Title

Sleep deprivation drives diverse changes in synaptic scaling in the Drosophila brain

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Publication Date

2022

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Sleep deprivation drives diverse changes in synaptic scaling in the *Drosophila brain*

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy in

Neuroscience

by

Jacqueline Taylor Weiss

2022

ABSTRACT OF THE DISSERTATION

Sleep deprivation drives diverse changes in synaptic scaling in the *Drosophila brain*

by

Jacqueline Taylor Weiss

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2022

Professor Jeffrey Michael Donlea, Chair

While sleep is conserved across evolution, the function or functions of sleep remain unknown. Notably, sleep loss impairs learning and memory throughout the animal kingdom. Previous work strongly suggests that sleep is required for maintenance of neural and behavioral plasticity, although mechanisms remain elusive. In this body of work, I present two studies (Chapters 2, 3) that characterize cell-type-specific changes in synapse abundance after sleep deprivation, first in the *Drosophila* Mushroom bodies (MB), an associative learning center. Then, I describe neurotransmitter cell type specific changes in synapse abundance throughout the entire central brain. The first study is published in *Current Biology*, while the second study will soon be submitted to *eLife*. Additionally, Chapter 1 is published in *Frontiers in Behavioral Neuroscience*.

In Chapter 1, I introduce roles for sleep in the maintenance of neural and behavioral plasticity in the contexts of development and memory acquisition and consolidation. Then, I discuss individual variations in response to sleep loss due to intrinsic and environmental factors. In Chapter 2, I show that sleep loss results in a net increase in synapse abundance in the MB using a genetic reporter of the presynaptic active zone protein Bruchpilot (BRP). Additionally,

sleep induction reduces BRP abundance throughout the MB lobes. For the first time, I then characterize cell-type-specific changes in synaptic abundance throughout the MB, finding that increased BRP abundance can be attributed to cholinergic Kenyon cells (KCs). Finally, using a fluorescent reporter for synaptic contacts, I show that KC output connections are not uniformly scaled by sleep loss, but depend on the identity of the postsynaptic partner. These changes in synapse abundance and connectivity may underlie learning and memory deficits induced by sleep loss.

In Chapter 3, I examine unclear whether the same cell-type specific trends in plasticity that we observe in the memory encoding MBs can be generalized to other neuropil regions of the central brain. This study examines changes in BRP abundance after sleep loss in each neuropil region in different neurotransmitter cell types. Consistent with my findings in the MB, I find that sleep deprivation upscales excitatory cholinergic synapses throughout each neuropil region, whereas other neurotransmitter cell types are less sensitive to sleep loss. Notably, BRP abundance is similarly affected within each neuropil region for a given neurotransmitter class. Our results are consistent with previous findings that excitatory and inhibitory neurons can be differentially affected by sleep loss. Sleep deprivation may therefore disrupt excitatory/inhibitory balance (E/I balance) in the brain, which may underlie some of the cognitive and behavioral consequences of insufficient sleep.

In sum, this work seeks to elucidate alterations in synaptic plasticity after sleep loss in the *Drosophila* brain. As sleep is evolutionarily conserved, our findings may inform the function(s) of sleep in animal models and humans. Here, I find that sleep loss does not uniformly affect synapse abundance across cell types. Certain cell types are especially vulnerable to the effects of sleep deprivation, which may underlie consequences of prolonged waking.

The dissertation of Jacqueline Taylor Weiss is approved.

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Gina Rochelle Poe

Stephen Lawrence Zipursky

Jeffrey Michael Donlea, Committee Chair

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2022

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ACKNOWLEDGEMENTS

I want to first offer my most sincere thanks to my primary chair and mentor, Jeff Donlea, for his support and guidance over the past six years. I feel very fortunate to have had a kind, patient mentor who built a lab full of great people. In addition to his guidance, I appreciate the intellectual and research independence Jeff has fostered over the years, giving me a chance to make mistakes and figure things out on my own. I'd like to thank Jeff for what seems to be an uncommonly enjoyable PhD experience, with opportunities to answer interesting questions as well as the chance to build my life outside of lab. As Jeff's first graduate student, I am honored to have had the occasion to help shape the lab, and I am excited to see what the future holds for the Donlea lab.

I would also like to thank the other committee members who have supported my graduate school journey over the past few years. Firstly, I would like to thank Dr. Larry Zipursky for providing his expertise in *Drosophila* genetics and neurobiology, and for creation of the STaR tool, without which this work would not exist. I would also like to extend my gratitude to Dr. David Krantz for his expertise in *Drosophila* genetics and neurobiology, and to Dr. Gina Poe for her wealth of knowledge in the fields of sleep, plasticity, and learning/memory. They have all been crucial in guiding my work, and I feel so fortunate to have been surrounded by such supportive and enthusiastic mentors.

Members of the Donlea lab have been integral in cultivating a supportive environment and providing me with many opportunities to learn. Jaison Omoto was a wonderful friend and mentor who happily shared his wealth of knowledge of *Drosophila* anatomy and genetics. I deeply admire Jaison's curiosity, scientific rigor, and kindness. Prabhjit Singh always impressed me with his grasp of biology and his scientific prowess. I have enjoyed our hours-long conversations and the humor he brings to lab every day. Perhaps I should also thank Chick-Fil-

A, for giving us sustenance and feeding my friendship with Jaison and Prabhjit. I would also like to thank the rest of the Donlea lab, who have created a supportive and fun place to work.

My family has provided me with immense support over the course of not only my PhD, but my entire life. My parents fostered my curiosity and encouraged my interests from a young age, which has propelled me to where I am today. I would also like to thank my sister, Laura, for being an overall awesome person and putting up with a sister like me – especially since she'll have to call me "Doctor" now. I also must thank my fiancé, Ricky, for his unwavering love and support over the past few years. I am very fortunate to have a partner who is so fun and supportive, yet inspires me to challenge myself. Graduate school has not always been easy, especially during a global pandemic, but I have cherished every day with Ricky by my side. Finally, I have to thank my dog, Cookie, who has provided me with endless joy over the past two years.

With regard to the work included herein, I would like to acknowledge that Chapter 1 consists of a manuscript published in *Frontiers in Behavioral Neuroscience* in 2022 entitled, "Roles for sleep in neural and behavioral plasticity: reviewing variation in the consequences of sleep loss." This work was co-authored by Jeffrey M. Donlea, who has consented to the inclusion of this work in this dissertation. Chapter 2 consists of a manuscript published in *Current Biology* in 2021 entitled "Sleep deprivation results in diverse patterns of synaptic scaling across the *Drosophila* mushroom bodies." This work would not have been possible without the guidance of last author Jeffrey M. Donlea, who has consented to inclusion of this work. Finally, Chapter 3 consists of a manuscript that will soon be submitted to *eLife* entitled, "Sleep deprivation drives brain-wide changes in cholinergic presynapse abundance in *Drosophila melanogaster*." This work would not have been possible without the substantial contributions of co-author Mei Blundell, who developed image registration and analysis protocols, and last author Jeffrey M. Donlea. All authors consented to inclusion in this manuscript.

Finally, I would like to recognize the funding sources that made this work possible. That includes the Interdepartmental Neuroscience Program as well as Jeff Donlea's various grants (NIH NS105967, HFSP CDA00026-2017-C). I was also bestowed a Jessamine K. Hilliard UCLA Neurobiology Graduate Student Grant to help support my research.

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Weiss, J.T. & Donlea, J.M. (2021). Sleep deprivation results in diverse patterns of synaptic scaling across the Drosophila mushroom bodies. Current Biology, 31(15), 3248-3261.

Weiss, J.T. & Donlea, J.M. (2022). Roles for sleep in neural and behavioral plasticity: reviewing variation in the consequences of sleep loss. Frontiers in Behavioral Neuroscience, 15:777799.

SELECTED POSTERS AND PRESENTATIONS

Weiss, J.T., Blundell, M. & Donlea J.M. Sleep deprivation drives brain-wide changes in synaptic abundance. Cold Spring Harbor Laboratories; 2021 October; Cold Spring Harbor, NY.

Weiss, J.T. Sleep deprivation results in diverse patterns of synaptic scaling across the Drosophila mushroom bodies. UCLA Synapse to Circuits Club; 2021 June; Los Angeles, CA.

Weiss, J.T. & Donlea J.M. Mapping synaptic scaling in the mushroom body during sleep loss. Neurobiology of Drosophila. Cold Spring Harbor Laboratories; 2019 October; Cold Spring Harbor, NY.

