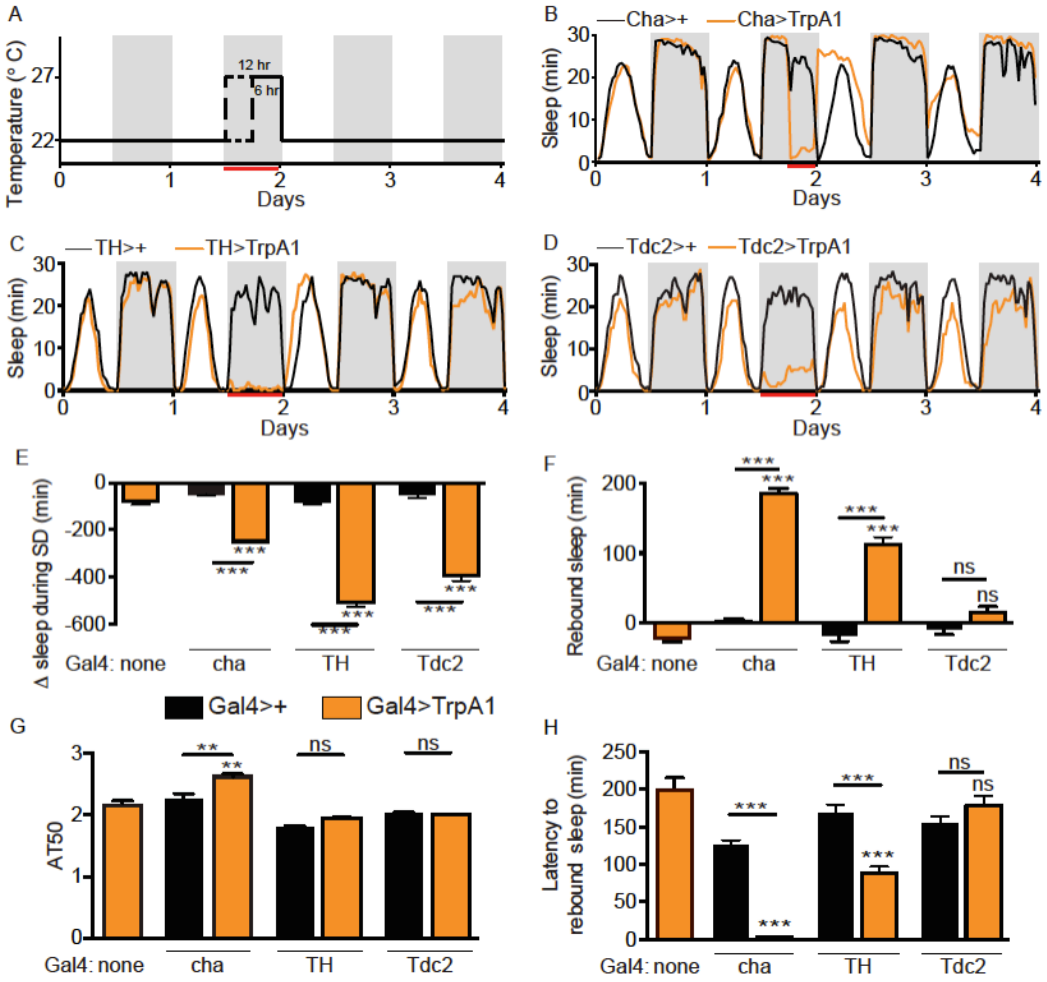

Figure 4.2 Cholinergic neurons selectively elicit homeostatic recovery sleep after thermogenetic activation. (**A**) Schematic for thermogenetic arousal: Animals were maintained at 22° C for 1 day, on the second day they were pulsed for the final 6 hrs (cha) or 12 hrs (TH and Tdc2) of night at 27° C, and were returned to 22° C for 2 days. (**B**) Activation of cholinergic neurons (cha>TrpA1) stimulated waking at night and rebound sleep the next morning. (**C, D**) Activation of dopaminergic (TH>TrpA1, C) or octopaminergic neurons (Tdc2>TrpA1, D) stimulated waking at night with reduced or no rebound sleep the next morning. (**E**) Sleep lost in Gal4>+ (black) vs Gal4>TrpA1 combinations (orange) during the heat pulse. (**F**) Rebound sleep in Gal4>+ vs Gal4>TrpA1 combinations after the heat pulse. (**G**) Arousal threshold was elevated following sleep deprivation of cha>TrpA1 but not TH>TrpA1 or Tdc2>TrpA1 animals. (**H**) Sleep latency was most severely reduced following sleep deprivation of cha>TrpA1 animals. N≥32 for each condition. Asterisks directly above or below bars in all figures reflect comparisons with TrpA1>+ control. In all figures, * p< .05, ** p< .01, *** p < 0.001 by one-way ANOVA with Bonferroni post-test unless otherwise stated. Error bars indicate s.e.m.

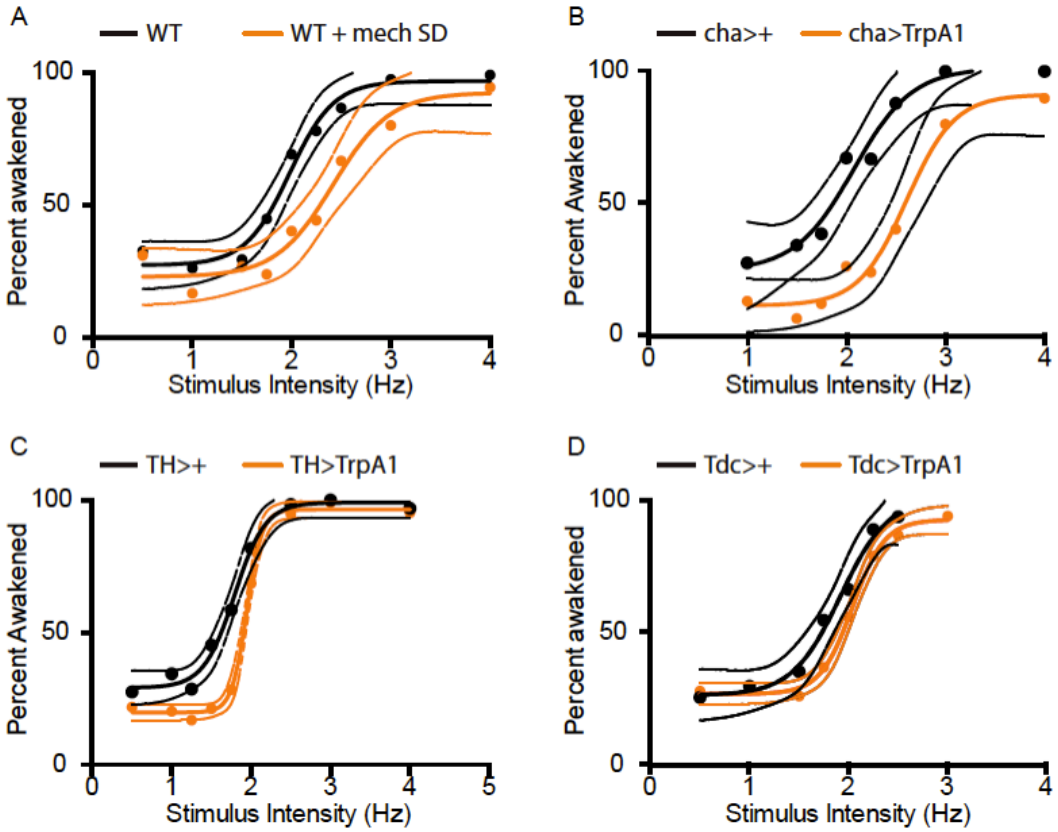

Figure 4.3 Sleep deprivation via mechanical deprivation or thermogenetic activation of cholinergic neurons increases arousal threshold and decreases sleep latency. (**A**) Mechanical sleep deprivation shifts arousal-response curve to the right. (**B**) A 6 hr heat pulse at night shifts arousal response curve to the right in cha>TrpA1 animals (orange) relative to cha>+ controls (black). (**C**, **D**) A 12 hr heat pulse sufficient to sleep deprive animals at night has no effect on arousal-response relationship of TH>TrpA1 (C) or Tdc2>TrpA1 animals (D) relative to controls. N≥28 per data point. Solid lines are fits to individual points; dashed lines are 95% confidence intervals.

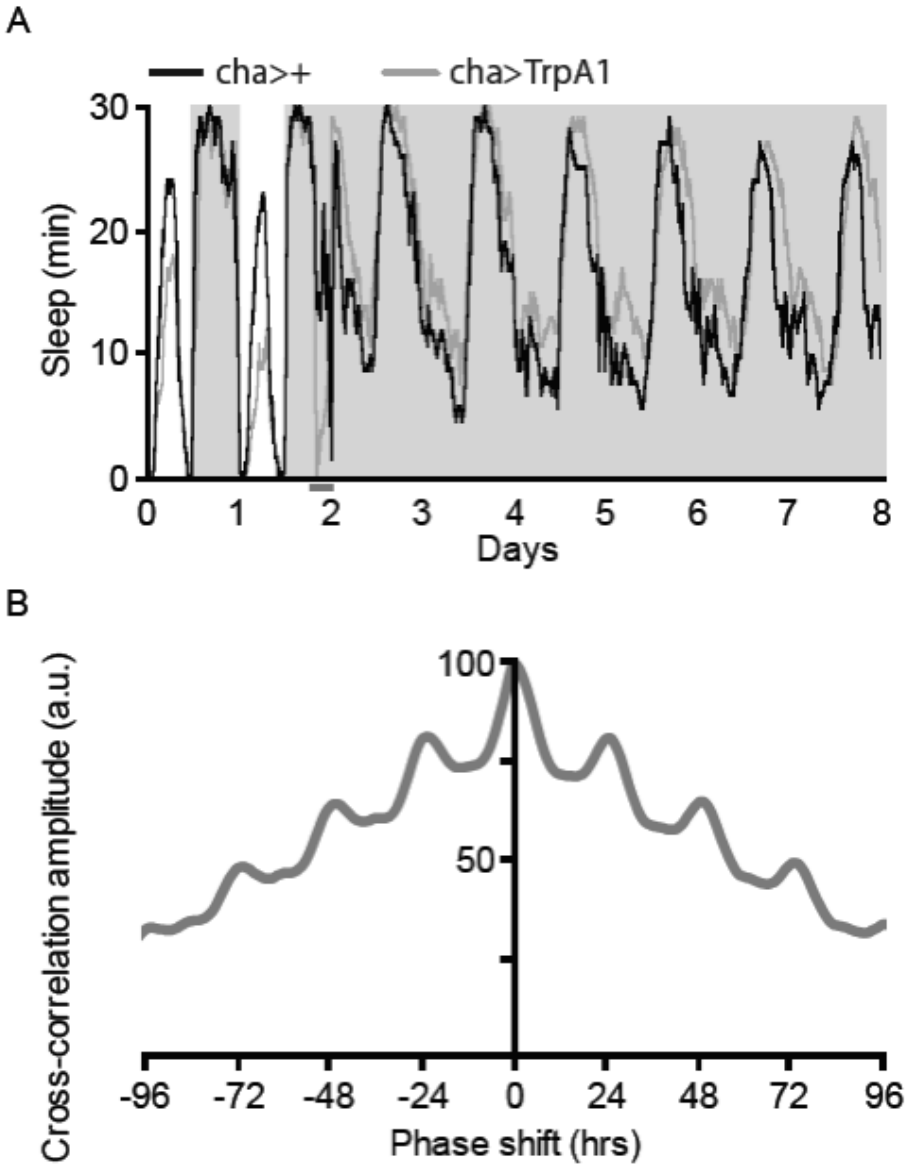

Figure 4.4 Thermogenetically-induced sleep homeostasis is not caused by a phase shift of the circadian clock. (**A**) cha>TrpA1 and cha>+ controls were heat pulsed for 4 hrs at night and then maintained in DD for 6 days. Sleep profiles of both groups show no obvious differences in timing. (**B**) Quantification of phase relationship between sleep cycles of cha>TrpA1 and split>+ animals after the heat pulse. Maximal amplitude of cross-correlation between both groups at 0 hrs indicates no significant phase shift following TrpA1 activation. N>32 per for each group.

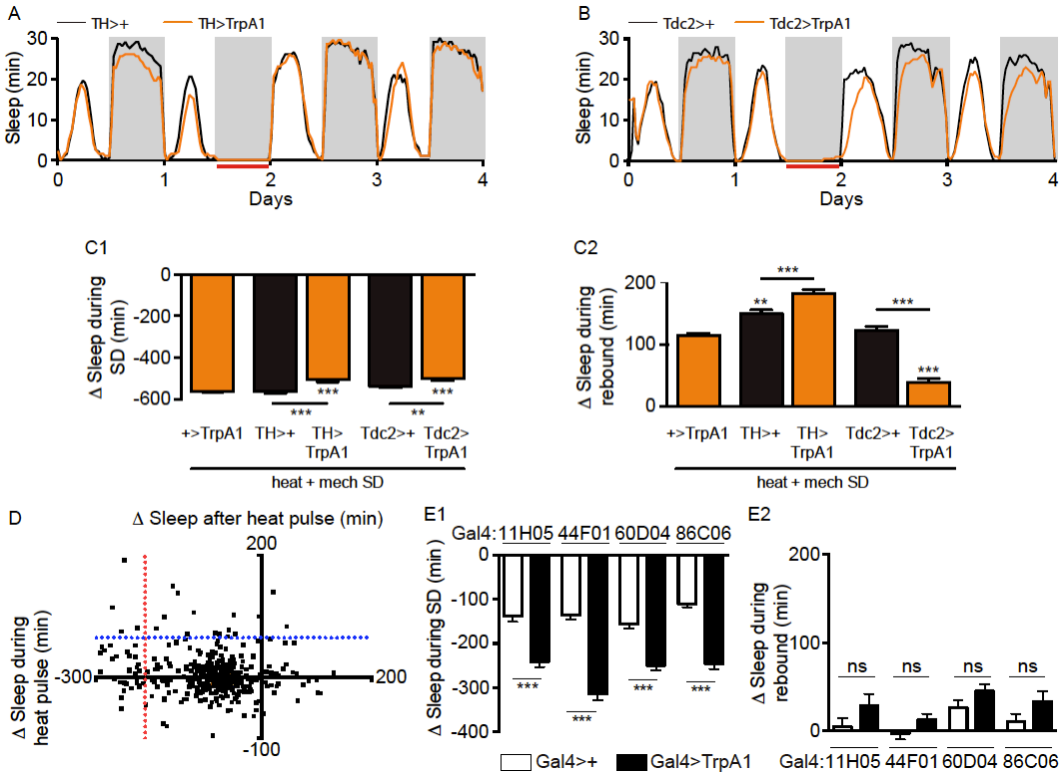

Figure 4.5 Homeostatic recovery sleep can be suppressed by prior activation of select arousal-promoting neurons. (**A,B**) Rebound sleep was normal in TH>TrpA1 (A) but suppressed in Tdc2>TrpA1 animals (B) relative to controls despite ~equivalent sleep deprivation by simultaneous mechanical perturbation and heat pulse for 12 hrs at night. (**C**) Quantification of sleep deprivation (C1) and subsequent rebound sleep (C2) resulting from 12 hr heat pulse alone or in combination with mechanical perturbation in Gal4>+ and Gal4>TrpA1 animals. N≥186 per group. (**D**) Screen for Gal4>TrpA1 combinations that cause sleep loss and subsequent homeostatic recovery sleep following thermogenetic stimulation for 6 hrs. Black dots are data points for individual Gal4>TrpA1 lines. Red vertical line represents two standard deviations less than mean sleep loss in Gal4>+ controls. Blue horizontal line represents two standard deviations greater than mean sleep rebound in Gal4>+ controls. N≥7 per data point. (**E**) Retest of 4 lines from the middle left quandrant in c shows that thermogenetic activation of Gal4>TrpA1 combinations caused sleep deprivation (E1) without subsequent recovery sleep (E2). N≥15 per group.

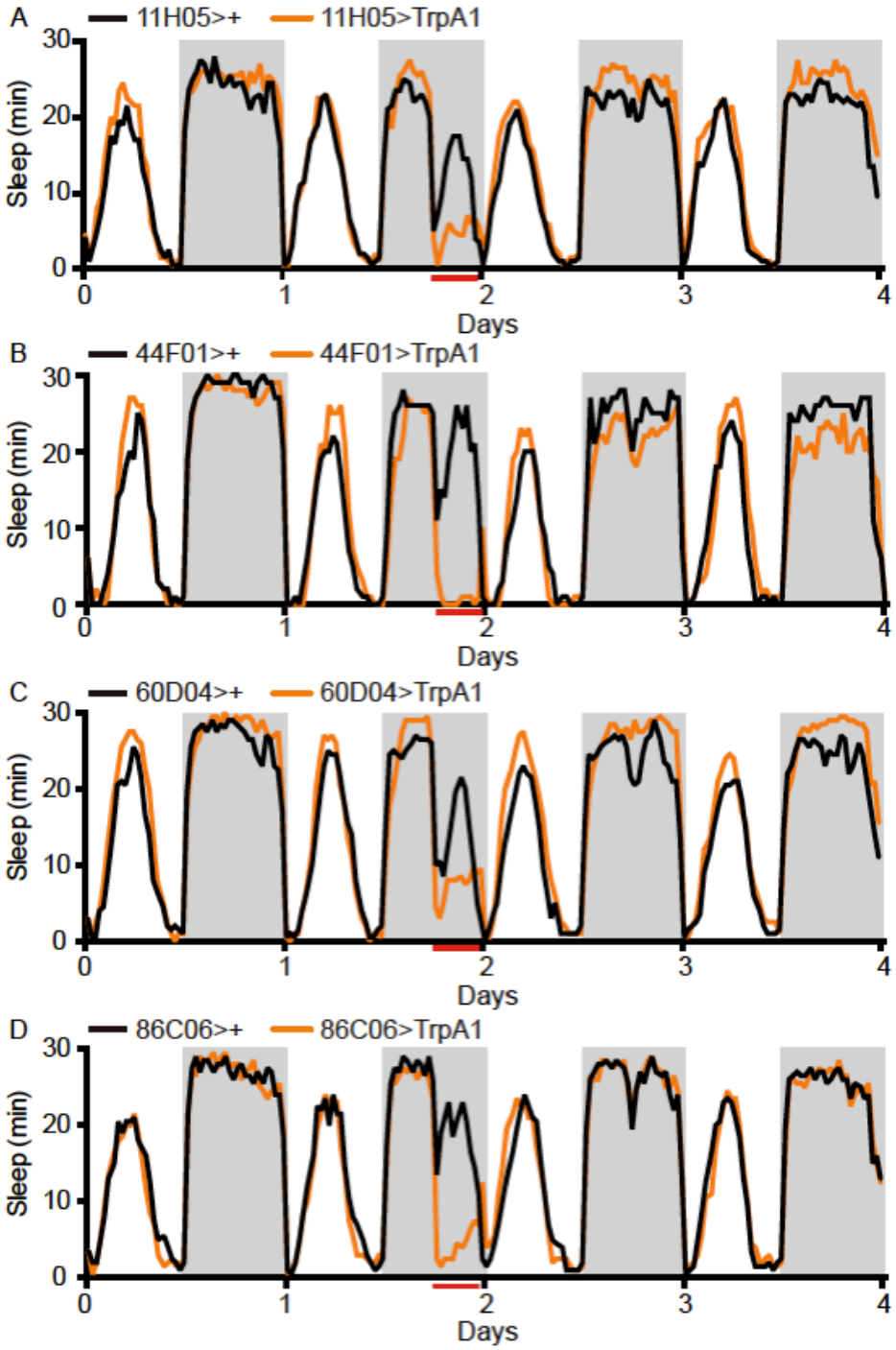

Figure 4.6 Sleep profiles of SDNRs (sleep deprivation no recovery sleep). (**A**-**D**) In each example, Gal4>TrpA1 animals and Gal4>+ controls were pulsed to 29 C for 6 hrs at night. No significant recovery sleep was observed the next day in any group. N≥15 for each group.