Weiss, J.T., Gupta, V., Skorput, A., Simineri, N., Fan, J., Yeh, P. & Yeh, H. Bumetanide mitigates ethanol-induced aberrant migration of MGE-derived GABAergic interneurons in mouse embryonic dorsomedial telencephalon. 44th Annual Society for Neuroscience Meeting; 2014 November; Washington, D.C.

CHAPTER ONE

Roles for Sleep in Neural and Behavioral Plasticity

1.1: Introduction

Sleep is a physiological state that has been conserved across evolution, even noted in invertebrates lacking a centralized brain (Hendricks et al., 2000; Shaw et al., 2000; Zhdanova et al., 2001; Raizen et al., 2008; Singh et al., 2014; Nath et al., 2017). Although sleep's physiological functions remain poorly understood, sleep loss has been associated with deleterious effects on health and cognition (Rechtschaffen and Bergmann, 1995; Dinges et al., 1997; Durmer and Dinges, 2005; Spiegel et al., 2005; Banks and Dinges, 2007; Knutson et al., 2007; Grandner et al., 2010, 2014). Sleep varies based on previous waking experience (Ganguly-Fitzgerald et al., 2006; Huber et al., 2007; Hanlon et al., 2009; Keene et al., 2010; Beckwith et al., 2017; Kirszenblat et al., 2019; Milinski et al., 2021), throughout the lifespan (Kales et al., 1967; Feinberg and Carlson, 1968; Cauter et al., 2000; Backhaus et al., 2007; Dijk et al., 2010; Feinberg and Campbell, 2010; Carrier et al., 2011; Vienne et al., 2016; Mander et al., 2017), and between species (Lyamin et al., 2008, 2017, 2018; Siegel, 2008; Lesku et al., 2012), suggesting that sleep has multiple functions. However, because sleep coincides with broad changes in neurophysiology and necessitates a loss of consciousness with reduced responsiveness to external threats, it is likely that sleep evolved, at least in part, to support brain function (Rasch and Born, 2013; Tononi and Cirelli, 2014). Notably, sleep is often elevated during periods of synaptic reorganization, including early development (Roffwarg et al., 1966; Shaw et al., 2000; Kayser et al., 2014), recovery from neural injury (Singh and Donlea, 2020; Stanhope et al., 2020), and memory consolidation (Walker et al., 2002; Ganguly-Fitzgerald et al., 2006). These findings each suggest that sleep supports plastic remodeling in the brain. Synaptic plasticity allows behavioral flexibility in response to external stimuli, and enables the processing and storage of information (Hughes, 1958; Zucker and Regehr, 2002; Cooke and Bliss, 2006). However, the underlying cellular and molecular mechanisms that support plasticity during sleep remain an area of intense investigation.

The impacts of sleep loss, interestingly, vary widely depending on age, environmental conditions, and genotype. While organisms typically recover from acute sleep disruptions relatively quickly, early-life sleep disruptions can prevent developmental plasticity during critical periods and result in long-lasting changes in circuit connectivity and behavior (Frank et al., 2001; Seugnet et al., 2011; Kayser et al., 2014). Conversely, some individuals withstand sleep loss with few consequences depending on the physiological conditions or genetic factors (Viola et al., 2007, 2012; Lyamin et al., 2008; Keene et al., 2010; Donlea et al., 2012; Lesku et al., 2012). In some cases, sleep disruption even provides an opportunity to weaken maladaptive memories (Poe, 2017). Examining the variables that can influence an individual's sensitivity to sleep loss could provide new insights into the core mechanisms of sleep-dependent plasticity

1.2: Sleep supports plasticity during development

Synaptic plasticity plays a crucial role in brain development, especially in the refining of neural connectivity through the process of pruning (Paolicelli et al., 2011). Defects in synaptic pruning during development are thought to contribute to atypical circuit function seen in neurodevelopmental disorders (Paolicelli et al., 2011; Konopaske et al., 2014; Tang et al., 2014; Cossío et al., 2017; Kim et al., 2017; Neniskyte and Gross, 2017). Daily sleep amounts peak in many species early in development, when the brain is undergoing significant plastic changes (Roffwarg et al., 1966; Jouvet-Mounier et al., 1969; Shaw et al., 2000; Kayser et al., 2014). Studies in humans have found that sleep disruption during development is associated with severe and lasting consequences for behavior and cognition (O'Brien et al., 2004; Halbower et al., 2006; Ednick et al., 2009). While these human studies provide a correlational link between impaired sleep and later cognition, several lines of animal studies described below indicate conserved roles for sleep in neurodevelopment of several species and begin to identify possible mechanisms by which sleep might influence brain development.

Rapid eye movement (REM) sleep is thought to play a particularly important role in development. Infants spend as much as 50% of their time asleep in REM, compared to 25% in adults (Roffwarg et al., 1966; Jouvet-Mounier et al., 1969). This period of increased REM sleep coincides with heightened formation and elimination of synapses in the developing mouse brain (Marks et al., 1995). Previous work found that REM deprivation, but not non-REM (NREM) deprivation, prevents the elimination of newly-formed dendritic spines in layer V pyramidal neurons in the developing mouse motor cortex (Li et al., 2017). Further, elimination of recent spines during REM facilitates the development of new spines at nearby sites. While most newly formed spines are eliminated, persistent spines are strengthened by REM sleep. Notably, similar findings were observed in the adult mouse brain following motor learning (Li et al., 2017).

A unique feature of REM sleep is the occurrence of myoclonic twitches, or spontaneous, discrete, spastic movements of the limbs (Tiriac et al., 2012; Blumberg et al., 2013; Sokoloff et al., 2020). These twitches occur throughout the mammalian lifespan, but are particularly abundant in infancy (Tiriac et al., 2012; Blumberg et al., 2013; Sokoloff et al., 2020, 2021). The development of myoclonic twitches depends on sensory feedback; the spatiotemporal organization of twitches is disrupted in newborn ErbB2 muscle- specific knockout mice which lack muscle spindles and exhibit impaired proprioception in adulthood (Blumberg et al., 2015). Muscle spindles are sensory receptors that relay changes in the length of muscles to the central nervous system and are necessary for intact proprioception (Kröger and Watkins, 2021). These findings suggest that twitches during sleep provide the developing brain with opportunities to refine immature sensorimotor maps and better coordinate limb movements. Twitching during early-life REM episodes, therefore, could facilitate the transformation of uncoordinated movements during infancy to the fine-tuned sensorimotor maps of an adult. Sensory feedback from twitching limbs are thought to contribute to motor learning and sensorimotor integration (Blumberg et al., 2013, 2020; Sokoloff et al., 2015; Rio-Bermudez and Blumberg, 2018; Glanz et al., 2021), as reafference from myoclonic twitches selectively activates brain regions such as

the thalamus, cortex, hippocampus and cerebellum in infant rats (Khazipov et al., 2004; Mohns and Blumberg, 2010; Tiriac et al., 2012; Sokoloff et al., 2015). Because reafference signals from self-movement are gated during waking, sleep disruptions that interfere with twitching and their corresponding neuronal activity may disrupt sensorimotor maturation (Tiriac and Blumberg, 2016). While these studies provide an important and promising link between early-life sleep episodes and the development of mature sensorimotor representations, the underlying synaptic mechanisms and long-term consequences of myoclonic twitch disruptions remain to be characterized in detail.

A vital role for sleep in early life plasticity is shared across sensory circuits. The study of ocular dominance plasticity (ODP) induced by monocular deprivation (MD) in cats, for example, is a canonical model of critical period plasticity during development that is reliant upon sleep. During an early critical period for visual development, occluding one eye leads to enhanced visual cortex responses to inputs from the non-deprived eye (Hubel and Wiesel, 1970). Sleep enhances ODP; NREM sleep deprivation prevents enhancement of cortical plasticity, suggesting that sleep is vital for consolidating experience-dependent changes in ocular dominance following monocular deprivation (Frank et al., 2001). More recent work has found that REM deprivation disrupts cortical plasticity after monocular deprivation as well, perhaps by disrupting replay-like patterns of activity in the visual cortex (Bridi et al., 2015). Additionally, REM sleep following MD is sufficient to prevent reversal of ODP following subsequent manipulations such as further SD (Bridi et al., 2015), cortical inactivation (Jha et al., 2005), and inhibition of NMDA receptors (Aton et al., 2009). The dependence of ODP on REM sleep parallels studies of sensorimotor development described above, suggesting a vital role for REM sleep in permitting developmental refinement across sensory systems. The consolidation of ODP is also reminiscent of hippocampal memory consolidation during sleep (Diekelmann and Born 2010; Rasch and Born, 2013). These studies suggest that sleep during development is necessary for the consolidation of plastic changes induced by waking experience, which likely

guide appropriate behavioral adaptations to a changing environment. Since ODP (along with other forms of developmental plasticity) occurs during a tightly restricted critical period of development, sleep disruptions early in life could have long-lasting effects on neurophysiology and behavior.