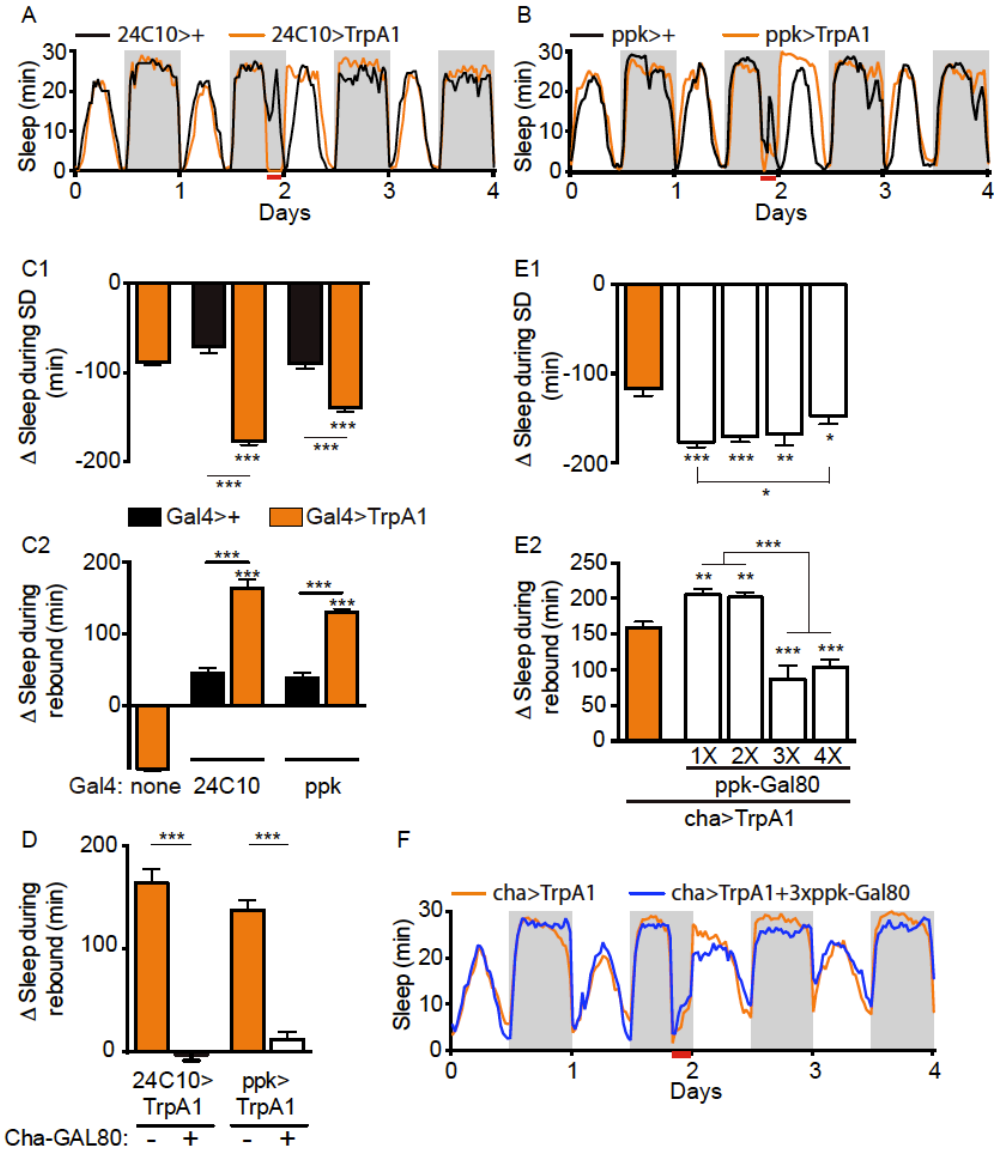

Figure 4.7 Homeostatic recovery sleep can be elicited by prior activation of select arousal-promoting neurons. (**A, B**) Sleep deprivation and subsequent homeostatic recovery sleep profiles of 24C10>TrpA1 and ppk>TrpA1 animals (orange) relative to Gal4>+ controls (black). Red bar, 4 hr heat pulse to 29º C at the end of the night. (**C**) Quantification of sleep deprivation (C1) and subsequent rebound sleep (C2) in A and B. N≥31 per group. (**D**) Repression of Gal4 activity in cholinergic neurons with cha-Gal80 blocked rebound sleep in 24C10>TrpA1 and ppk>TrpA1 animals. N≥42 per group. (**E**) Repression of Gal4 activity in ppk neurons with ppk-Gal80 reduced sleep deprivation (E1) and rebound (E2) in cha>TrpA1 animals. N≥29 per group. (**F**) Example sleep profile from an experiment in E. N≥74 for each condition.

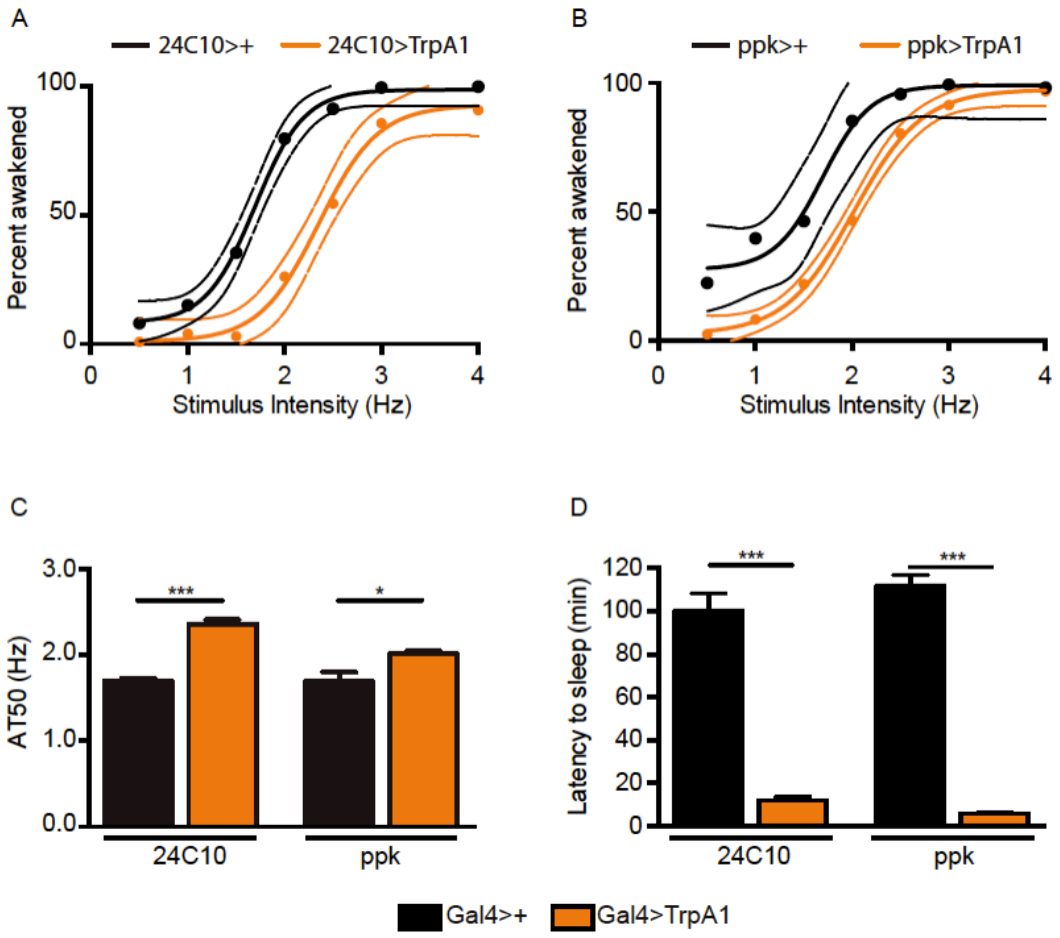

Figure 4.8 Gal4>TrpA1 combinations that induce rebound sleep exhibit elevated arousal threshold and reduced sleep latency following thermogenetic sleep deprivation for 4 hrs at 29 C. (**A**, **B**) Arousal response curves are right-shifted in 24C10>TrpA1 (**A**) and ppk>TrpA1 combinations (**B**) relative to Gal4>+ controls. (**C**) Quantification of AT50s from curves in A and B. N≥120 per group. (**D**) Latency to sleep is reduced in 24C10>TrpA1 and ppk>TrpA1 combinations relative to corresponding Gal4>+ controls following the heat pulse. N≥31 per group.

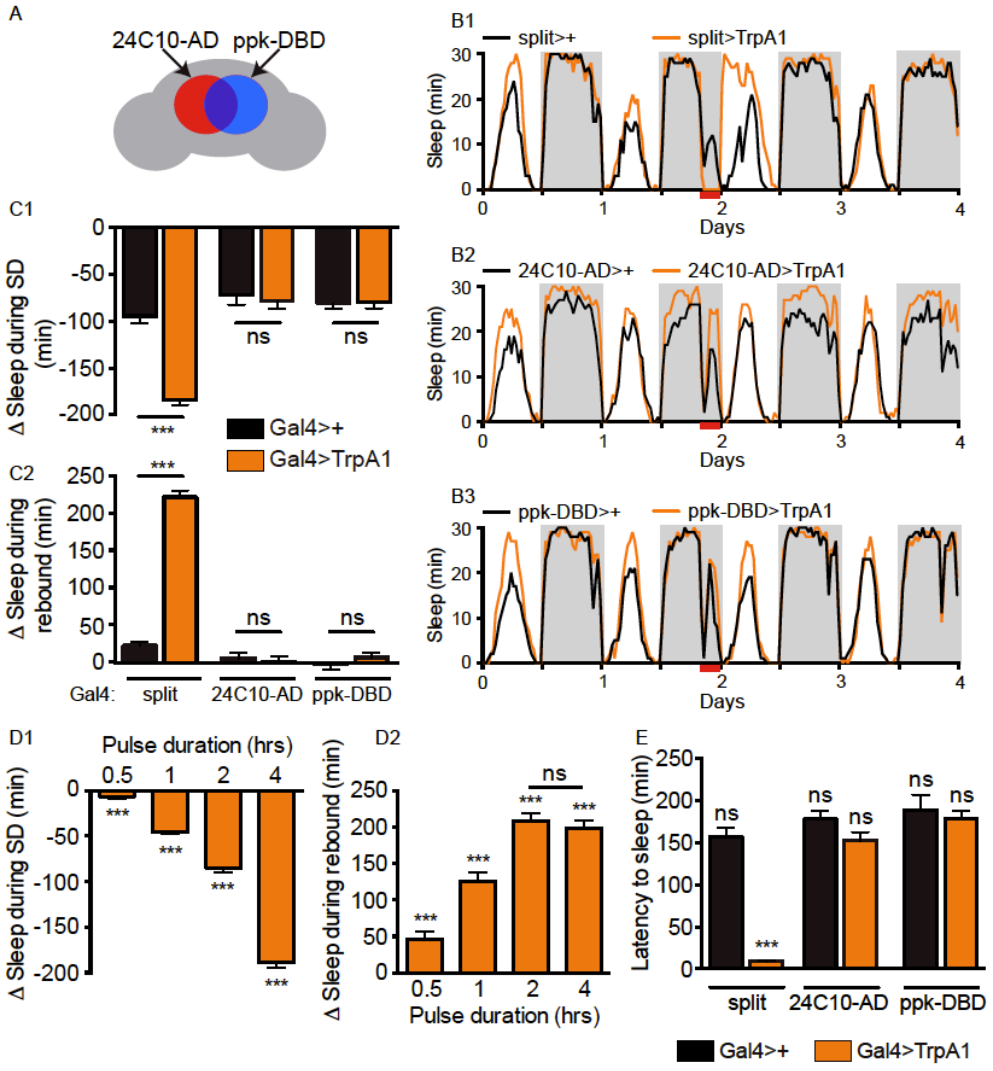

Figure 4.9 Thermogenetic activation of neurons at the intersection of 24C10 and ppk is sufficient to trigger sleep homeostasis. (**A**) Diagram of split Gal4 strategy: TrpA1 is produced in neurons (purple) in which functional Gal4 (i.e. AD + DBD) is reconstituted from intersecting expression of 24C10-AD (red) and ppk-DBD (blue). (**B**) Heat pulse to 29º C for 4 hrs at night was sufficient to induce sleep deprivation and subsequent homeostatic recovery sleep in split>TrpA1 animals (orange) but not in split>+ controls (black) (B1) or in 24C10-AD>TrpA1 (B2) or ppk-DBD>TrpA1 controls (B3). (**C**) Quantification of sleep deprivation (C1) and rebound (C2) in b. N≥31 per group. (**D**) Sleep deprivation increased in proportion to duration of 29º C heat pulse in split>TrpA1 animals (D1), whereas rebound sleep saturated after 2 hrs of prior pulsing (D2). N≥30 per condition. (**E**) Sleep latency after the heat pulse was severely reduced in split>TrpA1 animals but not in controls. N≥31 for each condition.

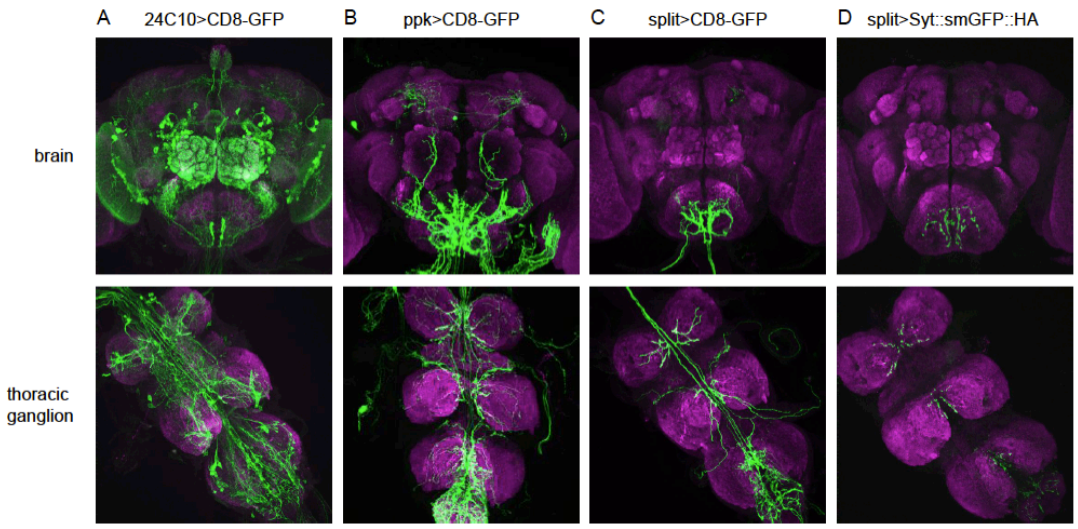

Figure 4.10 Expression patterns of Gal4 drivers capable of eliciting sleep homeostasis when coupled to TrpA1 activation. (**A-C**) Expression patterns of 24C10>CD8-GFP (A), ppk>CD8-GFP (B) and split>CD8-GFP (C). Left and right panels of each pair are brain and thoracic/abdominal ganglia, respectively. Anti-GFP staining is shown in green; neuropil stained with anti-nc82 is shown in magenta. (**D**) Expression pattern of split>Syt::smGFP::HA demonstrating synaptic terminals of split Gal4 neurons in the central brain (left panel) and thoracic/abdominal ganglia (right panel). Anti-HA staining is shown in green; neuropil stained with anti-nc82 is shown in magenta.

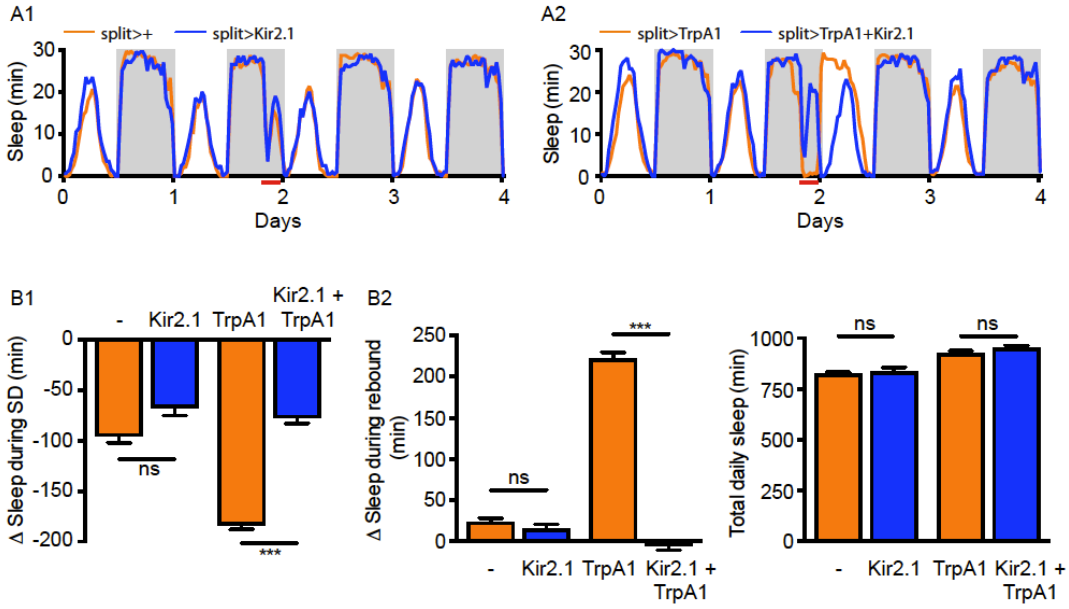

Figure 4.11 Electrically silencing split Gal4-expressing neurons blocks thermogenetic sleep deprivation and subsequent homeostatic recovery sleep without altering baseline sleep. (**A**) The sleep profiles of split>Kir2.1 animals are indistinguishable from split>+ controls (A1). Sleep profiles of split>TrpA1 animals alone or in combination with Kir2.1 show that hyperpolarization of split Gal4 neurons blocks thermogenetic sleep deprivation and sleep rebound (A2). (**B**, **C**) Quantification of effects in A on sleep deprivation (B1), rebound sleep (B2) and baseline sleep (C). N≥29 per group.

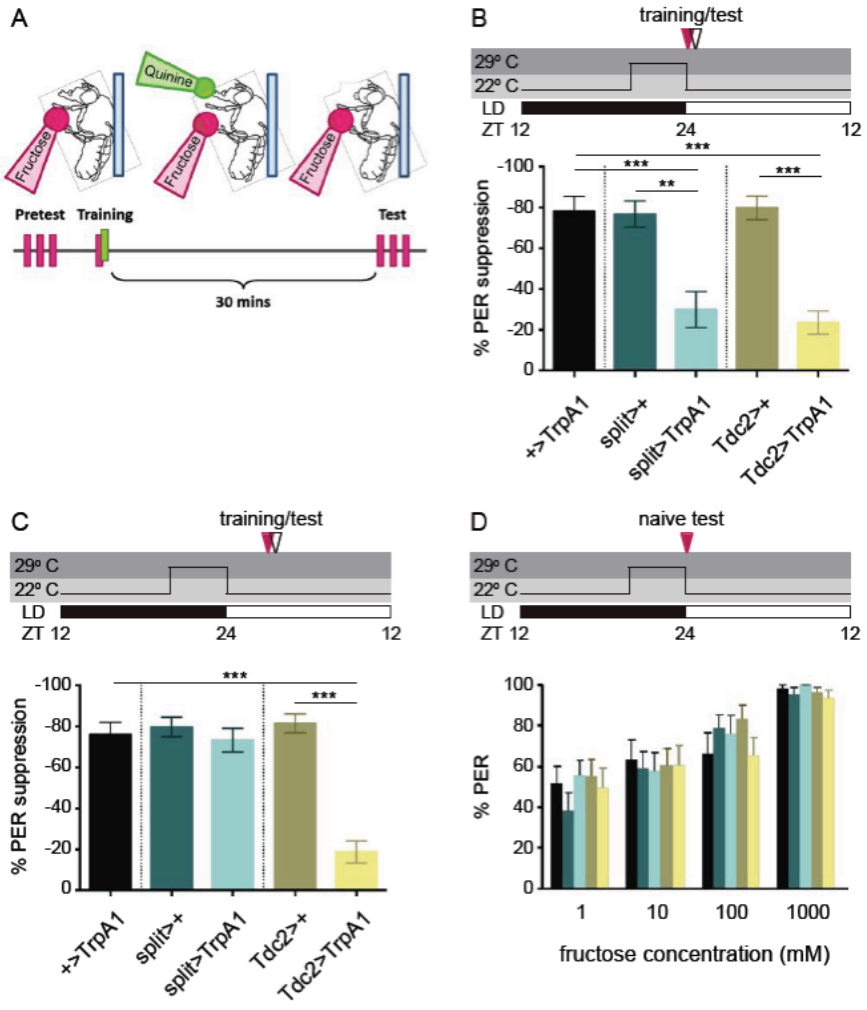

Figure 4.12 Sleep homeostasis protects against memory loss following sleep deprivation. (**A**) Aversive taste memory was induced by a single pairing of fructose (pink) and quinine (green). Flies were tested 30 minutes following training. (**B)** Animals were exposed to 29°C from ZT20-ZT24, returned to 22°C, and then immediately trained and tested as in A. Memory was reduced in split>TrpA1 and Tdc2>TrpA1 flies compared to controls harboring a single copy of either Gal4 or UAS-TrpA1 alone. N≥19 for each group. **(C)** Animals were exposed to 29°C from ZT20-ZT24, returned to 22°C for a 3 hr recovery period, then trained and tested for memory at ZT3. Memory in split>TrpA1 flies did not differ from controls. Memory was significantly reduced in Tdc2>TrpA1 flies compared to controls and to split>TrpA1 flies. N≥28 for each group. **(D)** Flies were tested for naïve PER following the temperature shift protocol used to induce sleep deprivation. No differences in PER response to fructose were detected at 1 mM, 10 mM, 100 mM or 1000 mM concentrations among any of the genotypes tested. N≥18 for each group. ** p< .01, *** p < 0.001 by non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's multiple comparison post-test. Error bars indicate s.e.m.

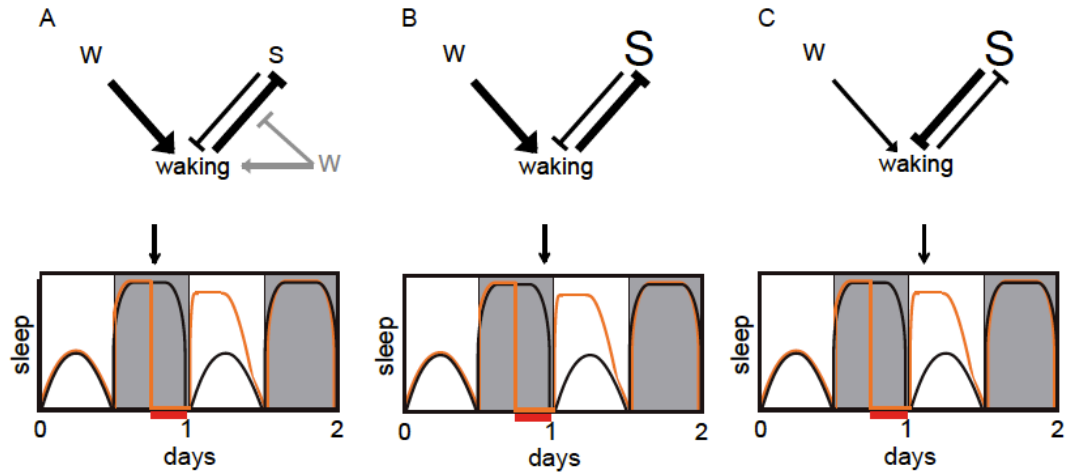

Figure 4.13 Simple model for hypothetical neural circuitry underlying sleep homeostasis in flies. (**A**) At least two classes of functionally distinct wake-promoting (W) neurons are proposed to exist: those that trigger subsequent sleep homeostasis (”privileged”, black) and those that inhibit it (gray). Privileged W neurons inhibit S neurons and thus suppress sleep either directly (not shown) or indirectly through induction of waking. In contrast, S neurons promote sleep by inhibiting waking. (**B**) Sustained inhibition of S neurons by privileged W neurons causes compensatory upregulation of S neuron activity. During sleep deprivation this upregulation is insufficient for S neurons to overcome the wake-promoting influence of W neurons. (**C**) But when stimulation of W neurons is withdrawn after sustained sleep deprivation, upregulated S neurons now have an enhanced ability to induce recovery sleep.

4.8 References

1. Gujar, N., Yoo, S.S., Hu, P., and Walker, M.P. (2011). Sleep deprivation amplifies reactivity of brain reward networks, biasing the appraisal of positive emotional experiences. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 4466-4474.

2. Yoo, S.S., Gujar, N., Hu, P., Jolesz, F.A., and Walker, M.P. (2007). The human emotional brain without sleep--a prefrontal amygdala disconnect. Current biology : CB *17*, R877-878.

3. Xie, L., Kang, H., Xu, Q., Chen, M.J., Liao, Y., Thiyagarajan, M., O'Donnell, J., Christensen, D.J., Nicholson, C., Iliff, J.J., Takano, T., Deane, R., and Nedergaard, M. (2013). Sleep drives metabolite clearance from the adult brain. Science *342*, 373-377.

4. Ju, Y.E., Lucey, B.P., and Holtzman, D.M. (2014). Sleep and Alzheimer disease pathology--a bidirectional relationship. Nat Rev Neurol *10*, 115-119.

5. Johnson, E.O., Roth, T., and Breslau, N. (2006). The association of insomnia with anxiety disorders and depression: exploration of the direction of risk. J Psychiatr Res *40*, 700-708.

6. Gangwisch, J.E., Heymsfield, S.B., Boden-Albala, B., Buijs, R.M., Kreier, F., Pickering, T.G., Rundle, A.G., Zammit, G.K., and Malaspina, D. (2007). Sleep duration as a risk factor for diabetes incidence in a large U.S. sample. Sleep *30*, 1667-1673.

7. Gottlieb, D.J., Punjabi, N.M., Newman, A.B., Resnick, H.E., Redline, S., Baldwin, C.M., and Nieto, F.J. (2005). Association of sleep time with diabetes mellitus and impaired glucose tolerance. Arch Intern Med *165*, 863-867.

8. Gangwisch, J.E., Heymsfield, S.B., Boden-Albala, B., Buijs, R.M., Kreier, F., Pickering, T.G., Rundle, A.G., Zammit, G.K., and Malaspina, D. (2006). Short sleep duration as a risk factor for hypertension: analyses of the first National Health and Nutrition Examination Survey. Hypertension *47*, 833-839.

9. Marin, J.M., Carrizo, S.J., Vicente, E., and Agusti, A.G. (2005). Long-term cardiovascular outcomes in men with obstructive sleep apnoea-hypopnoea with or without treatment with continuous positive airway pressure: an observational study. Lancet *365*, 1046-1053.

10. Ayas, N.T., White, D.P., Manson, J.E., Stampfer, M.J., Speizer, F.E., Malhotra, A., and Hu, F.B. (2003). A prospective study of sleep duration and coronary heart disease in women. Arch Intern Med *163*, 205-209.

11. Rechtschaffen, A., Gilliland, M.A., Bergmann, B.M., and Winter, J.B. (1983). Physiological correlates of prolonged sleep deprivation in rats. Science *221*, 182-184.

12. Rechtschaffen, A., and Bergmann, B.M. (2002). Sleep deprivation in the rat: an update of the 1989 paper. Sleep *25*, 18-24.

13. Ingiosi, A.M., Opp, M.R., and Krueger, J.M. (2013). Sleep and immune function: glial contributions and consequences of aging. Current Opinion in Neurobiology *23*, 806-811.

14. Saper, C., Scammell, T.E., and Lu, J. (2005). Hypothalamic regulation of sleep and circadian rhythms. Nature *437*, 1257-1263.

15. Sehgal, A., and Mignot, E. (2011). Genetics of sleep and sleep disorders. Cell *146*, 194-207.

16. Huber, R., Ghilardi, M., Massimini, M., and Tononi, G. (2004). Local sleep and learning. Nature *430*, 78-81.

17. Kattler, H., Dijk, D.J., and Borbely, A.A. (1994). Effect of unilateral somatosensory stimulation prior to sleep on the sleep EEG in humans. Journal of sleep research *3*, 159-164.

18. Landsness, E.C., Crupi, D., Hulse, B.K., Peterson, M.J., Huber, R., Ansari, H., Coen, M., Cirelli, C., Benca, R.M., Ghilardi, M.F., and Tononi, G. (2009). Sleep-dependent improvement in visuomotor learning: a causal role for slow waves. Sleep *32*, 1273-1284.

19. Hanlon, E.C., Faraguna, U., Vyazovskiy, V.V., Tononi, G., and Cirelli, C. (2009). Effects of skilled training on sleep slow wave activity and cortical gene expression in the rat. Sleep *32*, 719-729.

20. Vyazovskiy, V.V., Cirelli, C., Pfister-Genskow, M., Faraguna, U., and Tononi, G. (2008). Molecular and electrophysiological evidence for net synaptic potentiation in wake and depression in sleep - supplement. Nat Neurosci *11*, 200-208.

21. Vyazovskiy, V., Borbely, A.A., and Tobler, I. (2000). Unilateral vibrissae stimulation during waking induces interhemispheric EEG asymmetry during subsequent sleep in the rat. Journal of sleep research *9*, 367-371.

22. Moroni, F., Nobili, L., Curcio, G., De Carli, F., Tempesta, D., Marzano, C., De Gennaro, L., Mai, R., Francione, S., Lo Russo, G., and Ferrara, M. (2008). Procedural learning and sleep hippocampal low frequencies in humans. Neuroimage *42*, 911-918.

23. Nir, Y., Staba, R.J., Andrillon, T., Vyazovskiy, V.V., Cirelli, C., Fried, I., and Tononi, G. (2011). Regional slow waves and spindles in human sleep. Neuron *70*, 153-169.

24. Nobili, L., Ferrara, M., Moroni, F., De Gennaro, L., Russo, G.L., Campus, C., Cardinale, F., and De Carli, F. (2011). Dissociated wake-like and sleep-like electro-cortical activity during sleep. Neuroimage *58*, 612-619.

25. Vyazovskiy, V.V., Olcese, U., Hanlon, E.C., Nir, Y., Cirelli, C., and Tononi, G. (2011). Local sleep in awake rats. Nature *472*, 443-447.

26. Dijk, D.J., Beersma, D.G., and Daan, S. (1987). EEG power density during nap sleep: reflection of an hourglass measuring the duration of prior wakefulness. Journal of Biological Rhythms *2*, 207-219.

27. Bernstein, J.G., Garrity, P.A., and Boyden, E.S. (2012). Optogenetics and thermogenetics: technologies for controlling the activity of targeted cells within intact neural circuits. Current Opinion in Neurobiology *22*, 61-71.

28. Hendricks, J.C., Finn, S.M., Panckeri, K.A., Chavkin, J., Williams, J.A., Sehgal, A., and Pack, A.I. (2000). Rest in Drosophila is a sleep-like state. Neuron *25*, 129-138.

29. Shaw, P.J., Cirelli, C., Greenspan, R.J., and Tononi, G. (2000). Correlates of sleep and waking in Drosophila melanogaster. Science *287*, 1834-1837.

30. Deboer, T. (2015). Behavioral and electrophysiological correlates of sleep and sleep homeostasis. Curr Top Behav Neurosci *25*, 1-24.

31. Wu, M., Robinson, J.E., and Joiner, W.J. (2014). SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission. Current biology : CB *24*, 621-629.

32. Liu, Q., Liu, S., Kodama, L., Driscoll, M.R., and Wu, M.N. (2012). Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in Drosophila. Current biology : CB *22*, 2114-2123.

33. Ueno, T., Tomita, J., Tanimoto, H., Endo, K., Ito, K., Kume, S., and Kume, K. (2012). Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. Nature neuroscience *15*, 1516-1523.

34. Crocker, A., and Sehgal, A. (2008). Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. J Neurosci *28*, 9377-9385.

35. Crocker, A., Shahidullah, M., Levitan, I.B., and Sehgal, A. (2010). Identification of a neural circuit that underlies the effects of octopamine on sleep:wake behavior. Neuron *65*, 670-681.

36. Luan, H., Peabody, N.C., Vinson, C.R., and White, B.H. (2006). Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression. Neuron *52*, 425-436.

37. Aso, Y., Hattori, D., Yu, Y., Johnston, R.M., Iyer, N.A., Ngo, T.T., Dionne, H., Abbott, L.F., Axel, R., Tanimoto, H., and Rubin, G.M. (2014). The neuronal architecture of the mushroom body provides a logic for associative learning. Elife *3*, e04577.

38. Diekelmann, S., and Born, J. (2010). The memory function of sleep. Nature reviews. Neuroscience *11*, 114-126.

39. Joiner, W.J., Crocker, A., White, B., and Sehgal, A. (2006). Sleep in Drosophila is regulated by adult mushroom bodies. Nature *441*, 757-760.

40. Pitman, J., McGill, J.J., Keegan, K., and Allada, R. (2006). A dynamic role for the mushroom bodies in promoting sleep in Drosophila. Nature *441*, 753-756.

41. Berry, J.A., Cervantes-Sandoval, I., Chakraborty, M., and Davis, R.L. (2015). Sleep Facilitates Memory by Blocking Dopamine Neuron-Mediated Forgetting. Cell.

42. Haynes, P.R., Christmann, B.L., and Griffith, L.C. (2015). A single pair of neurons links sleep to memory consolidation in Drosophila melanogaster. Elife *4*.

43. Donlea, J.M., Thimgan, M.S., Suzuki, Y., Gottschalk, L., and Shaw, P.J. (2011). Inducing sleep by remote control facilitates memory consolidation in Drosophila. Science *332*, 1571-1576.

44. Keene, A.C., and Masek, P. (2012). Optogenetic induction of aversive taste memory. Neuroscience *222*, 173-180.

45. Masek, P., Worden, K., Aso, Y., Rubin, G.M., and Keene, A.C. (2015). A dopamine-modulated neural circuit regulating aversive taste memory in Drosophila. Current biology : CB *25*, 1535-1541.

46. Kirkhart, C., and Scott, K. (2015). Gustatory learning and processing in the Drosophila mushroom bodies. The Journal of neuroscience : the official journal of the Society for Neuroscience *35*, 5950-5958.

47. Cirelli, C., and Tononi, G. (2008). Is sleep essential? Plos Biol *6*, e216.

48. Lyamin, O., Pryaslova, J., Lance, V., and Siegel, J. (2005). Animal behaviour: continuous activity in cetaceans after birth. Nature *435*, 1177.

49. Rattenborg, N.C., Amlaner, C.J., and Lima, S.L. (2000). Behavioral, neurophysiological and evolutionary perspectives on unihemispheric sleep. Neuroscience and biobehavioral reviews *24*, 817-842.

50. Rattenborg, N.C., Lima, S.L., and Amlaner, C.J. (1999). Facultative control of avian unihemispheric sleep under the risk of predation. Behav Brain Res *105*, 163-172.

51. Lyamin, O.I., Mukhametov, L.M., and Siegel, J.M. (2004). Relationship between sleep and eye state in Cetaceans and Pinnipeds. Arch Ital Biol *142*, 557-568.

52. Suzuki, A., Sinton, C.M., Greene, R.W., and Yanagisawa, M. (2013). Behavioral and biochemical dissociation of arousal and homeostatic sleep need influenced by prior wakeful experience in mice. P Natl Acad Sci USA *110*, 10288-10293.

53. Martin PR, P.S., Swift RM. (2012). Pharmacology of drugs of abuse. In: Golan D, Tashjian AH, Armstrong EJ, Armstrong A, eds. Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy, 3rd edn, pp. 284–309. Philadelphia: Lippincott Williams & Wilkins. 284-309.