Ontogenetic changes in sleep are conserved; sleep amount and intensity are increased early in life for invertebrates, such as the fruit fly, just as they are in mammals (Jouvet-Mounier et al., 1969; Shaw et al., 2000). In *Drosophila*, 24 hours of sleep deprivation following eclosion leads to long-term learning deficits, whereas adults recover from the same duration of sleep loss after one night of recovery sleep (Seugnet et al., 2011). These chronic learning impairments are likely connected with altered dopamine signaling, and can be dampened either by blocking D1 receptor activity during early life sleep loss or by elevating dopamine signaling during the days after developmental sleep deprivation (Seugnet et al., 2011). Additionally, young sleep-deprived male flies, but not mature flies, show deficits in courtship behavior as adults (Seugnet et al., 2011; Kayser et al., 2014). These courtship deficits are accompanied by decreased size of an olfactory glomerulus associated with perception of social pheromones, caused by impaired developmental growth (Kayser et al., 2014). Similarly, one week of early life sleep disruption impairs later social bonding in adult prairie voles (Jones et al., 2019). In this study, sleep disruption occurred during the third and fourth weeks of life, which likely falls during a critical period for maturation of GABAergic circuits that contribute to sensory integration (Gogolla et al., 2014). Notably, early life sleep deprivation in prairie voles leads to an increase in parvalbumin immunoreactivity in the primary sensory cortex, a brain region relevant to social bonding (Jones et al., 2019). Chronic changes in parvalbumin signaling could disrupt sensory processing and social behavior by altering excitatory/inhibitory balance (Yizhar et al., 2011). Together, these studies demonstrate that early life sleep is vital for developmental growth of rapidly growing brain regions across many species, and that disrupted sleep during development can result in lasting effects on adult circuitry and behavior.

While human studies have not yet revealed a mechanistic understanding of how sleep promotes neural and cognitive development, animal models indicate that sleep's role in neurodevelopment is evolutionarily ancient. Model system studies, such as those in flies and mice discussed above, have begun to examine how sleep modulates synaptic connectivity in a variety of developing sensory circuits. Further studies in these systems may reveal interventions that facilitate healthy development during insufficient sleep (Seugnet et al., 2011; Kayser et al., 2014; Jones et al., 2019).

1.3: Sleep is required for memory encoding and consolidation

In a variety of species, sleep is required for several stages of memory formation and processing (Walker et al., 2002; Graves et al., 2003; McDermott et al., 2003; Ganguly-Fitzgerald et al., 2006; Seugnet et al., 2008; Krishnan et al., 2016). Indeed, sleep deprivation leads to impaired encoding (Walker et al., 2002; Yoo et al., 2007; Seugnet et al., 2008), consolidation (Graves et al., 2003; Diekelmann and Born, 2010), and retrieval (Gais et al., 2007; Lo et al., 2016; Montes-Rodríguez et al., 2019; Heckman et al., 2020) of recent associations. While even a brief nap restores memory in some assays (Seugnet et al., 2008; Ong et al., 2020), other learning and memory impairments persist after days of recovery sleep (Havekes et al., 2016; Wu et al., 2020; Yamazaki et al., 2020). While it is not clear why recovery from sleep loss varies between these conditions, studies have detected several types of longer-lasting cellular and molecular changes that persist after recovery sleep, including altered gene expression (Gaine et al., 2021), protein synthesis (Tudor et al., 2016; Lamon et al., 2021), and circuit connectivity (Weiss and Donlea, 2021). Interestingly, some types of memories seem to be more vulnerable to sleep loss than others. For example, procedural memories and memories acquired with a conscious motivation or reward benefit from sleep more than declarative or unmotivated memories (Stickgold and Walker, 2007; Diekelmann and Born, 2010). In *Drosophila*, sleep deprivation disrupts consolidation of appetitive sugar reward memories in fed flies, but in not

starved flies (Chouhan et al., 2021). Together, these studies indicate that sleep deprivation likely does not have a universal effect on learning and memory, but varies based on physiological, environmental, and behavioral factors.

While the negative impacts of sleep loss on memory formation are typically detrimental, it is possible that targeted sleep disruption could be used to prevent the consolidation of maladaptive memories. Some studies, for instance, suggest that sleep deprivation could be used following trauma to degrade fear memories in patients with post-traumatic stress disorder (PTSD). Studies by Vanderheyden et al., 2015 compared sleep patterns of rats that were susceptible to developing PTSD-like symptoms after trauma to those that were resilient. While susceptible rats exhibited an increase in REM sleep in the hours following the traumatic event, resilient rats slept little during this period (Vanderheyden et al., 2015). Heightened REM sleep following trauma could lead to consolidation and reactivation of the trauma memory, preventing fear extinction and resulting in generalization of the fear memory (Poe, 2017). Traumatic events drive activation of the mammalian locus coeruleus (LC) (Passerin et al., 2000; Naegeli et al., 2018), a collection of noradrenergic cells that promote LTP (Izumi et al., 1992; Thomas et al., 1996; Izumi and Zorumski, 1999) and are generally quiescent during REM sleep (Foote et al., 1980). Elevated LC activity during REM sleep following a traumatic event can contribute to enhancement of recently formed emotional memories as seen in PTSD (Wassing et al., 2019). Therefore, behavioral sleep deprivation or pharmacological REM suppression following a traumatic event could lead to interventions to prevent the development of PTSD (Vanderheyden et al., 2014, 2015; Poe, 2017). Conversely, given the importance of sleep in memory consolidation (Rasch and Born, 2013) and emotional processing (Palmer and Alfano, 2017; Tempesta et al., 2018), sleep loss following a traumatic event could prevent consolidation of fear extinction memory in other conditions (Pace-Schott et al., 2015). Recent human studies have produced mixed results (Porcheret et al., 2015; Kleim et al., 2016; Cohen et al., 2017), indicating that the role for sleep in consolidating and/or maintaining traumatic memories varies

with context or time elapsed since trauma. Further studies will be required to examine the therapeutic potential of sleep manipulations more clearly.

1.4: Sleep promotes synaptic plasticity and homeostasis

Although the primary function or functions of sleep are not understood, evidence suggests a strong relationship between sleep and plasticity (Tononi and Cirelli, 2014; Frank et al., 2001). Sleep loss leads to impairments in the plastic processes of learning and memory (Diekelmann and Born, 2010; Rasch and Born, 2013). One prominent hypothesis posits that sleep's function is the renormalization of synaptic strength via downscaling of synapses that are potentiated during wake, thereby constraining excitability and restoring signal-to-noise ratios for neuronal firing (Tononi and Cirelli, 2014). Learning about the environment during waking experience requires strengthening of synapses (Clem and Barth, 2006; Gruart et al., 2006; Tye et al., 2008). According to this synaptic homeostasis hypothesis, sleep deprivation leads to cognitive deficits due to saturation of synaptic connections (Tononi and Cirelli, 2014). Evidence supporting the role of synaptic downscaling during sleep exists in a variety of species (Gilestro et al., 2009; Vyazovskiy et al., 2009; Bushey et al., 2011). At the molecular level, synaptoneurosomes from the cortex and hippocampus of adult rats display increased protein levels of GluA1-containing AMPA receptors after spontaneous and forced wake than after sleep (Vyazovskiy et al., 2008). Sleep has been found to promote synaptic downscaling in the mouse forebrain by internalizing AMPA receptors via the immediate early gene Homer1 (Diering and Huganir, 2018). In addition, the size of the axon-spine-interface, an ultrastructural measure of synaptic strength, increases after several hours of wake compared to sleep in several mouse brain regions (Vivo et al., 2017, 2019; Spano et al., 2019). At the electrophysiological level, amplitude and/or frequency of miniature excitatory postsynaptic currents in several regions of the rodent brain increase during wake and after sleep loss, and decline following spontaneous sleep and recovery sleep (Liu et al., 2010; Bjorness et al., 2020; Khlghatyan et al., 2020).

Additionally, firing rates of hippocampal and cortical neurons have been shown to increase with wake and decrease with sleep (Vyazovskiy et al., 2008; Vyazovskiy et al., 2009; Huber et al., 2013; Lubenov and Siapas, 2008; Norimoto et al., 2018). Studies in *Drosophila* have also found increases in abundance of presynaptic and postsynaptic markers following sleep loss, consistent with the hypothesis of net potentiation during wake (Gilestro et al., 2009; Bushey et al., 2011; Huang et al., 2020; Weiss and Donlea, 2021). Additional work in the fruit fly has found that acute sleep induction is sufficient to reduce abundance of transcripts (Dissel et al., 2015) or protein (Weiss and Donlea, 2021) of synaptic components.

While evidence clearly suggests a role for sleep in synaptic downscaling in some circumstances, other studies have reported synaptic potentiation during sleep (Frank et al., 2001; Aton et al., 2013, 2014). Short periods of sleep loss decrease the number of dendritic spines in the CA1 region of the hippocampus due to increased activity of the actin-binding protein cofilin (Havekes et al., 2016). Suppressing cofilin activity in hippocampal neurons prevents spine loss and cognitive deficits following sleep deprivation, suggesting that disruption of synaptic potentiation during sleep deprivation can lead to defects in memory consolidation (Havekes et al., 2016). Similarly, sleep deprivation leads to decreased spine density in the dentate gyrus (Raven et al., 2019), and disrupts the formation of new spines following learning (Yang et al., 2014). These data indicate that, although evidence supports a general trend for synaptic downscaling during sleep, it is likely that different classes of synapses undergo different forms of plasticity during sleep or that sleep alters synaptic organization differently depending on the organism's developmental state and recent experience.

Several recent studies have sought to understand whether sleep loss differentially affects distinct classes of neurons within a single circuit or brain region. The *Drosophila* Mushroom Body (MB), which encodes olfactory associative memories, provides an ideal opportunity to examine the local effects of sleep loss on synapse organization. Heroic efforts have untangled the organization of the fly MB with the development of genetic drivers to label

each cell type, often with single-cell resolution (Aso et al., 2014a, 2014b) and serial reconstruction of electron micrographs have led to a detailed connectome of the MB circuitry (Li et al., 2020; Scheffer et al., 2020). These studies show that the *Drosophila* mushroom body (MB) is an associative learning center that is divided into 15 zones defined by non-overlapping arborization of several cell types, including cholinergic Kenyon Cells (KCs), reinforcing dopaminergic neurons (DANs), and mushroom body output neurons (MBONs) which mediate behavioral valence output (Aso et al., 2014a). Associative engrams can be localized to individual zones of the MB lobes, where plasticity in the connections between odor-encoding KCs and valence-driving MBONs determines the fly's behavioral response to odorant stimuli (Aso et al., 2014b; Hige et al., 2015; Owald et al., 2015). Since sleep loss prior to training can impair acquisition/short-term memory and disrupting sleep after training prevents memory consolidation (Ganguly-Fitzgerald et al., 2006; Seugnet et al., 2008), it is likely that sleep deprivation alters either synaptic connectivity or plasticity in MB circuits. Overnight sleep deprivation selectively upscales synapses of cholinergic memory-encoding Kenyon Cells (KCs), but not other cell types in the MB, including DANs or large, inhibitory interneurons (Weiss and Donlea, 2021). Further, not all types of KC output synapses were equally impacted by sleep loss; output connections from KCs to different classes of post-synaptic target neurons showed wide variations in abundance following sleep loss.

Interestingly, studies by Chouhan et al., 2021, found that flies housed without food did not require sleep after appetitive conditioning to form new memories, unlike fed flies. While appetitive memory is encoded in the KC>MBON-γ2α'1 circuit in fed flies and is sensitive to sleep loss, appetitive memory is encoded in KC>MBON-γ1pedc circuitry in starved flies, and remains intact with sleep loss (Chouhan et al., 2021). Additionally, Weiss et al., 2021 found that sleep loss led to decreased connectivity between KCs and MBON-γ2α'1, necessary for sleep-dependent memory consolidation, while KC>MBON-γ1pedc connections, dispensable for sleep-dependent memory consolidation, were unaffected. Sleep loss could therefore disrupt

consolidation of recent appetitive memories in fed flies by reducing overall connectivity between KCs and MBON-γ2α'1 (**Figure 1.1**). Because plasticity rules can differ widely between MB sub-circuits (Hige et al., 2015), environmental conditions during learning likely influence the strength, retention, and/or decay time of a particular association. These results suggest that different zones of the MB exhibit distinct plasticity rules during sleep, likely based on learning paradigm, internal state, and other previous experience.

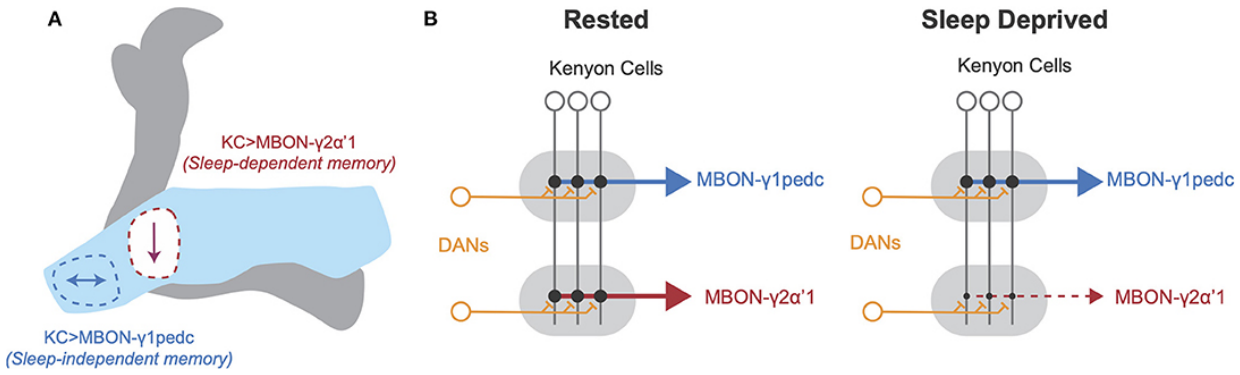

Figure 1.1: Schematic of local plasticity in *Drosophila* mushroom body after sleep loss. (A) Schematic illustration of Drosophila mushroom body. The γ lobe (light blue) contains the γ1 compartment, outlined in blue, and the γ2 compartment, outlined in red. Arrows represent changes in connectivity from Kenyon cells to MBON-γ1pedc (left, blue) and MBON-γ2α'1 (right, red). Appetitive memory encoded at KC>MBON-γ1pedc synapses is resilient to sleep loss, but appetitive memory encoded at KC>MBON-γ2α'1 synapses is impaired by sleep loss. (B) Schematic of connectivity between neuronal cell types in MB in rested (left) and sleep deprived brains (right). KC axons innervate tiled zones that each receive input from distinct DANs and provide input to unique MBONs. After SD, KC>MBON-γ1pedc connectivity is unchanged, but KC>MBON-γ2α'1 connectivity decreases. Based on findings from Weiss and Donlea (2021) and Chouhan et al. (2021).