54. Donlea, J.M., Pimentel, D., and Miesenbock, G. (2014). Neuronal machinery of sleep homeostasis in Drosophila. Neuron *81*, 860-872.

55. Grueber, W.B., Ye, B., Moore, A.W., Jan, L.Y., and Jan, Y.N. (2003). Dendrites of distinct classes of Drosophila sensory neurons show different capacities for homotypic repulsion. Current biology : CB *13*, 618-626.

56. Pfeiffer, B.D., Ngo, T.T., Hibbard, K.L., Murphy, C., Jenett, A., Truman, J.W., and Rubin, G.M. (2010). Refinement of tools for targeted gene expression in Drosophila. Genetics *186*, 735-755.

Chapter 5: *Nf1* is a sleep-promoting factor in *Drosophila melanogaster*

5.1 Summary

Approximately 1 in every 3,000 individuals is afflicted with disorders falling under the classification of neurofibromatosis caused by mutations in *Nf1*. Many of these disorders are cognitive in nature such as deficits in learning and memory. Here we utilize *Drosophila* as a model organism to probe the effects of loss of *Nf1* on sleep. We demonstrate that *Nf1* is required in a subset of cholinergic neurons to regulate sleep during both day and night. Additionally, we utilized CRISPR/Cas9-mediated homology directed repair to knock-in an epitope in the endogenous *Nf1* locus thus expanding the potential proteomic, biochemical and immunofluorescent experiments possible with this protein. We show that NF1 is expressed broadly throughout the brain but is enriched in cell bodies as opposed to neuropil. Finally, we show that NF1 protein is not detectable in the mushroom bodies of flies, a region of the brain that is critical for both learning/memory and for sleep behavior. Together, these studies suggest a role for *Nf1* in sleep regulation and a potential explanation for the cognitive learning/memory deficits observed in *Nf1*-deficient flies.

5.2 Introduction

Despite decades of study, the genetic basis of sleep regulation remains poorly understood. While it is well accepted that features of sleep are under genetic control [1, 2], only a few heritable mutations are known to

influence sleep in humans. Notably, genes that regulate circadian rhythms have been strongly implicated in various aspects of heritable sleep regulation [3, 4]. One gene in particular, *Nf1*, the gene which, when mutated, causes neurofibromatosis type 1, has been implicated in disorders with learning and sleep disturbances [5-11].

Mutations in *Nf1* cause disorders in approximately 1 in every 3,000 individuals [12]. It is thought that loss of functional NF1 protein sensitizes individuals to disease-causing secondary mutations. Since a primary pathology of *Nf1* mutations is cancer-related, much effort has gone into understanding the tumor-suppressing function of NF1 in cells. However, since many of the cancers associated with loss of *Nf1* are derived from dysregulated growth and differentiation of cells originating from the neural crest [13], there is a gap in knowledge related to symptoms that cannot be traced to neural crest action such as leukemia, bone deformities, skeletal lesions, learning disabilities and sleep disturbances.

Due to the cognitive effects of mutated *Nf1*, we hypothesized that loss of *Nf1* in *Drosophila* may have similar sleep effects as in humans. Previously, *Drosophila Nf1* has been shown to be involved in learning/memory through an adenylate cyclase/PKA pathway [14, 15] as well as disrupted circadian rhythms through Ras/MAPK [16]. Given the relationship between learning/memory and sleep [17], we hypothesized that *Nf1*-depleted *Drosophila* would exhibit deficits in sleep that could explain the learning/memory phenotype.

Here we show that *Drosophila Nf1* is required for normal sleep in a subset of cholinergic neurons. We describe the generation of an endogenous epitope-tagged allele of *Nf1* using CRISPR/Cas9. Using this allele, we show that NF1 is expressed broadly across the brain and is enriched in cell bodies as opposed to neuropil. Finally, we demonstrate that, in contrast to a previous report, NF1 is not expressed at detectable levels in the mushroom bodies of flies – a brain region critical for sleep and learning/memory.

5.3 Results

5.3.1 *Nf1* is required in cholinergic neurons for normal sleep

Given the many links between learning/memory and sleep [17, 18], we hypothesized that genetic disorders involving cognitive – especially learning – impairment would have underlying sleep phenotypes. *Nf1*, the gene frequently mutated in neurofibromatosis type-1, is conserved from *Drosophila* through humans with the *Drosophila* NF1 protein sharing about 60% identity with human NF1 [19]. Further, *Drosophila Nf1* mutants have cognitive deficits in learning/memory [14]. We decided to test our hypothesis that *Drosophila Nf1* is required for normal sleep by knocking down expression of *Nf1* in cholinergic neurons, which represent ~70% of the fly brain. In order to accomplish this task, we utilized the Gal4/UAS expression system [20] to couple the cholinergic driver, *cha-Gal4*, with a short hairpin RNAi directed against *Nf1*, HMC03551. Interestingly, we found that this knockdown was sufficient to reduce sleep in flies by more than 50% across both day and night (Figure

5.1A,B). It is interesting to note that we observe a significant decrease in sleep in our *UAS-HMC03551* alone control, suggesting leaky expression of the transgene. Sleep is disrupted in flies with depleted *Nf1* through the dual results of both shorter sleep bout durations and longer wake bout durations (Figure 5.1C,D). These data suggest that *Nf1* is required to promote sleep onset and maintenance since both aspects are perturbed in these flies. Since off-target effects of RNAi-mediated gene depletion are well known [21], we confirmed our results by knocking down *Nf1* in cholinergic neurons using a second RNAi, v109637, in conjunction with *UAS-Dicer* and found similar results (Figure 5.1E).

5.3.2 A Gal4 screen for neurons requiring *Nf1* for normal sleep behavior

Since cholinergic neurons comprise approximately 70% of the fly brain, we wanted to narrow down the region of the fly brain in which *Nf1* is required for normal sleep. By narrowing down our focus to the relevant neurons, we reasoned that we would be more capable of performing experiments to determine cell-autonomous effects of loss-of-*Nf1*. In order to determine which neurons in the fly brain require *Nf1*, we coupled the non-leaky *Nf1* RNAi, v109637, with *UAS-Dicer* and crossed the line to 492 different Gal4 lines generated from putative enhancers thought to be enriched for neuronal expression [22]. We measured sleep in 4-8 animals per group and found a broad distribution of sleep phenotypes (Figure 5.2A). Interestingly, after retesting promising lines, we found that a high percentage of Gal4 lines did not repeat and that many lines showed high variability between experiments (data

not shown) suggesting the possibility for complex underlying regulation of *Nf1*-mediated sleep regulation. We did, however, identify two specific Gal4 drivers, that, when coupled to *Nf1* RNAi, resulted in reproducible decreases in sleep compared to controls (Figure 5.2B). These Gal4 drivers, 71D04 and 87A08, appear not to share overlapping expression patterns when coupled to *UAS-CD8::GFP* (Figure 5.3A-D). 71D04 expresses in the *pars intercerebralis* neurons at the dorsal end of the brain, various cell bodies speckled throughout the CNS and thoracic ganglia, and in portions of glia as indicated by a sheath-like appearance covering various neuropil and nerve bundles. The 71D04 expression pattern is in stark contrast to the restricted expression pattern of 87A08. The 87A08 enhancer fragment only shows detectable expression in a subset of central complex neurons and a few visual system neurons with a distinct absence of expression in the thoracic ganglia.

Since the 87A08 enhancer fragment elicited a much weaker sleep phenotype (Figure 5.2B), we decided to focus on 71D04 to determine where *Nf1* is required for normal sleep. We noticed that projecting from near the cell bodies of the *pars intercerebralis* neurons were GFP-labeled projections that appeared within the vicinity of the PDF-expressing projections from the LNv clock neurons. Upon co-staining for GFP and PDF, we found that, indeed, these two sets of projections come in close proximity within the same optical sections by confocal microscopy (Figure 5.3E). This is a significant observation because of the tight regulation between circadian rhythms and sleep regulation. Additionally, a previous study demonstrated that *Drosophila*

Nf1 mutants have a severe circadian rhythm phenotype – almost complete arrhythmic behavior across circadian time [16]. While we have not yet been able to confirm that the putative connections between these neurons are relevant to the sleep phenotype we observe, it is interesting to speculate about the importance such a connection may pose.

In order to further narrow down which neurons *Nf1* is required in for normal sleep, we took an intersectional approach to this anatomical study. In order to do this, we utilized split Gal4 [23] to express the DNA binding domain (DBD) of Gal4 under control of the *cha* promoter and the transcriptional activation domain of Gal4 (AD) under control of the 71D04 enhancer. Upon measuring sleep in animals expressing *Nf1* RNAi under control of the split Gal4, we found a significant reduction in sleep compared to genetic controls (Figure 5.4A), albeit reduced compared to either *cha*- or 71D04-Gal4 lines. We then coupled the split Gal4 transgenes with *UAS-CD8::GFP* and visualized the expression pattern of the intersection between *cha*- and 71D04-Gal4s. The resulting expression pattern was more restricted than either 71D04 or *cha* alone, yet still reflected the majority of the 71D04 expression pattern. This result suggests that the majority neurons targeted by 71D04-Gal4 are cholinergic. It is worth noting that using the intersectional strategy, the resulting expression pattern of the split Gal4 lacked expression in glial cells as was observed in 71D04-Gal4.

5.3.3 Generation of an endogenous epitope-tagged *Nf1* allele using CRISPR

A previous study generated an antibody directed against *Drosophila* NF1 [24] which has been shown to work for both immunoblots and immunofluorescence [25]. However, availability of the antibody and the fact that immunoprecipitation is required for immunoblotting prompted us to generate an epitope-tagged endogenous allele of *Nf1*. In order to accomplish this goal, we utilized homology directed repair (HDR) after a CRISPR/Cas9-mediated double strand break [26, 27]. We took advantage of a gRNA target site that spans the endogenous *Nf1* lone translational start site to induce a double strand break (Figure 5.5A). Embryos were co-injected with a donor plasmid containing homologous sequence several hundred base pairs upstream and downstream of the predicted cut site. Within the donor plasmid, we added the HA epitope directly after the translational start site followed by a short linker sequence before the normal NF1 sequence. Our CRISPR-mediated HDR strategy resulted in ~21% of viable G0 flies giving rise to successful HDR offspring (Figure 5.5B). We confirmed the presence of the donor sequence in the correct genomic locus by performing PCR using primers specific for successful HDR (Figure 5.5C). Finally, we performed Western blot analysis of heads from flies with and without the HA-tagged NF1 protein and found the presence of a single band near the predicted size of full-length NF1 protein that was not present in otherwise genetically identical WT flies (>245kD; Figure 5.5D).

5.3.4 NF1 is broadly expressed in the brain but is not expressed in the mushroom bodies

Utilizing our newly generated *Nf1*^{HA} allele, we wanted to determine the endogenous expression pattern of NF1 protein in the fly brain. To accomplish this task, we stained WT and *Nf1*^{HA} brains with antibodies directed against HA. Upon visualization of these brains, we observed a predictably low signal from WT brains with only minor non-specific binding (Figure 5.6A). In the *Nf1*^{HA} brains, we found broad expression of NF1 throughout the fly brain (Figure 5.6B). Interestingly, we found that NF1 is predominantly expressed in the cell bodies of neurons as opposed to the neuropil.

In the literature, there are conflicting reports regarding the involvement of NF1 in the mushroom bodies in regards to learning and memory. One such study [28], utilizing broad and non-specific Gal4 drivers, suggested that NF1 is required in the mushroom bodies for learning and memory to occur. These results make intuitive sense given the critical role of the mushroom bodies in olfactory-mediated aversive learning and memory. However, another study [25] visualized the NF1 expression pattern in larval and adult brains using the previously generated NF1 antibody and did not detect any observable NF1 protein in these cells. Since the mushroom bodies have been implicated in cholinergic sleep regulation in *Drosophila* [29-31], we looked to see if we could observe NF1 expression in the adult Kenyon cells. Interestingly, we did not observe any detectable staining of NF1^{HA} in the Kenyon cells (Figure 5.6C)

and did not detect any changes in sleep after knocking down *Nf1* expression in the mushroom bodies (data not shown) suggesting that *Nf1* is not required in these cells for sleep regulation.

It is worth noting that, like many epitope tagged proteins, we observed a small decrease in NF1 function as correlated by a decrease in total daily sleep (Figure 5.6D). The decrease of ~120 minutes is suggestive that our $Nf1^{HA}$ allele is a mild hypomorph compared to untagged *Nf1*. While we do not believe this hypomorphic effect should preclude the use of the tagged allele, it is worth noting.

5.4 Discussion

NF1 is involved in numerous functions in the brain regardless of species. NF1 has been demonstrated to be involved in cognition, learning/memory, circadian rhythms, and development. In this study, we demonstrated a role for NF1 in *Drosophila* as a sleep promoting factor that, when depleted, results in ~50% reductions in sleep. Specifically, reducing *Nf1* in cholinergic neurons gives a robust phenotype. After performing a Gal4 screen composed of almost 500 Gal4 lines, we identified two non-overlapping expression patterns requiring *Nf1* for normal sleep. In one of these lines, we determined the cholinergic subset of neurons is responsible for the *Nf1* depletion phenotype. We then utilized the CRISPR/Cas9 system to knock-in an epitope into the endogenous *Nf1* locus in order to facilitate future proteomic and fluorescent assays. Using this $Nf1^{HA}$ allele, we found that NF1 is

expressed broadly throughout the fly brain and is enriched in cell bodies compared to neuropil. Finally, we found that NF1 protein is not expressed at detectable levels in the mushroom bodies, a region of the brain important for sleep and learning/memory.

It is interesting to postulate that learning/memory deficits in *Nf1* mutants may be due to insufficient sleep. There is a strong link between sleep loss and poor performance on learning/memory tasks [17] thus it is possible that these deficits in *Nf1* mutants can be explained solely by decreased sleep. It will be interesting to determine if these two phenotypes can be separated as distinct processes or if one is the direct result of the other.

It will also be interesting to determine downstream signaling cascades that mediate the *Nf1* sleep phenotype. Various reports indicate NF1 to be involved in Ras signaling as well as adenylate cyclase/PKA signaling [15]. PKA has been shown to be involved in the regulation of sleep in *Drosophila* [32] and is thus a tempting target for the hypothesized role of *Nf1* in sleep regulation. Future experiments will aim to address which signaling cascade is functionally disrupted in *Nf1* mutants to cause the observed sleep phenotype.

5.5 Methods

5.5.1 Fly stocks

Flies were grown at room temperature (20-22°C) on standard cornmeal media with yeast. The *Nf1* RNAi line v109637 and UAS-*Dicer* were always used together and were obtained from Vienna Drosophila Resource Center

[33]. HMC03551 [34, 35], all enhancer fragment Gal4 lines [22], *UAS-CD8::GFP*, *cha-Gal4*, $Nf1^{P3}$ (c00617) and balancer stocks were obtained from the Bloomington Stock Center. $Nf1^{P1}$ and $Nf1^{P2}$ were obtained from Dr. André Bernards. Male flies were used for all behavioral assays unless otherwise indicated.

5.5.2 Sleep and locomotor measurements

Individual 3-7 day old flies were placed in 5 mm x 65 mm Pyrex tubes containing a mixture of 2% agarose and 5% sucrose at one end as a food source. Animals were entrained for 2 days in 12 hr:12 hr light:dark conditions at 25°C, and infrared beam breaks were recorded in 1 min bins for the next 2-4 days using the Drosophila Activity Monitoring System (Trikinetics). Sleep analysis was performed as previously described using custom MATLAB (Mathworks) software [36].

5.5.3 Generation of epitope-tagged *Nf1* allele

To generate flies carrying the hemagglutinin tag (HA) at the ubiquitous N-terminus of NF1, we used CRISPR/Cas9-mediate homology directed repair [26, 27]. Briefly, we generated the gRNA plasmid by annealing the forward (5'-CTTCGCTTCTGGGTCATCTTGTTG-3') and reverse (5'-AAACCAACAAGATGACCCAGAAGC-3') oligos into pU6-BbsI-chi-RNA [26]. We generated the donor plasmid by TOPO cloning a PCR amplicon of the 5' region of the Nf1 gene with forward ('5-CGAGATCTTGGGTGGCGGTAAG-3')

and reverse (5'-ACAGAGCGGGTGAGGCAC-3') primers into pCR2.1 (Life Technologies). The HA tag was added immediately after the NF1 translational start site by site-directed mutagenesis using forward (5'-AAAACACCACAACAAGATGTACCCATACGATGTTCCAGATTACGCTGGCGGAAGCCGAACCCAGAAGCCAGGCGAGTGG-3') and reverse (5'-CCACTCGCCTGGCTTCTGGGTTCGGCTTCCGCCAGCGTAATCTGGAACATCGTATGGGTACATCTTGTTGTGGTGTTTT-3') primers. After digestion with EcoRI and SpeI, the 604bp fragment containing the HA tag was subcloned into the same sites in pCR2.1 to create the final donor plasmid.

Transgenic animals were generated after injection of both gRNA and donor plasmids into y^{1},M(vas-Cas9)ZH-2A,w^{1118} (Bloomington line 51323) (Rainbow Transgenic Flies, Inc.). Up to 10 F2 lines were established per G0 fly and were screened for the 39bp band shift (242bp vs 203bp) indicating successful homology-directed repair by PCR and gel electrophoresis using forward (5'-AAGATGCCACTCAACCAATTGCC-3') and reverse (5'-ACGCAACTACGGAAAAGCGTTTG-3') primers. Putatively successfully engineered lines were confirmed with the forward (5'-ACCTATTACTGCTCGAAATGCTG-3') and reverse (5'-AGCGTAATCTGGAACATCGTATGGG) primers that are specific for correct integration.

5.5.4 Generation of transgenic animals

For 71D04-AD, the 71D04 enhancer fragment was cloned into the SpeI and EcoRI sites of a modified mENTRY plasmid (Life Technologies) from P(GMR71D04-GAL4)attP2 flies using forward (5'-ATAACTAGTTGCCTCGCTGGGCATGGCCCATATT-3') and reverse (5'-ATATAGAATTCGCTACACAGCCAGGTAAATCGTACG-3') primers containing SpeI and EcoRI restriction sites, respectively. The 71D04 enhancer fragment was then inserted into pBPp65ADZpUw [37] using Gateway recombination (Life Technologies). The 71D04-AD plasmid was injected (Rainbow Transgenic Flies, Inc.) into the M(vas-int.Dm)ZH-2A;;P(CaryP)attP2 line and transgenic animals were outcrossed into the *iso31* w^{1118} background for at least 3 generations.

For *cha*-DBD, the 7.4kb *cha* promoter fragment of *ChaT* [38] was cloned into the SpeI and XbaI sites of mENTRY. The *cha* promoter was then inserted into pBPZpGAL4DBDUw [37] by Gateway recombination. Transgenic animals were generated by injecting the *cha*-DBD plasmid into the y^{1},w^{*},P(y[+t7.7]=nos-phiC31int.NLS)X;;PBac(y[+]-attP-9A)VK00027 (Rainbow Transgenic Flies, Inc.).

5.5.5 Immunohistochemistry

Immunohistochemistry was performed as described previously [36]. Briefly, 3-7 day-old brains were dissected in ice-cold PBS, fixed in 4% paraformaldehyde, and blocked in PBST (PBS and 0.3% Triton X-100) with 5% normal donkey serum (Jackson Laboratory) prior to staining. Brains were

then incubated with 1:1,000 rabbit anti-GFP (Invitrogen) and 1:50 mouse anti-nc82 or 1:50 mouse anti-PDF (Developmental Studies Hybridoma Bank) antibodies overnight at 4°C and washed five times in PBST. Brains were then incubated with 1,000 Alexa 488 anti-rabbit (Life Technologies) and 1:1,000 Alexa 568 anti-mouse (Life Technologies) antibodies for 2-4 hours at room temperature prior to washing five times in PBST and coverslip mounting in Vectashield (Vector Labs). Images were taken at 40x magnification on a Leica SP5 confocal microscope at 0.5-0.75 µm intervals and reassembled for display as maximum projections using Fiji [39].

5.5.6 Western blot analysis

16 male and female heads from 3-7 day old flies were lysed in sample buffer (20mM HEPES, pH7.5, 100mM KCl, 10mM EDTA, 50mM NaF, 0.1% Triton X-100, 10% glycerol, 1mM DTT, 1X Roche Complete Protease Inhibitors). Lysates were cleared of particulate debris by centrifugation at 5,000g for 15 minutes at 4°C prior to protein quantification. Lysate were resolved on 15-well 4-12% NuPAGE SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were probed using 1:500 mouse anti-HA (Covance) and 1:10,000 mouse anti-actin primary antibodies (EMD Millipore) followed by 1:5,000 or 1:10,000 anti-mouse HRP antibodies (VWR), respectively. Visualization of bound secondary antibodies was achieved using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

5.5.7 Statistics

Replicates (n values) represent the number of biological replicates for each experimental condition. Bar graphs depict the mean ± SEM, except for box and whisker plots, which depict the median (line), 25^{th} to 75^{th} percentiles (box) and minimum/maximum values (whiskers). Unless otherwise indicated, datasets that approximate to a normal Gaussian distribution were analyzed by one-way ANOVA followed by the Tukey-Kramer test for multiple comparisons. For datasets with non-Gaussian distributions, comparisons were performed using Kruskal-Wallis test followed by Dunn's multiple comparisons test. All statistical tests were two-sided and performed using Prism 6.0f for Mac OS X (GraphPad Software).

5.6 Acknowledgements

Work presented in Chapter 5 will be submitted for publication upon completion of follow-up studies. The dissertation author was the primary author of this work in collaboration with Glen Seidner and William Joiner.

5.7 Figures

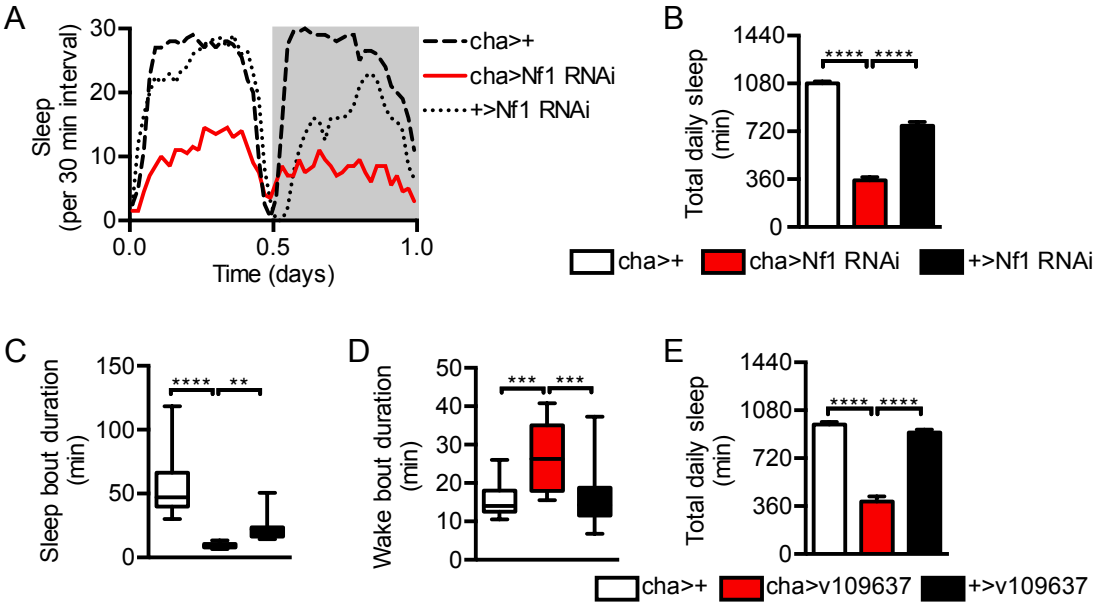

Figure 5.1 Knockdown of *Nf1* in cholinergic neurons reduces sleep. (**A**) Representative sleep traces of male flies with (red trace) and without (black traces) *Nf1* knockdown in cholinergic neurons using the HMC03551 *Nf1* RNAi (A-D). (**B**) Quantification of total daily sleep shows decreased sleep in *Nf1* knockdown animals. (**C**) *Nf1* knockdown results in shorter sleep bout durations and longer wake bout durations (**D**). For A-D, n = 32 per group. (**E**) Total daily sleep is reduced in animals expressing a second *Nf1* RNAi, v109637, compared to controls. For E, n = 30-32 per group.

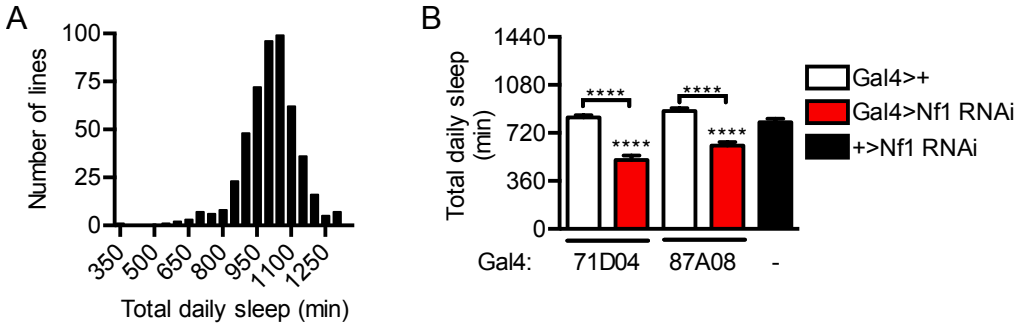

Figure 5.2 Gal4 screen for populations of neurons requiring *Nf1* for normal sleep. (**A**) Histogram depicting median sleep values (4-8 flies per line) for each Gal4 line tested with *Nf1* RNAi. (**B**) Total daily sleep for the two strongest phenotypes from the screen, 71D04-Gal4 and 87A08-Gal4, with and without *Nf1* RNAi. For B, n = 28-32 per group.

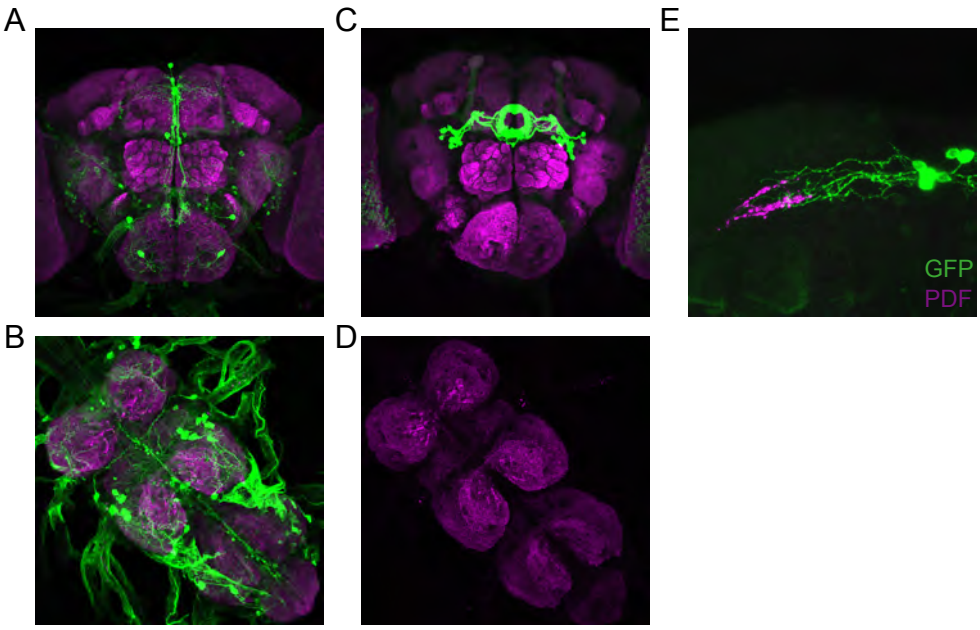

Figure 5.3 Expression patterns of 71D04-Gal4 and 87A08-Gal4. (**A**) Expression pattern of 71D04>CD8::GFP in the central brain and (**B**) thoracic ganglia. (**C**) Expression pattern of 87A08>CD8::GFP in the central brain and (**D**) thoracic ganglia. In A-D, green indicates GFP whereas magenta indicates nc82. (**E**) Projections emanating from near the PI neurons come in close contact with PDF-expressing projections. In E, green indicates GFP and magenta indicates PDF.

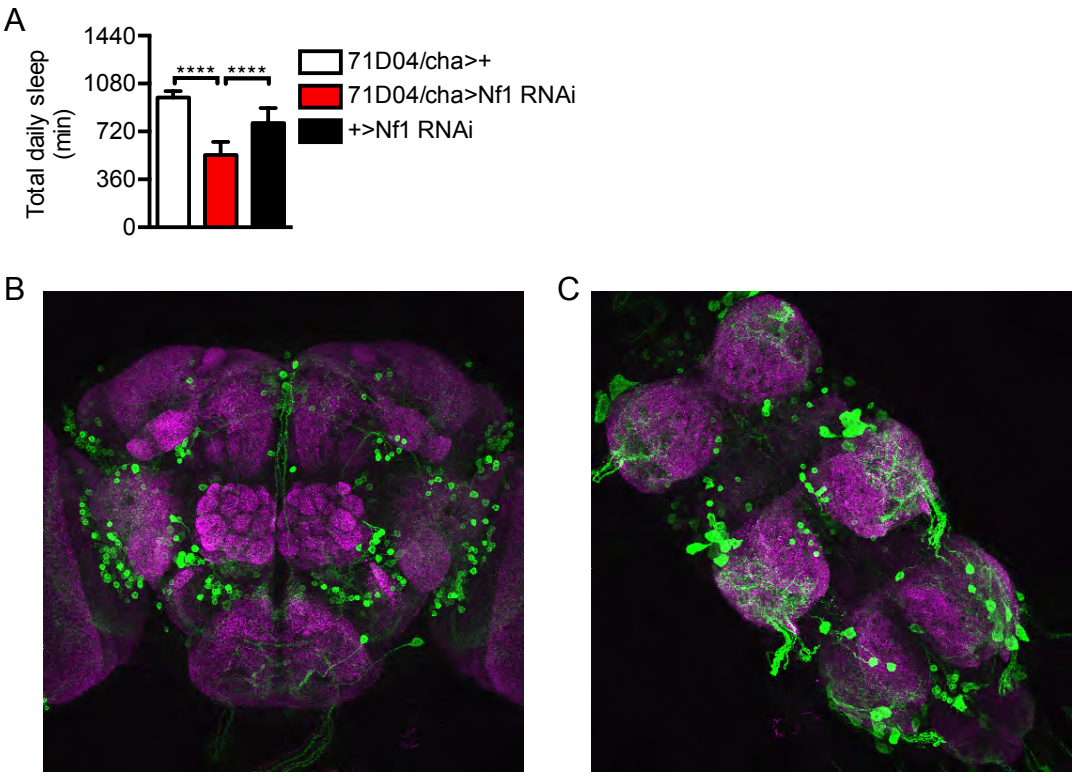

Figure 5.4 Relevant 71D04 neurons are cholinergic. (**A**) Total daily sleep for animals expressing *Nf1* RNAi (HMC03551) under control of 71D04-AD and *cha*-DBD. For A, n ≥ 8 per group. (**B**) Expression pattern of 71D04-AD with *cha*-DBD expressing UAS-CD8::GFP in the central brain and (**C**) thoracic ganglia. In B and C, green indicates GFP and magenta indicates nc82.

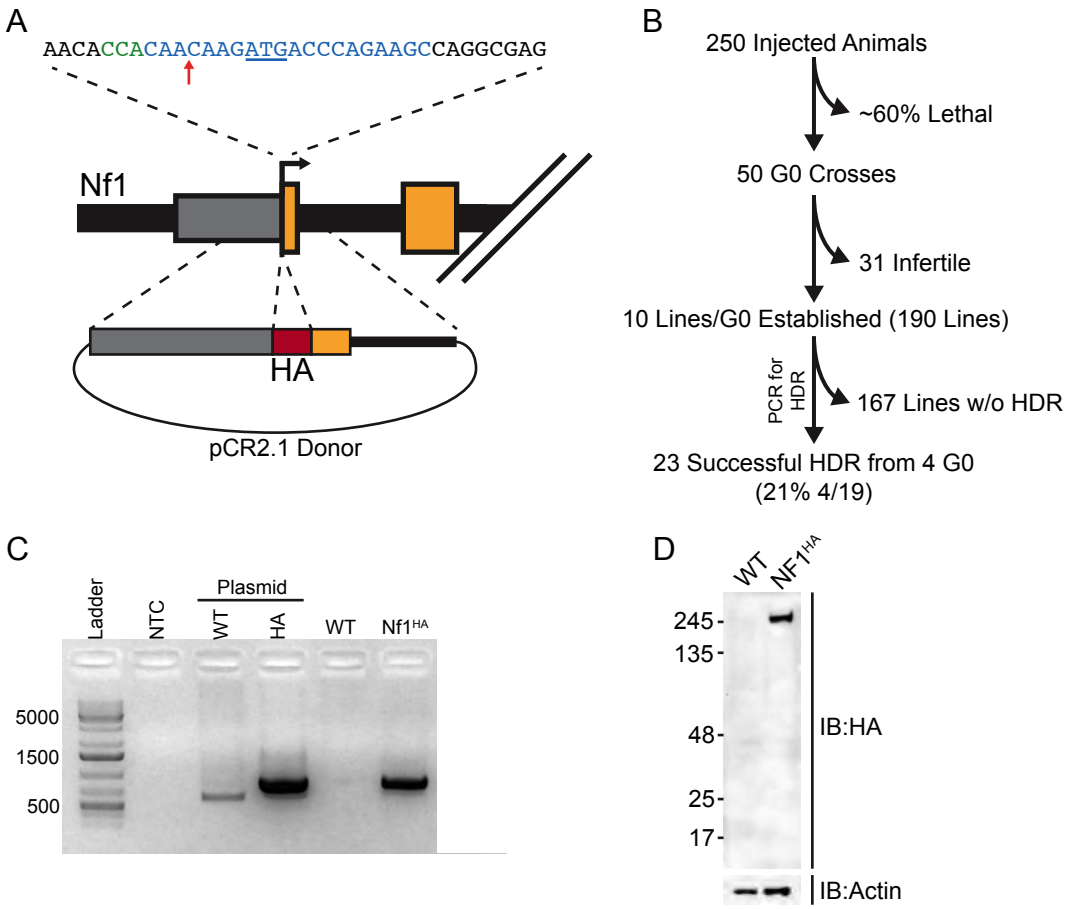

Figure 5.5 Generation of *Nf1*^{HA} flies through CRISPR/Cas9-mediated homology directed repair. (**A**) Schematic of epitope tagging strategy for the endogenous *Nf1* locus. The gRNA was directed against the sequence shown in blue (top) where the predicted cut site is indicated with a red arrow, translational start site is underlined and PAM is shown in green. The donor vector (bottom) shared homology with >250bp on each side of the cut site. The gRNA target sequence was disrupted with the HA sequence (red) immediately following the translational start site. Intergenic sequence is indicated by the bold black line; 5'UTR is indicated by the gray bar; and CDS is indicated by the orange bar. (**B**) Flow chart of the screening process used from injection (top) to identification of successful HDR lines (bottom). (**C**) Gel electrophoresis of PCR products from successful HDR (Nf1^{HA}) indicated by the presence of a ~800bp band. WT flies do not produce a band. Note the presence of a non-specific band from the negative control plasmid of isolated WT genomic sequence vs the positive control plasmid sequence. (**D**) Representative Western blot of HA-blotted WT vs *Nf1*^{HA} flies. The single band migrates to approximately the correct predicted size of NF1 (> 250Kd).