Supporting the idea of region and circuit specific changes in plasticity with SD, Puentes-Mestril et al., 2021 examined the effects of sleep loss on ribosome-bound transcripts for activity-dependent regulators of plasticity in excitatory pyramidal neurons and inhibitory parvalbumin-expressing interneurons. While both classes of neurons show increases in plasticity-mediating transcripts in the cortex following sleep loss, SD had little effect on abundance of these transcripts in both cell types in the hippocampus (Puentes-Mestril et al., 2021). Additional work suggests that certain cell types in the mouse hippocampus likely have privileged roles in memory consolidation during sleep (Delorme et al., 2021). Sleep deprivation leads to activation of inhibitory somatostatin-expressing (Sst+) interneurons in the hippocampus, likely due to inputs from increasingly active cholinergic neurons (Delorme et al., 2021). Both pharmacological activation of cholinergic neurons and chemogenetic activation of Sst+ cells in the dorsal hippocampus in the absence of SD leads to deficits in sleep-dependent memory consolidation (Delorme et al., 2021). Notably, both Delorme et al., 2021, and Weiss and Donlea, 2021 found that sleep deprivation enhances cholinergic signaling onto GABAergic interneurons in learning/memory-related circuits, which likely increases inhibition onto memory-encoding neurons (**Figure 1.2**). Enhanced hippocampal inhibition due to increased Sst+ activity during SD correlates with impairment of memory consolidation by disrupting long-term potentiation (LTP) (Vecsey et al., 2012; Havekes et al., 2016), the reactivation of memory-encoding cells (Stefanelli et al., 2016; Clawson et al., 2021), or hippocampal oscillations (Puentes-Mestril et al., 2019). Similarly, while some inhibition from the *Drosophila* APL interneurons onto KCs is necessary to maintain spatial and temporal sparseness of odor encoding (Lei et al., 2013; Lin et al., 2014), excess inhibition would likely prevent encoding of new odor associations and reactivation of existing memory traces. Interestingly, GABAergic signaling from dorsal paired medial (DPM) and anterior paired lateral (APL) promotes sleep at night, suggesting that these interneurons may be recruited by increased KC activity during SD to promote sleep and sparsen KC representations (Haynes et al., 2015). These studies in both mice and *Drosophila* suggest

that increased cholinergic signaling disrupts learning and memory after sleep deprivation, and that inhibitory drive onto memory-encoding neurons could be recruited to compensate. While these studies find complementary effects of sleep loss in the fly and mouse, these results use different approaches; Weiss and Donlea (2021) measure synaptic active zone reporters in the fly MB while Delorme et al., (2021) and Puentes-Mestril et al., (2021) quantify hippocampal transcript levels of activity-dependent immediate early genes. Additional studies will be required to directly test the relationship between connectivity changes and cell-type specific changes in activity. Ultimately, characterizing the subsets of synapses, cell types, and circuits that are most sensitive to sleep loss will help elucidate the mechanisms by which SD impairs behaviors such as learning and memory.

Sleep not only balances synaptic connectivity, but also influences neuronal firing patterns. In the rodent frontal cortex, fast spiking pyramidal cells show decreased activity during NREM sleep, while slow firing neurons increase their firing rate (Watson et al., 2016). Similar findings were observed in the mouse primary visual cortex, and these changes in firing rates were disrupted by a period of brief sleep deprivation (Clawson et al., 2018). Pyramidal neurons that are active during sleep spindles, oscillatory activity that promotes plasticity underlying memory formation (Rasch et al., 2013; Cairney et al., 2018), are increasingly active over the course of slow-wave sleep (SWS), whereas spindle-inactive pyramidal neurons show decreased activity during SWS (Niethard et al., 2021). These results indicate that sleep can increase the signal-to-noise ratio of neuronal responses by increasing the activity of sparsely firing neurons with the highest selectivity while reducing noise by decreasing activity of faster spiking, less selective neurons (Clawson et al., 2018). Interestingly, sleep during early-life ocular dominance plasticity in mice is vital for firing rate homeostasis, indicating a potential life-long role for sleep in normalizing neuronal activity (Hengen et al., 2016; Pacheco et al., 2021).

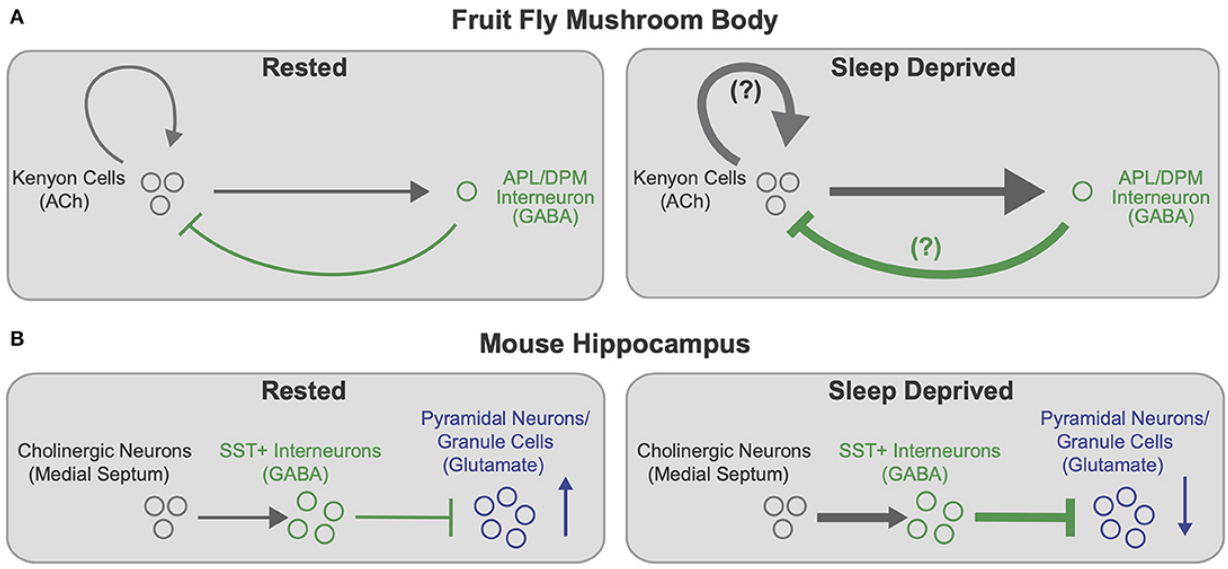

Figure 1.2: Cell type specific effects of sleep loss on memory-encoding circuits.

(A) Schematic of connectivity between memory-encoding KCs and APL/DPM interneurons in the Drosophila MB in rested (left) and sleep deprived (right) flies. Cholinergic KCs activate GABAergic interneurons, which provide feedback inhibition onto KCs. KCs also synapse back onto other KCs. After SD (right), KC>APL connectivity strengthens, presumably increasing inhibition back onto KCs. KC>KC synapses may also strengthen, further contributing to increased KC>APL connectivity. Increased inhibition from APL/DPM after SD could dampen KC>KC excitation and promote recovery sleep. Based on findings from Weiss and Donlea (2021). (B) Schematic of hippocampal circuitry including cholinergic neurons in the medial septum to the mouse hippocampus in rested (left) and sleep deprived (right) mice. Cholinergic neurons activate GABAergic SST+ interneurons in the hippocampus, which inhibit memory-encoding pyramidal neurons/granule cells (principal neurons). After SD (right), enhanced cholinergic signaling increasingly activates SST+ interneurons, thereby heightening inhibition and reducing activity of hippocampal pyramidal neurons and granule cells. Based on findings from Delorme et al. (2021).

1.5: Ethological context affects an individual's resilience to sleep loss

While sleep contributes to many forms of experience-dependent plasticity as described above, individuals can show a wide variation in their responses to sleep loss. Sleep is homeostatically regulated across many species, but both extrinsic and intrinsic factors can influence the responses of an organism to specific sleep challenges. Food-deprived *Drosophila*, for instance, typically reduce their sleep, presumably to maximize foraging opportunities (Keene et al., 2010; Thimgan et al., 2010; Yurgel et al., 2019). While acute sleep-deprivation is typically accompanied by impaired memory and a homeostatic increase in sleep, flies that lose sleep overnight during food deprivation can retain intact memory formation and show little, if any, sleep rebound (Thimgan et al., 2010). Similarly, socially naïve male flies will also forego sleep when paired overnight with a female fly (Beckwith et al., 2017; Machado et al., 2017). This effect can be replicated by activating pheromone sensing neurons or courtship control circuits and, like starvation-induced arousal, is not followed by a sleep rebound.

Similarly, the ability to temporarily offset the need for sleep has also been found in vertebrate species. Fur seals suppress REM sleep for days or weeks when foraging in seawater, accompanied by little to no REM rebound (Lyamin et al., 2018). Migratory frigate birds can reduce the time that they spend asleep by over 90% for ~10 days while continuously in flight over the Pacific Ocean compared to their sleeping patterns on land (Rattenborg et al., 2016). Similarly, Arctic male sandpipers suppress sleep for a roughly three week period annually while they compete for mating partners (Lesku et al., 2012). During mating season, the sun never sets in the high Arctic, allowing males to engage in unlimited visual courtship displays. Because mating success is correlated with the amount of time that male sandpipers spend awake, there is likely selective pressure for genetic factors that can allow male sandpipers to withstand prolonged sleep loss without accruing cognitive deficits or sleep drive.

Constant sunlight during this period likely interacts with social and reproductive cues, enabling males to forego sleep for an extended period. Social behaviors can also drive contexts in which mammals can delay the need for sleep. Whales and dolphins, for example, can nearly fully suppress sleep for up to a month after giving birth with no recorded physiological consequences (Lyamin et al., 2005).

Importantly, vertebrate sleep stages are characterized by electrophysiological signatures measured with electroencephalography (EEG), whereas *Drosophila* sleep is defined by behavioral criteria such as quiescence and increased arousal threshold (Hendricks et al., 2000; Shaw et al., 2000). Recent work has begun to investigate whether sleep in *Drosophila* is composed of distinct stages (Yap et al., 2017; Raccuglia et al., 2019; Tainton-Heap et al., 2021), which may account for variations in plasticity and responses to sleep loss discussed above. While mechanistic studies are not feasible in many of the species mentioned here, the range of contexts in which sleep need can be temporarily offset provides exciting opportunities to understand when sleep is required for plasticity (**Table 1.1**).

Species	Manipulation	Sleep response	Behavioral response	References
Drosophila melanogaster	Sleep deprivation	Decreased sleep, homeostatic rebound	Impaired learning, STM and LTM	Ganguly-Fitzgerald et al., 2006; Seugnet et al., 2008; Li et al., 2009
	Starvation	Decreased sleep, no rebound	Intact memory	Keene et al., 2010; Thimgan et al., 2010; Yurgel et al., 2019
	Stimulants	Decreased sleep	Not measured	Hendricks et al., 2000; Shaw et al., 2000; Andretic et al., 2005
	Courtship	Decreased sleep, no rebound	Not measured	Beckwith et al., 2017; Machado et al., 2017
Frigatebirds	Migration	Decreased sleep in flight, rebound on land	Not measured	Rattenborg et al., 2016
Sandpipers	Mating season	Decreased sleep	Mating success positively correlated with amount of sleep loss	Lesku et al., 2012
Cetaceans	Postpartum	Little to no sleep	Not measured	Lyamin et al., 2005, 2007
Fur seals	In seawater	Greatly reduced REM, no REM rebound	Not measured	Lyamin et al., 2018

Table 1.1: Summary of experimental or ethologically-relevant conditions that reduce sleep in several species.

1.6: Intrinsic factors affect an individual's resilience to sleep loss

Resilience to sleep loss can also be influenced by intrinsic factors that vary between individuals. Human subjects exhibit reliable, stable responses to repeated episodes of sleep loss, suggesting that sensitivity to sleep loss can be a durable trait over time (Dennis et al., 2017; Yamazaki and Goel 2019). Naturally occurring genetic polymorphisms coincide with an individual's response to sleep loss in flies and humans (Viola 2007, 2012; Donlea et al., 2012; Satterfield et al., 2015). In two of these studies, the same genetic alleles correlated with reduced cognitive impairments and dampened homeostatic sleep pressure after prolonged waking, indicating that the identified loci could contribute to protecting neural functions during sleep loss (Viola et al., 2007; Donlea et al., 2012). Interestingly, the identified human alleles in *per3* and *tnfα* that protected individuals from the consequences of sleep loss did not predominate in the subject populations, consistent with the possibility that these alleles are accompanied with susceptibility to other physiological challenges.

Brain structure can also influence sensitivity to sleep loss; variation in functional connectivity between brain regions and hippocampal structure can predict the cognitive impact of sleep loss in human subjects (Yeo et al., 2015; Saletin et al., 2016). While the neural and molecular mechanisms that connect these variations with susceptibility to sleep are not yet known, studies of model systems provide some insights into pathways that might provide protection from insufficient sleep. *Drosophila* and mouse studies have identified genetic pathways, including circadian rhythm (Mang et al., 2016; Ehlen et al., 2017), and metabolic

factors (Thimgan et al., 2010, 2015), that can be manipulated to prevent rebound sleep following extended waking. It is important to note that each of these interventions can temporarily delay the accumulation of sleep debt, but it is unclear how long their protection persists and whether other consequences build as a result. Nonetheless, further examination of the external contexts and internal factors that can confer resilience to sleep loss may provide new insight into the neural functions of sleep and identify controllable interventions to facilitate rapid recovery from sleep loss.

1.7: Conclusions on the link between sleep and plasticity

In many contexts, sleep is vital for individuals to learn and adapt their behavior to best fit their environmental conditions. Sleep facilitates brain development and circuit refinement, and early life disruptions in sleep can result in long-lasting behavioral changes. Throughout the lifespan, sleep also impacts whether new memories can be effectively acquired and consolidated. While understanding the mechanisms that contribute to sleep-dependent plasticity remain an area of intense interest, many studies have already identified molecular and synaptic connectivity changes that occur during sleep to facilitate memory formation. More clearly identifying these mechanisms and developing strategies to manipulate them could open opportunities to support cognitive processing during sleep loss. Finally, individuals exhibit varying responses to sleep loss due to intrinsic and environmental factors. Understanding the benefits and detriments of variations in sleep, as well as the biological basis for interindividual differences, will help resolve the function(s) of sleep and elucidate how sleep patterns affect future behavior.

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CHAPTER TWO

Sleep Deprivation Results in Diverse Patterns of Synaptic Scaling across the *Drosophila* Mushroom Bodies

2.1: Abstract

Sleep is essential for a variety of plastic processes, including learning and memory. However, the consequences of insufficient sleep on circuit connectivity remain poorly understood. To better appreciate the effects of sleep loss on synaptic connectivity across a memory-encoding circuit, we examined changes in the distribution of synaptic markers in the Drosophila mushroom body (MB). Protein-trap tags for active zone components indicate that recent sleep time is inversely correlated with Bruchpilot (BRP) abundance in the MB lobes; sleep loss elevates BRP while sleep induction reduces BRP across the MB. Overnight sleep deprivation also elevated levels of dSyd-1 and Cacophony, but not other pre-synaptic proteins. Cell-type-specific genetic reporters show that MB-intrinsic Kenyon cells (KCs) exhibit increased pre-synaptic BRP throughout the axonal lobes after sleep deprivation; similar increases were not detected in projections from large interneurons or dopaminergic neurons that innervate the MB. These results indicate that pre-synaptic plasticity in KCs is responsible for elevated levels of BRP in the MB lobes of sleep-deprived flies. Because KCs provide synaptic inputs to several classes of post-synaptic partners, we next used a fluorescent reporter for synaptic contacts to test whether each class of KC output connections is scaled uniformly by sleep loss. The KC output synapses that we observed here can be divided into three classes: KCs to MB interneurons; KCs to dopaminergic neurons; and KCs to MB output neurons. No single class showed uniform scaling across each constituent member, indicating that different rules may govern plasticity during sleep loss across cell types.

2.2: Introduction

In a variety of species, sleep supports the capacity for new learning and is vital for the consolidation of recently formed memories[1–6]. In *Drosophila melanogaster*, overnight sleep loss is sufficient to impair acquisition of new associative memories that are encoded in the Mushroom bodies (MBs) [3], and sleep disruptions that follow learning can prevent memory

consolidation [2,7]. Interestingly, sleep is often elevated or intensified during conditions of heightened synaptic reorganization, including early development [8–10], recovery from neural injury [11,12], and memory consolidation [1,2]. Together, these results indicate that sleep may support plastic remodeling in the brain. The consequences of sleep disruptions on synaptic connectivity, however, are not clearly understood. One hypothesis proposes that sleep permits the homeostatic downscaling of synapses throughout the brain, suggesting that sleep loss may impact cognition by saturating synaptic connections across plastic circuits [13–15]. This model is supported by several studies that have found an increase in the size or number of synaptic processes after extended waking in both flies and mice [16–19]. Additionally, sleep deprivation increases the overall abundance of several synaptic proteins in whole fly brains, suggesting a trend towards synaptic overgrowth during sleep loss [20,21]. Conversely, acute sleep induction can result in a net decrease of synaptic protein[21] and transcripts[22] in fly brain homogenates. Other experiments, however, indicate that sleep deprivation may either weaken or prevent the expansion of synaptic connections in some circuits [9,23–25]. These previous studies demonstrate that sleep disruption alters synaptic abundance or size in several neuronal cell types, but it is unclear whether sleep loss uniformly affects all classes of neurons or synapses within a given circuit.