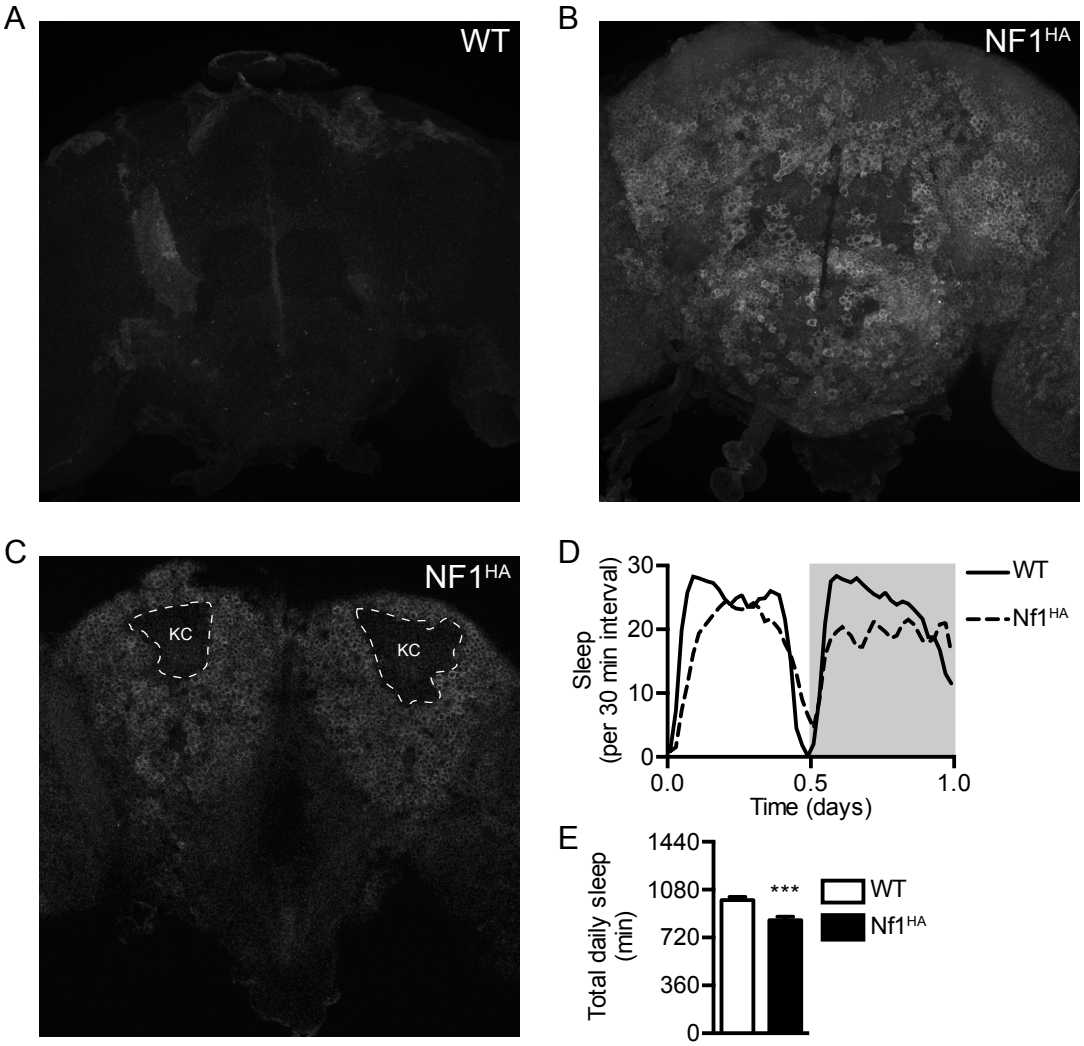

Figure 5.6 The endogenous expression pattern of NF1^{HA} is broad but distinctly absent in Kenyon cells. (**A**) Representative image of a max-projection from a HA-stained WT brain. Note the absence of specific staining. (**B**) Representative image of a max-projection from a HA-stained *Nf1^{HA}* brain. NF1 protein is broadly distributed across the brain but appears enriched in cell bodies compared to neuropil. (**C**) Representative image of a substack of images through the Kenyon cells of *Nf1^{HA}* brains stained against HA. Note the distinct absence of NF1 protein in the Kenyon cells. (**D**) Representative sleep trace and (**E**) quantification of total daily sleep for WT vs *Nf1^{HA}* flies.

5.8 References

1. De Gennaro, L., Marzano, C., Fratello, F., Moroni, F., Pellicciari, M.C., Ferlazzo, F., Costa, S., Couyoumdjian, A., Curcio, G., Sforza, E., Malafosse, A., Finelli, L.A., Pasqualetti, P., Ferrara, M., Bertini, M., and Rossini, P.M. (2008). The electroencephalographic fingerprint of sleep is genetically determined: a twin study. Ann Neurol *64*, 455-460.

2. Ambrosius, U., Lietzenmaier, S., Wehrle, R., Wichniak, A., Kalus, S., Winkelmann, J., Bettecken, T., Holsboer, F., Yassouridis, A., and Friess, E. (2008). Heritability of sleep electroencephalogram. Biol Psychiatry *64*, 344-348.

3. Toh, K.L., Jones, C.R., He, Y., Eide, E.J., Hinz, W.A., Virshup, D.M., Ptacek, L.J., and Fu, Y.H. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science *291*, 1040-1043.

4. Xu, Y., Padiath, Q.S., Shapiro, R.E., Jones, C.R., Wu, S.C., Saigoh, N., Saigoh, K., Ptacek, L.J., and Fu, Y.H. (2005). Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome. Nature *434*, 640-644.

5. Stine, S.B., and Adams, W.V. (1989). Learning problems in neurofibromatosis patients. Clin Orthop Relat Res, 43-48.

6. North, K., Joy, P., Yuille, D., Cocks, N., Mobbs, E., Hutchins, P., McHugh, K., and de Silva, M. (1994). Specific learning disability in children with neurofibromatosis type 1: significance of MRI abnormalities. Neurology *44*, 878-883.

7. Brewer, V.R., Moore, B.D., 3rd, and Hiscock, M. (1997). Learning disability subtypes in children with neurofibromatosis. J Learn Disabil *30*, 521-533.

8. Stradling, J.R., Huddart, S., and Arnold, A.G. (1981). Sleep apnoea syndrome caused by neurofibromatosis and superior vena caval obstruction. Thorax *36*, 634-635.

9. Leschziner, G.D., Golding, J.F., and Ferner, R.E. (2013). Sleep disturbance as part of the neurofibromatosis type 1 phenotype in adults. Am J Med Genet A *161A*, 1319-1322.

10. Licis, A.K., Vallorani, A., Gao, F., Chen, C., Lenox, J., Yamada, K.A., Duntley, S.P., and Gutmann, D.H. (2013). Prevalence of Sleep

Disturbances in Children With Neurofibromatosis Type 1. J Child Neurol *28*, 1400-1405.

11. Marana Perez, A.I., Duat Rodriguez, A., Soto Insuga, V., Dominguez Carral, J., Puertas Martin, V., and Gonzalez Gutierrez Solana, L. (2014). Prevalence of sleep disorders in patients with neurofibromatosis type 1. Neurologia.

12. Lammert, M., Friedman, J.M., Kluwe, L., and Mautner, V.F. (2005). Prevalence of neurofibromatosis 1 in German children at elementary school enrollment. Arch Dermatol *141*, 71-74.

13. Brannan, C.I., Perkins, A.S., Vogel, K.S., Ratner, N., Nordlund, M.L., Reid, S.W., Buchberg, A.M., Jenkins, N.A., Parada, L.F., and Copeland, N.G. (1994). Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. Genes Dev *8*, 1019-1029.

14. Guo, H.F., Tong, J., Hannan, F., Luo, L., and Zhong, Y. (2000). A neurofibromatosis-1-regulated pathway is required for learning in Drosophila. Nature *403*, 895-898.

15. Hannan, F., Ho, I., Tong, J.J., Zhu, Y., Nurnberg, P., and Zhong, Y. (2006). Effect of neurofibromatosis type I mutations on a novel pathway for adenylyl cyclase activation requiring neurofibromin and Ras. Hum Mol Genet *15*, 1087-1098.

16. Williams, J.A., Su, H.S., Bernards, A., Field, J., and Sehgal, A. (2001). A circadian output in Drosophila mediated by neurofibromatosis-1 and Ras/MAPK. Science *293*, 2251-2256.

17. Stickgold, R. (2005). Sleep-dependent memory consolidation. Nature *437*, 1272-1278.

18. Tononi, G., and Cirelli, C. (2014). Sleep and the Price of Plasticity: From Synaptic and Cellular Homeostasis to Memory Consolidation and Integration. Neuron *81*, 12-34.

19. Guo, H.F., The, I., Hannan, F., Bernards, A., and Zhong, Y. (1997). Requirement of Drosophila NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. Science *276*, 795-798.

20. Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

21. Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P.S. (2003). Expression profiling reveals off-target gene regulation by RNAi. Nat Biotechnol *21*, 635-637.

22. Jenett, A., Rubin, G.M., Ngo, T.T., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B.D., Cavallaro, A., Hall, D., Jeter, J., Iyer, N., Fetter, D., Hausenfluck, J.H., Peng, H., Trautman, E.T., Svirskas, R.R., Myers, E.W., Iwinski, Z.R., Aso, Y., DePasquale, G.M., Enos, A., Hulamm, P., Lam, S.C., Li, H.H., Laverty, T.R., Long, F., Qu, L., Murphy, S.D., Rokicki, K., Safford, T., Shaw, K., Simpson, J.H., Sowell, A., Tae, S., Yu, Y., and Zugates, C.T. (2012). A GAL4-driver line resource for Drosophila neurobiology. Cell Rep *2*, 991-1001.

23. Luan, H., Peabody, N.C., Vinson, C.R., and White, B.H. (2006). Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression. Neuron *52*, 425-436.

24. The, I., Hannigan, G.E., Cowley, G.S., Reginald, S., Zhong, Y., Gusella, J.F., Hariharan, I.K., and Bernards, A. (1997). Rescue of a Drosophila NF1 mutant phenotype by protein kinase A. Science *276*, 791-794.

25. Walker, J.A., Tchoudakova, A.V., McKenney, P.T., Brill, S., Wu, D., Cowley, G.S., Hariharan, I.K., and Bernards, A. (2006). Reduced growth of Drosophila neurofibromatosis 1 mutants reflects a non-cell-autonomous requirement for GTPase-Activating Protein activity in larval neurons. Genes Dev *20*, 3311-3323.

26. Gratz, S.J., Cummings, A.M., Nguyen, J.N., Hamm, D.C., Donohue, L.K., Harrison, M.M., Wildonger, J., and O'connor-Giles, K.M. (2013). Genome Engineering of Drosophila with the CRISPR RNA-Guided Cas9 Nuclease. Genetics *194*, 1029-1035.

27. Gratz, S.J., Ukken, F.P., Rubinstein, C.D., Thiede, G., Donohue, L.K., Cummings, A.M., and O'connor-Giles, K.M. (2014). Highly Specific and Efficient CRISPR/Cas9-Catalyzed Homology-Directed Repair in Drosophila. Genetics *196*, 961-971.

28. Buchanan, M.E., and Davis, R.L. (2010). A distinct set of Drosophila brain neurons required for neurofibromatosis type 1-dependent learning and memory. J Neurosci *30*, 10135-10143.

29. Joiner, W.J., Crocker, A., White, B., and Sehgal, A. (2006). Sleep in Drosophila is regulated by adult mushroom bodies. Nature *441*, 757-760.

30. Pitman, J.L., McGill, J.J., Keegan, K.P., and Allada, R. (2006). A dynamic role for the mushroom bodies in promoting sleep in Drosophila. Nature *441*, 753-756.

31. Yi, W., Zhang, Y., Tian, Y., Guo, J., Li, Y., and Guo, A. (2013). A subset of cholinergic mushroom body neurons requires Go signaling to regulate sleep in Drosophila. Sleep *36*, 1809-1821.

32. Hendricks, J.C., Williams, J.A., Panckeri, K., Kirk, D., Tello, M., Yin, J.C., and Sehgal, A. (2001). A non-circadian role for cAMP signaling and CREB activity in Drosophila rest homeostasis. Nat Neurosci *4*, 1108-1115.

33. Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., Couto, A., Marra, V., Keleman, K., and Dickson, B.J. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature *448*, 151-156.

34. Ni, J.-Q., Liu, L.-P., Binari, R., Hardy, R., Shim, H.-S., Cavallaro, A., Booker, M., Pfeiffer, B.D., Markstein, M., Wang, H., Villalta, C., Laverty, T.R., Perkins, L.A., and Perrimon, N. (2009). A Drosophila Resource of Transgenic RNAi Lines for Neurogenetics. Genetics *182*, 1089-1100.

35. Ni, J.Q., Zhou, R., Czech, B., Liu, L.P., Holderbaum, L., Yang-Zhou, D., Shim, H.S., Tao, R., Handler, D., Karpowicz, P., Binari, R., Booker, M., Brennecke, J., Perkins, L.A., Hannon, G.J., and Perrimon, N. (2011). A genome-scale shRNA resource for transgenic RNAi in Drosophila. Nat Methods *8*, 405-407.

36. Wu, M., Robinson, J.E., and Joiner, W.J. (2014). SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission. Curr Biol *24*, 621-629.

37. Pfeiffer, B.D., Ngo, T.-T.B., Hibbard, K.L., Murphy, C., Jenett, A., Truman, J.W., and Rubin, G.M. (2010). Refinement of Tools for Targeted Gene Expression in Drosophila. Genetics *186*, 735-755.

38. Salvaterra, P.M., and Kitamoto, T. (2001). Drosophila cholinergic neurons and processes visualized with Gal4/UAS-GFP. Brain Res Gene Expr Patterns *1*, 73-82.

39. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P.,

and Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods *9*, 676-682.

Chapter 6: SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission

6.1 Summary

Although sleep is conserved throughout evolution, the molecular basis of its control is still largely a mystery. We previously showed that the *quiver/sleepless* (*qvr*/*sss*) gene encodes a membrane-tethered protein that is required for normal sleep in *Drosophila*. SLEEPLESS (SSS) protein functions, at least in part, by upregulating the levels and open probability of Shaker (Sh) potassium channels to suppress neuronal excitability and enable sleep. Consistent with this proposed mechanism, loss-of-function mutations in *Sh* phenocopy *qvr*/*sss* null mutants. However, sleep is more genetically modifiable in *Sh* than in *qvr*/*sss* mutants, suggesting that *sss* may regulate additional molecules to influence sleep. Here we show that SSS also antagonizes nicotinic acetylcholine receptors (nAChRs) to reduce synaptic transmission and promote sleep. Mimicking this antagonism by RNAi knockdown of specific nAChR subunits is sufficient to restore sleep to *qvr*/*sss* mutants. Regulation of nAChR activity by SSS occurs post-transcriptionally since the levels of nAChR mRNAs are unchanged in *qvr*/*sss* mutants. Together, our data point to an evolutionarily conserved, bi-functional role for SSS and its homologs in controlling excitability and synaptic transmission in fundamental processes of the nervous system such as sleep.

6.2 Introduction

Sleep is an essential process that is highly conserved across evolution, yet its functions and underlying mechanisms of control are poorly understood. One of the most conserved features of sleep is its association with large-scale changes in brain activity relative to the waking state, suggesting that modulation of neuronal activity may be central to the regulation of sleep. In support of this hypothesis, homologous genes in mammals and flies encoding ion channels and ionotropic receptors have been shown to be necessary for normal sleep [1-4]. The requirement for potassium (K) channels in the Shaker (Sh) family is particularly notable. In both mammals and flies, loss-of-function mutations in *Sh* or its orthologs cause reduced sleep [2, 4]. Although little is known about whether modulators of Sh-type channels might control sleep in mammals, loss-offunction mutations in either of two genes that upregulate Sh, *hyperkinetic* (*Hk*) and *quiver*/*sleepless* (*qvr*/*sss*), result in reduced sleep in flies [5-7]. Unlike Hk, which is a cytosolic protein, SSS is anchored by glycosylphosphatidylinositol (GPI) to the outer leaflet of the plasma membrane, where it may associate with the extracellular surfaces of other membrane proteins. Evidence suggests that one such protein is Sh itself. For example, SSS and Sh are expressed in many of the same regions of the fly brain; SSS can form a stable complex with and upregulate levels of Sh protein; and SSS can increase the activation kinetics and decrease C-type inactivation kinetics of Sh channels in excised patches of transfected cells [6-8].

However, differences between the sleep phenotypes of *Sh* and *qvr*/*sss* mutants suggest that the two genes may not act in exactly the same signaling pathways. For example, over several generations selection pressure appears to favor the accumulation of genetic modifiers in populations of *Sh* mutants to compensate for loss of sleep [4]. In contrast, loss of sleep in *qvr*/*sss* null mutants is not lost over the same time span, if at all (unpublished data), signifying that the *qvr*/*sss* phenotype cannot be easily overcome by other naturally occurring alleles in the genome. Furthermore, although *Sh* mutants exhibit homeostatic recovery sleep following periods of sleep deprivation [4], this process is impaired in *qvr*/*sss* mutants [6]. Collectively, these differences suggest that SSS may have other downstream effectors, which, in combination with Sh, control sleep.

Clues about the identities of such effectors may be gleaned from the predicted structure of SSS. Modeling of the tertiary structure reveals that SSS is a member of a large family of proteins, including snake a-neurotoxins, which possess a “three-finger” fold that has also been referred to as the ly6 domain [7]. Intriguingly, many of the targets of a-neurotoxins have been identified as ion channels or acetylcholine signaling pathways. For example, FS-2 blocks voltage-gated Ca channels [9]; fasciculin-I blocks acetylcholinesterase [10]; MT2 acts on muscarinic acetylcholine receptors [11]; and abungarotoxin inhibits nicotinic acetylcholine receptors (nAChRs) [12]. Although ly6 domain-encoding homologs of *qvr*/*sss* have also been identified in the genomes of

nonvenomous animals [13, 14], in most cases the endogenous binding partners of the corresponding proteins have not been identified.

In mammals, however, evidence strongly suggests that two of these proteins, lynx1 and lynx2, are endogenous antagonists of nAChRs. For example, lynx1 and lynx2 can complex with and accelerate the desensitization rates of α4β2 nAChRs [15, 16]. Consistent with these effects, *lynx1* knockout mice have extended critical periods for ocular dominance plasticity that requires nAChR activity [17], and *lynx2* knockout mice have increased nicotine-evoked EPSPs in prefrontal cortical pyramidal neurons [18].

As a result of such studies, we hypothesized that the ly6 domain-containing protein SSS may also antagonize nAChRs to reduce synaptic transmission. Here we present evidence to support this hypothesis and demonstrate that the resulting receptor inhibition is required for normal sleep in *Drosophila*. Specifically, we show that genetic reduction of nAChR signaling restores sleep to *qvr*/*sss* mutants. We also show that SSS regulates nAChRs post-transcriptionally. Finally, we show that the SSS, Dα3, and Sh all co-express in the same region of the brain suggesting a cell-autonomous role for SSS acting as a bifunctional regulator of neuronal activity. Collectively our data suggest the existence of a dual role for SSS-like molecules in reducing excitability and synaptic transmission to control essential nervous system functions like sleep.

6.3 Results

6.3.1 *qvr*/*sss* genetically interacts with nAChR subunits to control sleep

After determining that acute antagonism of nAChR activity could restore sleep to sss^{P1} mutants (data not shown) [19], we asked which nAChR subunits might be aberrantly upregulated in the absence of SSS. To address this question we first determined which nAChR subunits are expressed in the adult fly brain. In mammals, nAChRs are homopentamers of alpha subunits or heteropentamers of alpha and beta subunits. In the fly genome there are 7 genes encoding alpha subunits (*Dα1-7*) and 3 genes encoding beta subunits (*Dβ1-3*). The combinations of receptor subunits that can form functional channels in flies is unknown, largely because it has been difficult to measure activity of cloned fly nAChRs in heterologous expression systems [20]. Using quantitative PCR (qPCR) to measure the levels of nAChR transcripts, we found that all but one subunit is enriched in the adult fly brain relative to the body (Figure 6.2A).