The *Drosophila* Mushroom Body (MB) provides an ideal structure to characterize how sleep loss may differentially impact distinct types of synaptic contacts within a plastic circuit. The MB is a core associative neuropil that is conserved across arthropod species and is required for the acquisition and encoding of olfactory memories [26–28]. In the fruit fly, olfactory information is relayed to the MBs via secondary projection neurons, which synapse onto ~2,200 Kenyon cells (KCs) in each brain hemisphere [29–32]. KC axons extend through fasciculated bundles that comprise five distinct MB lobes: α, β, γ, α', and β' [33]. Each axonal lobe of the MBs is divided into compartments that are each innervated by distinct dopaminergic neuron (DAN) types (~21 total) and connect to unique MB output neurons (~22 total types) [29]. Associative memories are

encoded in the synaptic connections between odor-encoding KCs and valence-encoding MB output neurons (MBONs), with reinforcement signals provided by compartment-specific DANs [34–41]. DANs, as a result, encode unconditioned stimuli during learning, and MBONs mediate behavioral output. Additionally, two modulatory interneurons, APL and DPM, project throughout the MB lobes. Both neurons likely receive synaptic inputs from and provide recurrent connections back onto many KCs, but each plays a functionally distinct role in MB functions: APL facilitates sparse coding of odor cues and memory storage [42–44], while DPM supports recurrent activity during memory storage and promotes sleep [45–49]. Because of the high degree of interconnectivity within and between cell types, the MB provides an optimal system to test whether sleep loss alters all circuit components to a similar degree, whether one particular connection type may be especially sensitive to sleep loss, or if different constituents may exhibit distinct patterns of reorganization with insufficient sleep. Distinguishing between these models may open opportunities to understand how sleep loss degrades memory encoding in the MB and to develop interventions that maintain plasticity during prolonged waking.

While KCs in the MB γ lobe individually exhibit increased pre-synaptic terminal volume with sleep deprivation [16], the effects of sleep loss on other cell types in the MB have not been systematically examined. Because activity within subpopulations of MB neurons can regulate sleep [34,50–53], understanding the effects of sleep loss on MB connectivity may inform our understanding not only of the cognitive consequences of sleep loss, but also of mechanisms that encode sleep need. Here, we quantify the effects of sleep loss on the abundance of pre-synaptic proteins across neuron types in the MB. We observe a net increase following sleep loss in the abundance of protein-trap reporters for the pre-synaptic proteins Bruchpilot (BRP), dSYD-1, and Cacophony [54–58], but not other synaptic components. Using cell type-specific genetic reporters, we find that the increase in BRP can be localized to MB-intrinsic KCs and not to other neuronal populations in the MB. Because KCs synapse upon many other cell types in the MB, we also tested the effect of sleep loss on the abundance of synaptic contacts between

KCs and many of their post-synaptic partners. These experiments find an assortment of responses in synaptic contacts between KCs and different post-synaptic partners, suggesting that sleep deprivation does not uniformly scale all KC output synapses. Instead, sleep loss results in a variety of plastic effects on different classes of KC output synapses, with some increasing their contacts, others weakening their connections, and a final portion remaining unchanged. Our results indicate that different circuit motifs within the MB may be differentially affected by sleep loss, and identify particularly plastic connections that may contribute to impaired memory and increased homeostatic sleep drive following prolonged waking.

2.3: Materials and Methods

2.3.1: Fly strains and environment

Fly stocks were fed standard cornmeal media (per 1L H20: 12g agar, 29g Red Star yeast, 71g cornmeal, 92g molasses, 16mL methyl paraben 10% in EtOH, 10mL propionic acid 50% in H_20) at 25˚C with 60% relative humidity and entrained to a daily 12hr light, 12hr dark schedule. All flies were reared in environmentally-controlled chambers at 25˚C and 60% relative humidity on a 12hr light:12hr dark schedule. *Brp*$^{MI2978\text{-}GFSTF}$, *dSyd-1*$^{MI05387\text{-}GFSTF}$, *Rim*$^{MI03470\text{-}GFSTF}$, *Syt1*$^{MI02197\text{-}GFSTF}$, *OK107*-Gal4, *R13F02*-Gal4, *R19B03*-Gal4, *R58E02*-Gal4, *GH146*-Gal4, *C316*-Gal4, *R12G04*-Gal4, *R25D01*-Gal4, *R66C08*-Gal4, *R71D08*-Gal4, *nsyb* GRASP effectors (*w**; P{w^{+mC}=lexAop-*nSyb*-spGFP$_{1\text{-}10}$}2, P{w^{+mC}=UAS-CD4-spGFP$_{11}$}2; MKRS/TM6B) and *rab3*^{mCherry} were obtained from the Bloomington Drosophila Stock Center, *TH*-Gal4 was provided by Dr. David Krantz (UCLA), STaR effector flies (w^{-}; 20xUAS-RSR.PEST, 79C23S-RSRT-STOP-RSRT-smGFP_V5-2A-LexA/cyo) were provided by Dr. Orkun Akin (UCLA), and *cac*^{sfGFP} was a gift from Dr. Kate O'Connor-Giles (Brown University). All MB split-Gal4 fly stocks (*MB011B*, *MB185B*, *MB371B*, *MB434B*, *MB463B*, *MB543B*, *MB594B*, and *MB607B*) were generated by the lab of Dr. Gerald Rubin [29,34] and generously provided by the HHMI Janelia Research Campus.

2.3.2: Behavior

Sleep was measured as previously described [108]. 3-7 day old adult female flies were housed individually in 65mm borosilicate glass tubes (5mm diameter) containing fly food coated with paraffin wax on one end and a foam plug in the other. Locomotor activity was measured using Drosophila Activity Monitors from Trikinetics (Waltham MA, USA) and sleep was analyzed using Visual Basic macros in Microsoft Excel [108] or SCAMP analysis scripts in MATLAB [109]. Baseline sleep was monitored in all groups, and sleep deprivation was performed using mechanical stimulation via the SNAP method [108]. For starvation experiments, flies either remained on standard fly media for control treatment or were transferred into fresh tubes containing 2% agar in H2O at ZT0, 24h prior to dissection.

2.3.3: Immunochemistry and confocal imaging

Flies were anesthetized on ice, then brains were dissected in PBS and fixed in either 4% paraformaldehyde in PBS for 30 minutes or in 3% glyoxal for 25 minutes (all brains from an individual experiment were treated identically). Following fixation, brains were washed in PBS and PBTX (PBS + 0.3% Triton-x100) and incubated in 3% Normal Goat Serum in PBTX for one hour. For GFP and mCherry immunostaining, brains were incubated in primary antibody overnight followed by secondary antibody for roughly 24 hours. Immunostaining for V5 used a 48-hour incubation period in mouse anti-V5 conjugated with DyLight550 (Bio-Rad). After antibody incubation, brains were washed in PBS and mounted on slides using Vectashield fluorescence mounting medium from Electron Microscopy Services (Burlingame CA, USA). All specimens were imaged on a Zeiss 880 laser scanning confocal microscope using a 40x water immersion objective. Matching imaging settings were used for each brain within individual experiments.

Primary antibodies used: chicken anti-GFP at 1:1000 (Molecular Probes), rabbit anti-DsRed at 1:1000 (Clontech), mouse anti-GFP at 1:100 (Sigma), mouse anti-V5 conjugated with DyLight550 at 1:400 (Bio-Rad).

Secondary antibodies used: goat anti-chicken Alexa 488, goat anti-rabbit Alexa 546, goat anti-mouse Alexa 488 (Molecular Probes). All secondary antibodies were used at a 1:1000 dilution.

Quantification of mushroom body fluorescent signal intensity used an average intensity projection over 4 z-slices of the lobe of interest, followed by manual outlining of the labeled lobe to measure mean GFP or anti-V5 intensity in Fiji [110].

2.3.4: Statistics

Statistical comparisons were made using t-Tests or One- or Two-Way ANOVAs as appropriate; figure legends describe the statistical tests used for each panel. Where needed, post-hoc pairwise analysis measured the effect of sleep manipulations on each MB lobe using Sidak's multiple comparisons tests. All statistical comparisons were conducted using GraphPad Prism 8 (San Diego CA, USA). Sample sizes for each experiment are depicted in figure panel or in the appropriate figure legend. All group averages shown in data panels depict mean ± SEM.