To determine which of these subunits might be responsible for the increased waking time of *qvr/sss* mutants, we reduced expression of each alpha or beta subunit in *sss*-expressing neurons by RNAi knockdown. Consistent with our hypothesis that nAChR activity is upregulated in *qvr*/*sss* mutants, knockdown of *Dα3* in particular and to a lesser extent, *Dβ3*, partly restored sleep to sss^{P1} mutants (Figure 6.1A,B) but had no effect on sleep in control animals with normal levels of *qvr*/*sss* expression (Figure 6.2B). qPCR analysis of *Dα3* transcripts from heads of pan-neuronal RNAi knockdown flies

confirms a ~65% reduction in *Dα3* expression levels (Figure 6.2C). These results suggest that excessive nAChR activity may be at least partly responsible for the increased waking time of sss^{P1} mutants. These results also indicate that nAChR activity does not normally need to be reduced by withdrawal of endogenous cholinergic signaling to allow wild-type animals to sleep.

To determine whether upregulation of nAChR activity in *qvr*/*sss*-expressing neurons could reduce sleep, we coupled *sss*-Gal4 to a P-element (d08339) inserted ~40 bp upstream of the predicted transcriptional start site of the *Dα3* gene. Since this P-element carries UAS sequences pointing in both directions along the X chromosome, animals bearing it in combination with *sss*-Gal4 should express elevated levels of *Dα3* transcript. When we tested these animals we found that they expressed very high levels of *Dα3* mRNA and slept less than controls (Figures 6.2D, 6.1C). Thus, upregulation of Dα3 nAChRs in *qvr*/*sss*-expressing neurons is sufficient to account for at least part of the low-sleeping phenotype of sss^{P1} mutants.

6.3.2 nAChRs are post-transcriptionally regulated by SSS and co-localize with SSS and Sh in the mushroom bodies

To determine if SSS can indirectly regulate nAChRs by transcriptional feedback, we performed qPCR on Dα3 and Dβ3, two of the nAChR subunits that genetically interacted with *qvr/sss* (Figure 6.1A,B). Just as we previously showed for regulation of *Sh* transcript [7], levels of fly brain nAChR transcripts

are unchanged in *sss*^{P1} mutants. We also found that fly brain nAChR transcripts are similarly unchanged in *Sh*^{mns} mutants (Figure 6.3A). Collectively, these data support a post-transcriptional role for regulation of nAChR activity by SSS, although we cannot exclude the possibility that upregulation of nAChR transcript in *qvr/sss* mutants occurs in a small subset of the neurons in our whole brain preparations and is therefore undetectable in our assays.

If, however, SSS is acting directly on both K channels and nAChRs to suppress neuronal activity and promote sleep, then SSS and both effector proteins should be expressed in the same set of neurons. To determine if this is indeed the case, we labeled *qvr/sss*-expressing neurons with UAS-*tdTomato* expressed under the control of *sss*-Gal4. In the same brains we also stained for the presence of Sh and Dα3 using established antibodies for each molecule [7, 21]. We found overlapping expression of all 3 molecules in the mushroom bodies (MBs) (Figure 6.3B), which others and we previously showed to be important sleep-regulatory loci [22, 23]. Our cell labeling was likely to be specific for the intended antigens since Sh labeling disappeared in Sh mutants [7]; Dα3 labeling was significantly reduced when antibody was pre-incubated with lysate from COS cells transfected with Dα3 cDNA (Figure 6.4A-C); and Dα3 labeling increased in brains of animals in which *sss*-Gal4 was combined with UAS-*Dα3* (i.e. P-element d08339) (Figure 6.4D). Thus, the sleep-regulating proteins SSS, Sh and Dα3 are expressed together in a known sleep-regulating locus in the fly brain.

To determine whether the MBs might contribute to sleep regulation by *qvr/sss*, we first coupled the *sss*-Gal4 driver to UAS-*sss* in a sss^{P1} mutant background. As we previously demonstrated [7], we found that the *qvr/sss* transgene was capable of fully rescuing the loss of sleep in sss^{P1} mutants. However, when we blocked expression of the *qvr/sss* transgene with MB-Gal80, a repressor of Gal4 activity that expresses in the MBs, restoration of sleep to otherwise genetically identical animals was reduced (Figure 6.3C). These data suggest that *qvr/sss* utilizes the MBs to regulate sleep, though they do not exclude the possibility that other brain regions may also be involved.

6.4 Discussion

We previously demonstrated that SSS couples Sh levels and gating kinetics to reduced membrane excitability to allow sleep in *Drosophila*. In this study, we show that SSS interacts with and antagonizes nAChRs to promote sleep as well, and that the activity of *sss*-expressing neurons is both necessary and sufficient for this process in *Drosophila*. The molecular bi-functionality of SSS is unexpected since K channels and nAChRs are functionally and structurally unrelated. For example, Sh-type channels are gated by voltage, have 6 transmembrane domains and multimerize to form functional tetramers. In contrast, nAChRs are gated by synaptic release of acetylcholine, have 4 transmembrane domains and multimerize to form functional pentamers.

It is unclear which structural features SSS recognizes on each class of membrane protein, especially in the case of Sh, which is thought to expose little surface area outside the plasma membrane. X-ray structures of a-cobtratoxin (a-Cbtx) bound to *Lymnaea stagnalis* acetylcholine binding protein (LS-AChBP) show interactions between two loops of a-Cbtx and both the agonist binding pocket and the cis-loop of AChBP [24]. Using this information and an NMR structure of water-soluble lynx1, Lyukmanova et. al, modeled a possible interaction of lynx1 with the same site, although the loops of lynx1 were shorter than those of a-Cbtx, and did not form as many contacts with the AChBP [25].

It is also unclear whether such interactions, even if translated to SSS, would result in acute and direct antagonism of nAChR activity or, alternatively, reduction in targeting of receptors at the cell surface. Both potential mechanisms of action could account for the reduced α4/β2 activity we observed in cells transfected with *qvr*/*sss* [19]. In addition, it is unknown whether a single SSS molecule can interact with both Sh and nAChRs simultaneously. However, our data suggest that all three proteins are co-expressed in some regions of the brain, particularly the mushroom bodies, which others and we have shown to play an important role in controlling sleep [22, 23, 26]. We previously showed that SSS is enriched in these structures as well as the antennal nerves, superior protocerebrum and the lobula plate of the optic lobes [7], and here we show that MB-Gal80 reduces the ability of *sss*-Gal4>UAS-*sss* to restore sleep to sss^{P1} mutants. Nonetheless, the contribution

of the MBs relative to other brain loci in regulating sleep via SSS, Sh and Dα3 still needs to be determined.

A particularly intriguing feature of SSS is its ability to reduce both membrane excitability and synaptic transmission, which endows the protein with unusual gain control over neuronal activity. In the present context we propose that SSS reduces the activity of wake-promoting neurons through two pathways to permit sleep (Figure 6.5). In one, it enhances Sh K channel protein levels and open channel probability to reduce neuronal excitability. In another pathway, it inhibits nAChR signaling to reduce synaptic transmission. In *qvr/sss* mutants the processes are reversed in wake-promoting neurons: K channel activity is reduced, leading to increased excitability, and nAChR activity is increased, leading to increased synaptic transmission. In these as well as *Sh* mutants, reduction of nAChR expression by RNAi knockdown or of nAChR activity by pharmacological antagonism reduces the activity of wake-promoting neurons within a range in which sleep can once again occur (Figure 6.5).

In summary, we have identified a role for SSS in regulating synaptic transmission, in addition to its established role in regulating membrane excitability. Both functions appear to be important for the ability of SSS to regulate sleep in *Drosophila*. In this study, we demonstrated the ability of lynx1 to substitute for SSS as well as to form complexes with effectors of SSS, namely Sh-type K channels and nAChRs, suggests that lynx1 and perhaps other mammalian ly6 proteins possess similar, multifunctional roles in

controlling neuronal activity. Potassium channels, nAChRs and their modulators are key regulators or targets for molecular intervention in various human disorders, including ataxias, congenital deafness, epilepsy, cardiac arrhythmias, type II diabetes, autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, cognitive decline in Alzheimer's Disease, loss of motor coordination in Parkinson's Disease, nicotine addiction and its associated risk of developing cancer and cardiovascular disease from smoking [27-33]. Understanding the functions of ly6 proteins may provide insights into these disorders as well as new screening strategies for more selective and efficacious pharmacotherapeutic regulators of neuronal function.

6.5 Methods

6.5.1 Fly stocks and transgenic fly lines

sss^{P1} , *sss*-Gal4 and UAS-*sss* flies were described previously [6, 7]. UAS-*Dα3* (stock d03389) was from the Exelixis collection at Harvard. Other fly lines were obtained from the Bloomington Stock Center (UAS-*dicer* [24650]; UAS-*tdTomato* [32221]; UAS-nAChR RNAi's [28688, 27493, 27671, 31985, 25943, 25835, 27251, 31883, 28038 and 25927]). UAS-*lynx1* flies were generated by targeting UAS-*lynx1* in pUAST-attB to the attP site of y^{1},w^{67c23};;attP2 flies (Rainbow Transgenics, Camarillo, CA) and outcrossing transgenic animals into a w^{1118} iso31 background for 2 generations.

6.5.2 Behavior assays

1-5 day old flies were loaded into glass tubes containing 5% sucrose and 2% agarose and entrained to a 12hr:12hr light:dark (LD) cycle for 2 days before measuring sleep/wake patterns using the *Drosophila* Activity Monitoring System (Trikinetics). Sleep was defined as 5 minutes of inactivity and measured as previously described [34]. Experiments were carried out at 25ºC.

6.5.3 Quantitative PCR

For each sample, 30-50 brains or 7-12 heads from 5-9 day old flies were lysed in Trizol (Life Technologies), and first strand cDNA was synthesized from extracted RNA using High Capacity cDNA Reverse Transcription (ABI). Quantitative PCR was performed on each cDNA sample using the primers listed in Table 6.1, and results were normalized to levels measured for RP49. Relative expression was further normalized to levels of *Dα3* transcript measured in w^{1118} controls. All primer pairs were validated for amplification efficiencies (R^2) greater than 0.98.

6.5.4 Molecular biology

We generated UAS-*lynx1* by subcloning the previously described full-length mouse EST into pUAST-attB between the EcoRI and NotI sites.

6.5.5 Immunohistochemistry

3-7 day old female brains were dissected in ice cold PBS before fixation in 4% paraformaldehyde. Brains were blocked in PBST (PBS, 0.3% Triton X-

100) containing 5% normal donkey serum (Jackson Laboratory) and 5% normal goat serum (Life Technologies). Brains were incubated with 1:200 rat anti-Sh [7] pre-cleared with Sh^{Df} lysate followed sequentially by incubation with 1:250 rabbit anti-Dα3 bleed 88 [21]. Brains were washed with PBST and stained with 1:1000 Alexa 633 anti-rabbit (Life Technologies) and 1:1000 anti-rat Alexa 488 (Jackson ImmunoResearch). After additional washes, brains were equilibrated and mounted in Vectashield (Vector Labs). Images were taken at 40X magnification on a Leica SP5 confocal microscope using 0.5μm stack intervals. 5μm Z-projection images were generated, rotated and brightness/contrast adjustments were made across the entirety of the images using Fiji [35].

6.6 Acknowledgements

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6.7 Figures

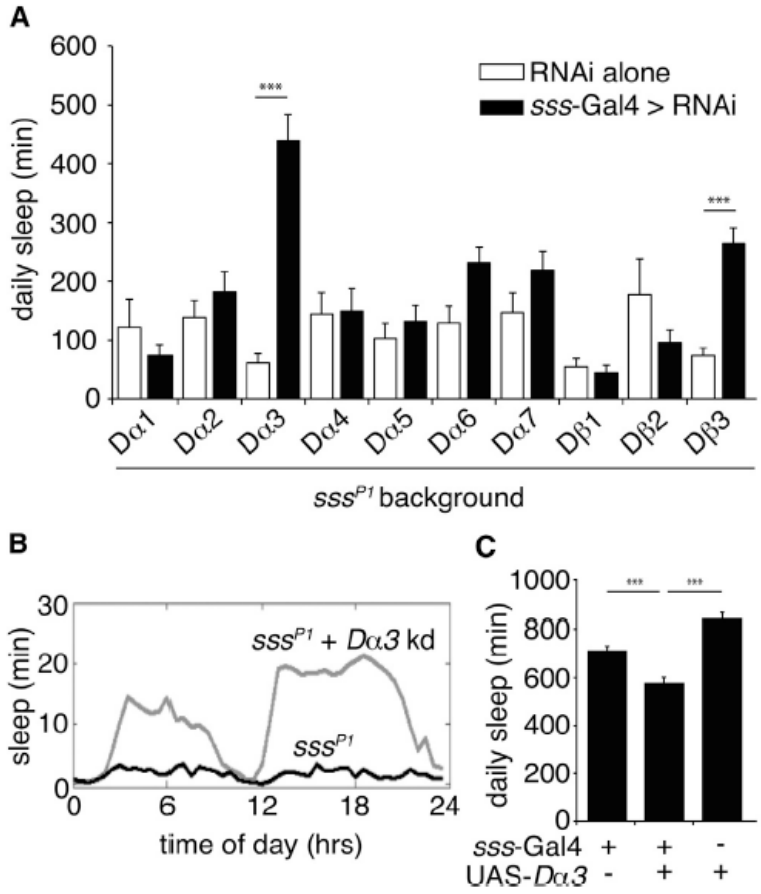

Figure 6.1 RNAi knockdown of nAChR subunits restores sleep to *qvr/sss* mutants. (**A**) Sleep in *sss*-Gal4/+;UAS-RNAi/UAS-*dicer* animals compared to UAS-RNAi/+ controls (all in a sss^{P1} background). The low sleeping phenotype of *sss* mutants is rescued by knockdown of *Dα3* and to a lesser extent *Dβ3*. N ≥ 26 for each group. (**B**) 24 hr sleep profiles of sss^{P1} alone or *sss* animals in which *Da3* has been knocked down in *sss* neurons. (**C**) Overexpression of *Dα3* in *sss* neurons reduces total daily sleep. N ≥ 15 for each group. *** $p<0.001$ by one-way ANOVA with Bonferroni post-test. Error bars indicate s.e.m.

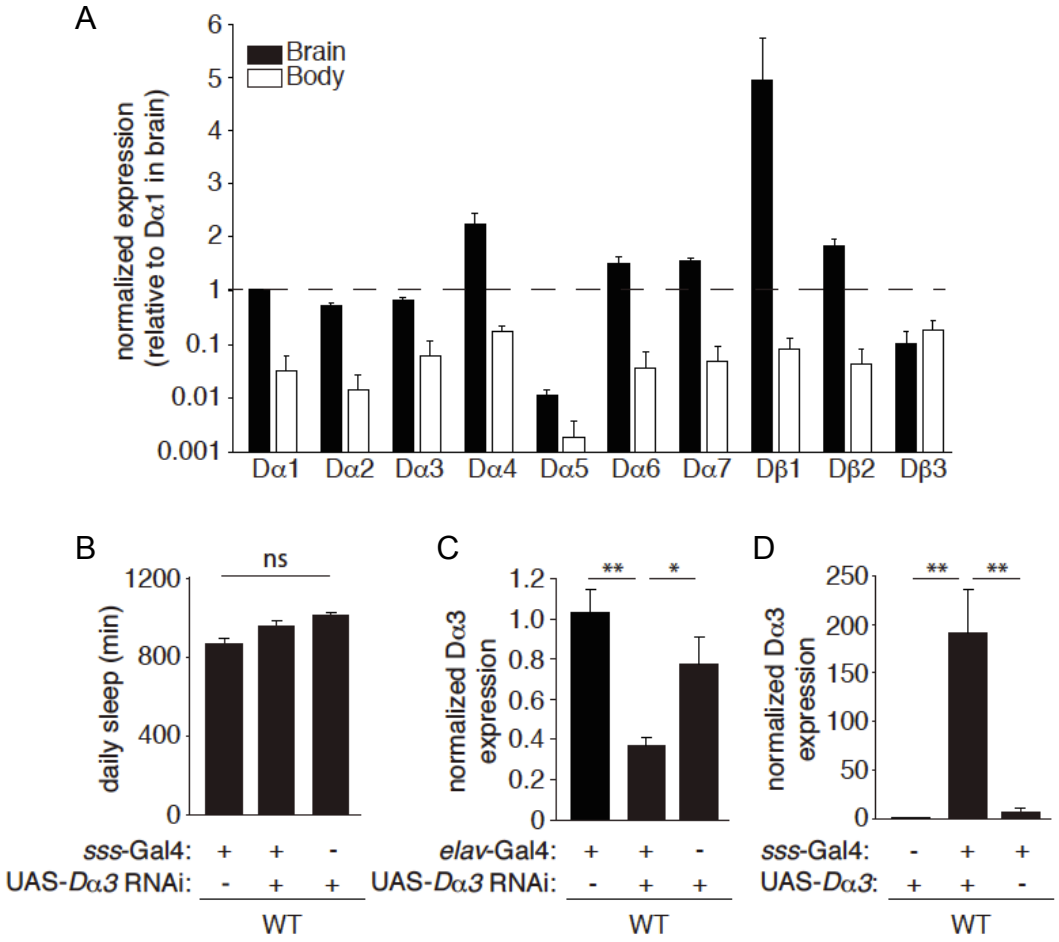

Figure 6.2 qPCR analysis of nAChR subunit levels and manipulation of *Dα3* expression. (**A**) nAChR subunit levels in adult fly brains (black bars) compared to bodies (white bars). All values have been normalized to expression of Dα1 in brain. N = 3 for each sample. (**B**) Daily sleep is unchanged in *Dα3* RNAi knockdown animals compared to controls. N = 16 for each group. (**C**) qPCR analysis of *Dα3* transcripts from heads of pan-neuronal RNAi knockdown animals compared to genetic controls. N ≥ 6 for each group. (**D**) qPCR analysis of *Dα3* transcripts from heads of *sss*-Gal4>UAS-*Dα3* (d08339) compared to genetic controls. N ≥ 3 for each group. ns, not significant; * $p < 0.05$; ** $p < 0.01$ by one-way ANOVA with Bonferroni post-test. Error bars indicate s.e.m. UAS-*Dicer* is present in A-C.

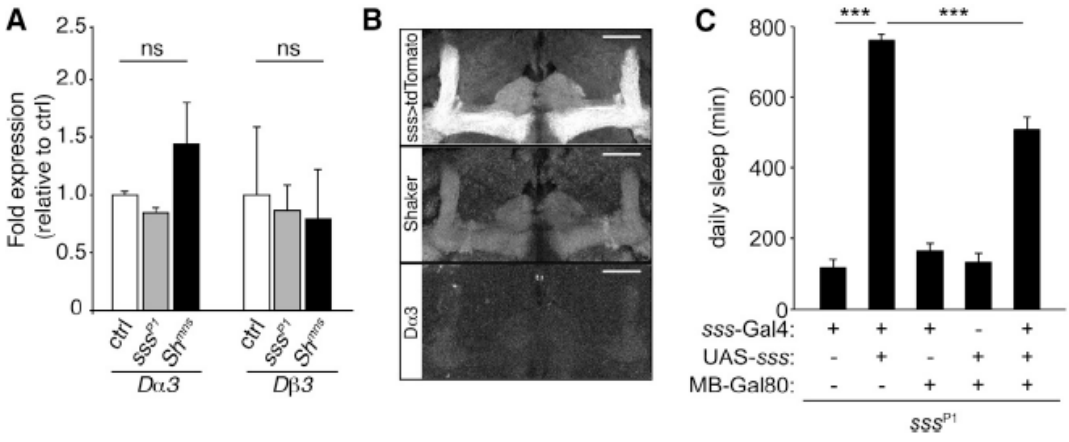

Figure 6.3 *Dα3* and *Dβ3* are not transcriptionally regulated by SSS or Sh but co-express with them in the mushroom bodies. (**A**) Quantitative PCR analyses of *Dα3* and *Dβ3* transcripts show no dependence on *qvr*/*sss* or *Sh* for normal expression. N = 3 for each group. ns, not significant by one-way ANOVA with Bonferroni post-test. Error bars indicate s.e.m. (**B**) Coexpression of *sss*, Sh and Da3 in the mushroom bodies. Representative immunostaining (N = 8) of tdTomato expressed under the control of *sss*-Gal4 (upper panel), native Sh protein (center panel), and native Dα3 protein (lower panel). Scale bar = 25mm. (C) MB-Gal80 suppresses rescue of sleep by *sss*-Gal4>UAS-*sss* in sss^{P1} mutants. N ≥ 30 for all groups. *** $p<0.001$ by one-way ANOVA with Bonferroni post-test. Error bars indicate s.e.m.

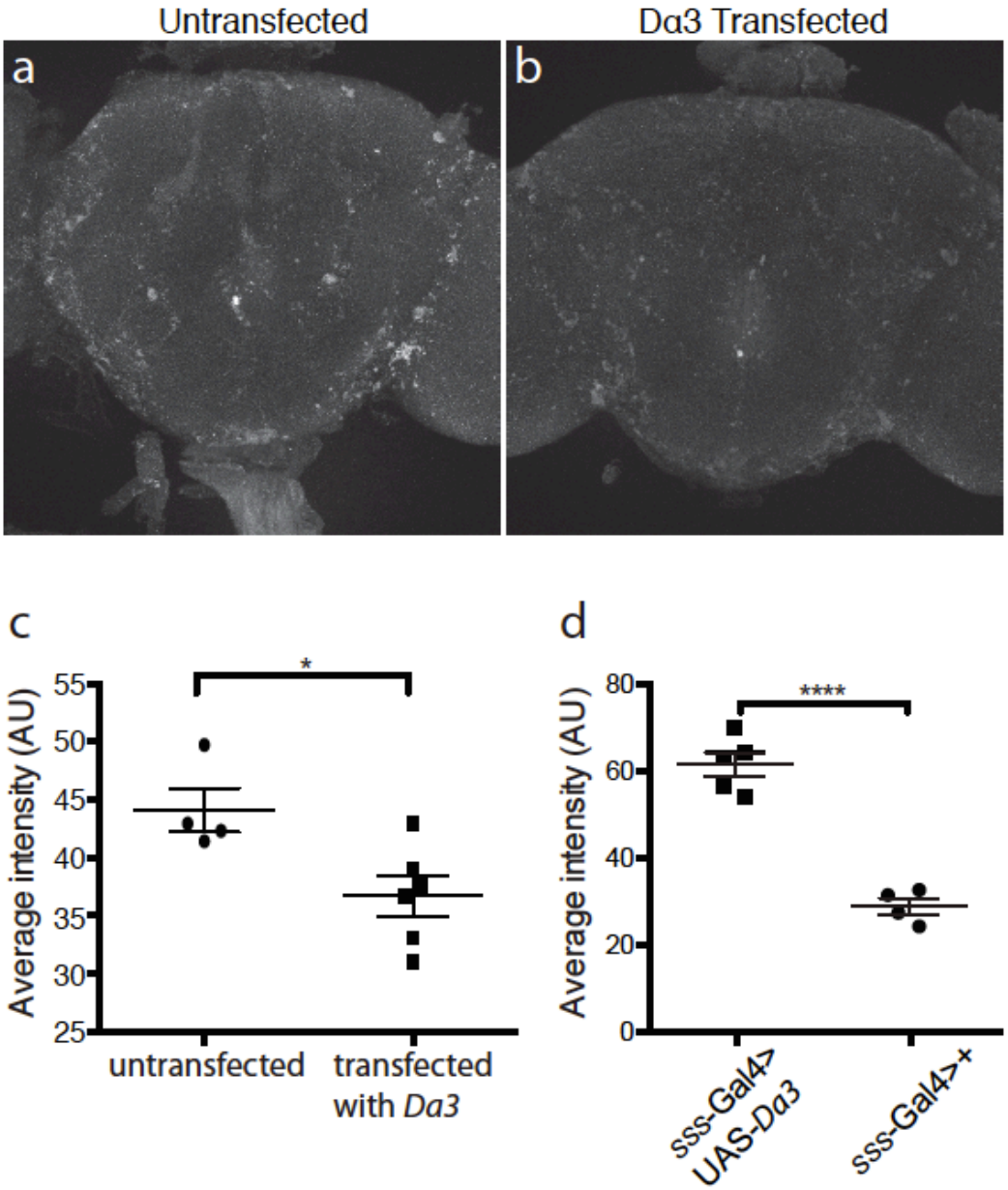

Figure 6.4 Quantification of staining in fly brains shows that Dα3 antibody is specific for Dα3 protein. (**A**) Dα3 antibody was pre-incubated with cell lysate from untransfected COS cells prior to staining. (**B**) Dα3 antibody was pre-adsorbed to cell lysate from HEK cells transfected with *Dα3* cDNA prior to staining. (**C**,**D**) Quantitation of integrated whole-brain pixel intensity following Dα3 staining of wild-type flies in A and B (C) or in *sss*-Gal4>UAS-*sss* flies (D). Anti-rabbit Alexa 568 was used as a secondary antibody for a-d. Laser intensity and gain were kept constant between pairs. * $p < 0.05$; **** $p < .001$ by unpaired t-test.

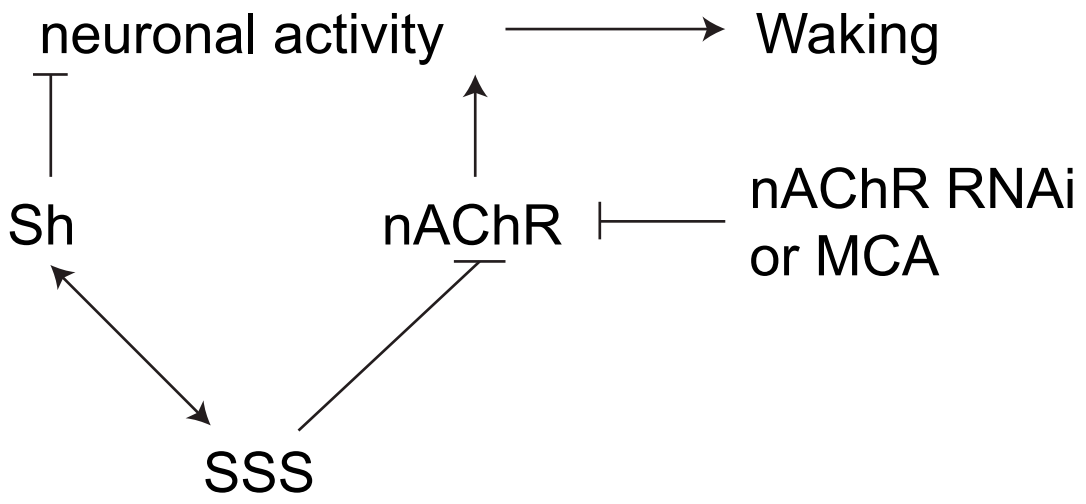

Figure 6.5 Model for control of sleep by SSS in wake-promoting neurons. SSS reduces neuronal activity in arousal-promoting neurons through two pathways to permit sleep. SSS enhances Sh K channel activity to reduce intrinsic excitability (left side). SSS also suppresses nAChR activity to reduce synaptic transmission (right side). Pharmacological or genetic antagonism of nAChR activity functionally substitutes for SSS to restore sleep to *sss*^{P1} mutants (right side). Bi-directional arrow (left side) reflects mutual dependence of Sh and SSS for elevated expression levels of both proteins.

Table 6.1 Primers used for qPCR.

Primer	Sequence
nAcRalpha-7e-QF	5' - GTGCAGAGGGAAATGAGATG - 3'
nAcRalpha-7e-QR	5' - CGGTCGTACCAGTTTGTTGTAG - 3'
nAcRalpha-18C-QF	5' - TGGCAGAATGCTGGTCTATG - 3'
nAcRalpha-18C-QR	5' - GCTCCAGGCTGTTGTAGTTG - 3'
nAcRbeta-21C-QF	5' - AACGTGTCCCTGGAAATGG - 3'
nAcRbeta-21C-QR	5' - TATTGTGACGGTTGCCACAC - 3'
nAcRalpha-30D-QF	5' - AACAAGCCAAGGACATGGAC - 3'
nAcRalpha-30D-QR	5' - TAGGTGGACAGCAGATGGTTC - 3'
nAcRalpha-34E-QF	5' - CAGCAGCACGCAAATATTAAAC - 3'
nAcRalpha-34E-QR	5' - GGGATCCAAAAGATCGTGTAAC - 3'
nAcRbeta-64B-QF	5' - AGCCATGTCCCTGGAGTAAG - 3'
nAcRbeta-64B-QR	5' - ACCAAGCGCTCTTCATCTTC - 3'
nAcRalpha-80B-QF	5' - GCACATGGATGAACAACAGG - 3'
nAcRalpha-80B-QR	5' - GTATGGTTCGTCGCAACAAG - 3'
nAcRbeta-96A-QF	5' - TGTGGCATTGGAGTTTGTTG - 3'
nAcRbeta-96A-QR	5' - CACAACGGGCCTAATCAATC - 3'
nAcRalpha-96Aa-QF	5' - GCAACTACAATCGCCTCATC - 3'
nAcRalpha-96Aa-QR	5' - TCGTTCCATTCCTGTTCCAC - 3'
nAcRalpha-96Ab-QF1	5' - AACAACTCGGACCGTCTCAC - 3'
nAcRalpha-96Ab-QR	5' - TCGGATGGCACATAAAGCTC - 3'
rp49-QF	5' - CAAGATCGTGAAGAAGCG - 3'
rp49-QR	5' - GTTGGGCATCAGATACTGTC - 3'

6.8 References

1. Agosto, J., Choi, J., Parisky, K., Stilwell, G., Rosbash, M., and Griffith, L. (2008). Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in Drosophila. Nat Neurosci *11*, 354-359.

2. Douglas, C.L., Vyazovskiy, V., Southard, T., Chiu, S.Y., Messing, A., Tononi, G., and Cirelli, C. (2007). Sleep in Kcna2 knockout mice. BMC Biol *5*, 42.

3. Lancel, M. (1999). Role of GABAA receptors in the regulation of sleep: initial sleep responses to peripherally administered modulators and agonists. Sleep *22*, 33-42.

4. Cirelli, C., Bushey, D., Hill, S., Huber, R., Kreber, R., Ganetzky, B., and Tononi, G. (2005). Reduced sleep in Drosophila Shaker mutants. Nature *434*, 1087-1092.

5. Bushey, D., Huber, R., Tononi, G., and Cirelli, C. (2007). Drosophila Hyperkinetic mutants have reduced sleep and impaired memory. J Neurosci *27*, 5384-5393.

6. Koh, K., Joiner, W.J., Wu, M.N., Yue, Z., Smith, C.J., and Sehgal, A. (2008). Identification of SLEEPLESS, a sleep-promoting factor. Science *321*, 372-376.

7. Wu, M., Joiner, W., Dean, T., Yue, Z., Smith, C., Chen, D., Hoshi, T., Sehgal, A., and Koh, K. (2010). SLEEPLESS, a Ly-6/neurotoxin family member, regulates the levels, localization and activity of Shaker. Nat Neurosci *13*, 69-75.

8. Dean, T., Xu, R., Joiner, W., Sehgal, A., and Hoshi, T. (2011). Drosophila QVR/SSS modulates the activation and C-type inactivation kinetics of Shaker K(+) channels. J Neurosci *31*, 11387-11395.

9. Yasuda, O., Morimoto, S., Jiang, B., Kuroda, H., Kimura, T., Sakakibara, S., Fukuo, K., Chen, S., Tamatani, M., and Ogihara, T. (1994). FS2. a mamba venom toxin, is a specific blocker of the L-type calcium channels. Artery *21*, 287-302.

10. Dajas, F., Bolioli, B., Castello, M.E., and Silveira, R. (1987). Rat striatal acetylcholinesterase inhibition by fasciculin (a polypeptide from green mamba snake venom). Neurosci Lett *77*, 87-91.

11. Toomela, T., Jolkkonen, M., Rinken, A., Jarv, J., and Karlsson, E. (1994). Two-step binding of green mamba toxin to muscarinic acetylcholine receptor. FEBS Lett *352*, 95-97.

12. Changeux, J.P., Kasai, M., and Lee, C.Y. (1970). Use of a snake venom toxin to characterize the cholinergic receptor protein. Proc Natl Acad Sci U S A *67*, 1241-1247.

13. Galat, A. (2011). Common structural traits for cystine knot domain of the TGFbeta superfamily of proteins and three-fingered ectodomain of their cellular receptors. Cell Mol Life Sci *68*, 3437-3451.

14. Hijazi, A., Masson, W., Auge, B., Waltzer, L., Haenlin, M., and Roch, F. (2009). boudin is required for septate junction organisation in Drosophila and codes for a diffusible protein of the Ly6 superfamily. Development *136*, 2199-2209.

15. Miwa, J.M., Ibanez-Tallon, I., Crabtree, G.W., Sanchez, R., Sali, A., Role, L.W., and Heintz, N. (1999). lynx1, an endogenous toxin-like modulator of nicotinic acetylcholine receptors in the mammalian CNS. Neuron *23*, 105-114.

16. Ibanez-Tallon, I., Miwa, J.M., Wang, H.L., Adams, N.C., Crabtree, G.W., Sine, S.M., and Heintz, N. (2002). Novel modulation of neuronal nicotinic acetylcholine receptors by association with the endogenous prototoxin lynx1. Neuron *33*, 893-903.

17. Morishita, H., Miwa, J.M., Heintz, N., and Hensch, T.K. (2010). Lynx1, a cholinergic brake, limits plasticity in adult visual cortex. Science *330*, 1238-1240.

18. Tekinay, A.B., Nong, Y., Miwa, J.M., Lieberam, I., Ibanez-Tallon, I., Greengard, P., and Heintz, N. (2009). A role for LYNX2 in anxiety-related behavior. Proc Natl Acad Sci U S A *106*, 4477-4482.

19. Wu, M., Robinson, J.E., and Joiner, W.J. (2014). SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission. Curr Biol *24*, 621-629.

20. Lansdell, S.J., Collins, T., Goodchild, J., and Millar, N.S. (2012). The Drosophila nicotinic acetylcholine receptor subunits Dalpha5 and Dalpha7 form functional homomeric and heteromeric ion channels. BMC Neurosci *13*, 73.

21. Chamaon, K., Smalla, K.H., Thomas, U., and Gundelfinger, E.D. (2002). Nicotinic acetylcholine receptors of Drosophila: three subunits encoded

by genomically linked genes can co-assemble into the same receptor complex. J Neurochem *80*, 149-157.

22. Joiner, W.J., Crocker, A., White, B., and Sehgal, A. (2006). Sleep in Drosophila is regulated by adult mushroom bodies. Nature *441*, 757-760.

23. Pitman, J.L., McGill, J.J., Keegan, K.P., and Allada, R. (2006). A dynamic role for the mushroom bodies in promoting sleep in Drosophila. Nature *441*, 753-756.

24. Bourne, Y., Talley, T.T., Hansen, S.B., Taylor, P., and Marchot, P. (2005). Crystal structure of a Cbtx-AChBP complex reveals essential interactions between snake alpha-neurotoxins and nicotinic receptors. EMBO J *24*, 1512-1522.

25. Lyukmanova, E.N., Shenkarev, Z.O., Shulepko, M.A., Mineev, K.S., D'Hoedt, D., Kasheverov, I.E., Filkin, S.Y., Krivolapova, A.P., Janickova, H., Dolezal, V., Dolgikh, D.A., Arseniev, A.S., Bertrand, D., Tsetlin, V.I., and Kirpichnikov, M.P. (2011). NMR structure and action on nicotinic acetylcholine receptors of water-soluble domain of human LYNX1. J Biol Chem *286*, 10618-10627.

26. Yuan, Q., Joiner, W.J., and Sehgal, A. (2006). A sleep-promoting role for the Drosophila serotonin receptor 1A. Curr Biol *16*, 1051-1062.

27. Kass, R.S. (2005). The channelopathies: novel insights into molecular and genetic mechanisms of human disease. J Clin Invest *115*, 1986-1989.

28. Ashcroft, F.M. (2006). From molecule to malady. Nature *440*, 440-447.

29. Robbins, C.A., and Tempel, B.L. (2012). Kv1.1 and Kv1.2: similar channels, different seizure models. Epilepsia *53 Suppl 1*, 134-141.

30. Lam, J., and Wulff, H. (2011). The Lymphocyte Potassium Channels Kv1.3 and KCa3.1 as Targets for Immunosuppression. Drug Dev Res *72*, 573-584.

31. Quik, M., and Wonnacott, S. (2011). alpha6beta2* and alpha4beta2* nicotinic acetylcholine receptors as drug targets for Parkinson's disease. Pharmacol Rev *63*, 938-966.

32. Picciotto, M.R., and Zoli, M. (2008). Neuroprotection via nAChRs: the role of nAChRs in neurodegenerative disorders such as Alzheimer's and Parkinson's disease. Front Biosci *13*, 492-504.

33. Picciotto, M.R., and Kenny, P.J. (2013). Molecular mechanisms underlying behaviors related to nicotine addiction. Cold Spring Harb Perspect Med *3*, a012112.

34. Shaw, P.J., Cirelli, C., Greenspan, R.J., and Tononi, G. (2000). Correlates of sleep and waking in Drosophila melanogaster. Science *287*, 1834-1837.

35. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods *9*, 676-682.