2.4: Results

2.4.1: BRP abundance in mushroom body lobes is inversely related with recent sleep time

To examine the consequences of sleep loss on MB synaptic connections, we first observed the abundance of GFP-tagged Bruchpilot expressed from a MiMIC protein trap insertion into an intron of the Brp locus [56,57]. Brp is a core component of pre-synaptic active zones [55,58], and pre-synaptic Brp protein levels correlate closely with active zone size and release probability [59–61]. We mechanically deprived *brp*$^{MI02987\text{-}GFSTF}$/+ flies of sleep overnight **(Figure S2.1A)** then dissected their brains and imaged BRP::GFP fluorescence using confocal

microscopy. The BRP::GFP signal increased by ~32-46% in each MB lobe from sleep-deprived *brp*$^{MI02987\text{-}GFSTF}$/+ flies compared to controls **(Figures 2.1A-B)**, supporting previous reports of increased pre-synaptic terminal size in MB neurons [16]. We also tested the effects of acutely inducing sleep by activating sleep-promoting neurons in a dorsal stratum of the Fan-shaped Body [62]. Flies expressing *brp*$^{MI02987\text{-}GFSTF}$ along with the warm-sensitive cation channel TrpA1 under the control of *R23E10*-Gal4 were heated to 31˚C for 6h to acutely increase their sleep from ZT0-6 **(Figure 2.1C)** [63,64]. Sleep induction reduced BRP::GFP fluorescence in the MB γ, α, β, and β' lobes of experimental flies (*R23E10*-Gal4>UAS-*TrpA1*/*brp*$^{MI02987\text{-}GFSTF}$) compared to siblings that were maintained at 25˚C **(Figures 2.1D-E)**. A 6h exposure to 31˚C did not significantly alter either sleep or BRP::GFP fluorescence in *brp*$^{MI02987\text{-}GFSTF}$/+ controls relative to siblings housed at 25˚C **(Figures 2.1D, 2.1F)**. Together, these results support the hypothesis that prolonged waking results in a net synaptic expansion in the MBs, and that sleep broadly facilitates the downscaling of these connections.

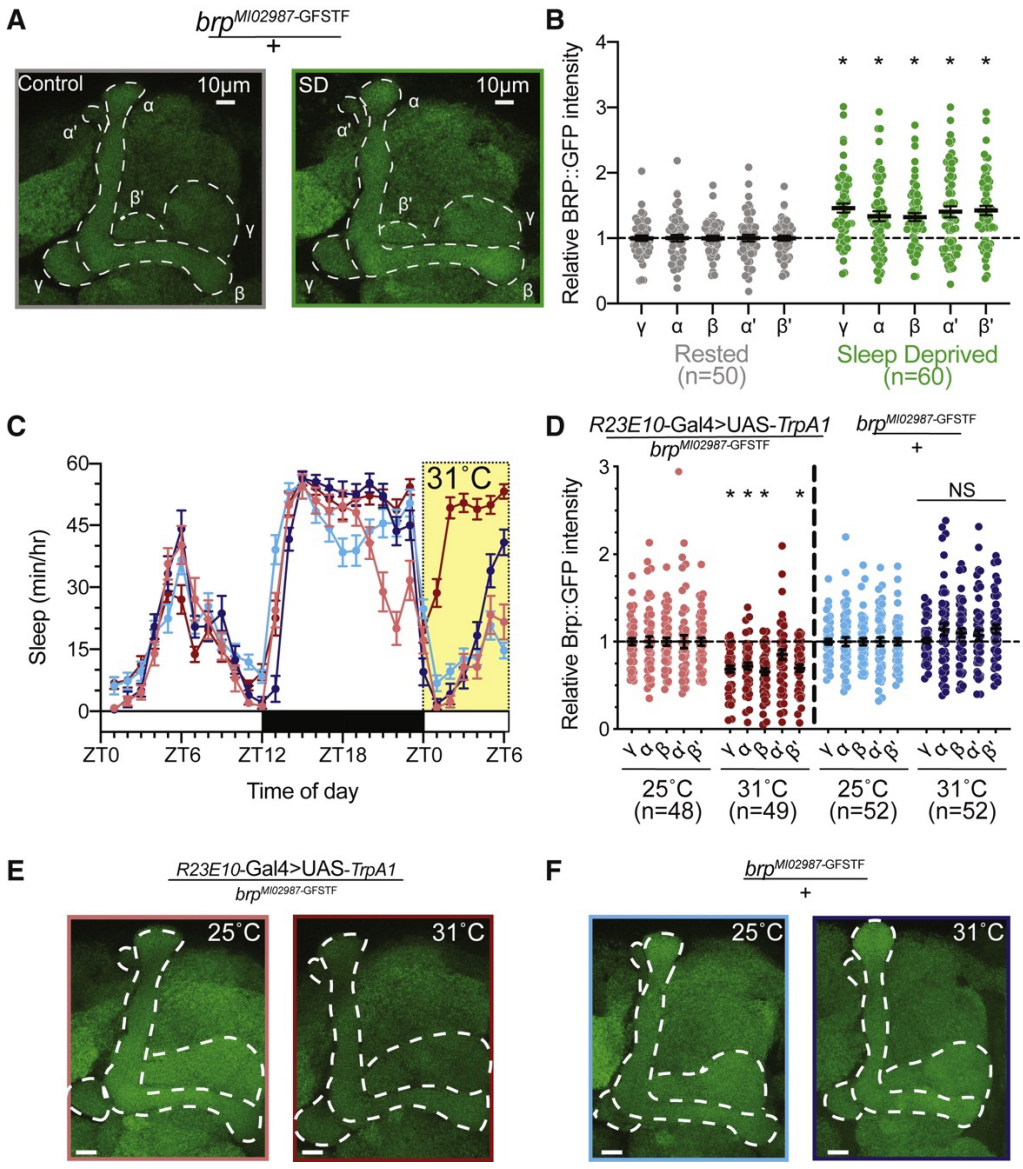

Figure 2.1: Sleep bidirectionally regulates Brp abundance in the mushroom body

(A) Representative images of endogenous Brp (green) labeled with GFP in *brp*$^{MI02987\text{-}GFSTF}$/+ flies following 12 hours of rest (left) or 12 hours of overnight sleep deprivation (right). The lobes of the MB are outlined in white.

(B) Quantification of Brp::GFP intensity throughout the MB lobes of *brp*$^{MI02987\text{-}GFSTF}$/+ flies after 12-hr of overnight sleep loss (green) normalized to rested controls (gray). Two-way ANOVA finds a significant effect of SD ($F_{(1,108)}$=20.62, p<0.0001, n=50-60 hemispheres/group). Pairwise

comparisons using Sidak's multiple comparisons test found significant increases in Brp::GFP in each MB lobe after sleep deprivation relative to rested siblings ($p \leq 0.002$ for each test).

(C) Hourly sleep traces at 25˚C (light shading) and 31˚C (dark shading) for *R23E10*-Gal4>UAS-*TrpA1*/*brp*$^{MI02987\text{-}GFSTF}$ (red) and *brp*$^{MI02987\text{-}GFSTF}$/+ flies (blue). Thermogenetic activation of dFB neurons in *brp*$^{MI02987\text{-}GFSTF}$-expressing flies (*R23E10*-Gal4>UAS-*TrpA1*/*brp*$^{MI02987\text{-}GFSTF}$; dark red) increased sleep time compared to siblings that remained at 25˚C (light red) and brpMI02987-GFSTF/+ genetic controls that were housed at 25˚C (light blue) or shifted to 31˚C (dark blue). Flies were temperature shifted from ZT0-6 (yellow shading).

(D) Quantification of Brp::GFP intensity for groups shown in Figure 1C. Sleep induction in *R23E10*-Gal4>UAS-*TrpA1*/*brp*$^{MI02987\text{-}GFSTF}$ flies that were shifted to 31˚C (dark red) led to a significant decrease in Brp intensity in α, β, β', and γ lobes compared to siblings that remained at 25˚C (light red). Exposure to 31˚C did not change Brp::GFP in *brp*$^{MI02987\text{-}GFSTF}$/+ genetic controls (25˚C shown in light blue, 31˚C in dark blue). Two-way Repeated Measures ANOVA found a significant group-by-lobe interaction ($F_{(12,784)}=3.796$, $p<0.0001$, n=48-52 hemispheres/group).

(E) Representative images of endogenous Brp::GFP labeled in *R23E10*-Gal4>UAS-*TrpA1*/*brp*$^{MI02987\text{-}GFSTF}$ flies that were housed at 25˚C (left) or given a 6-h exposure at 31˚C (right).

(F) Representative images of endogenous Brp::GFP labeled in *brp*$^{MI02987\text{-}GFSTF}$/+ flies that were housed at 25˚C (left) or shifted to 31˚C for 6-h (right).

See also **Figure S2.1** for sleep traces from experimental groups shown in Figure 3.1A-B.

Scale bars depict 10 μm; error bars represent SEM for all panels.

2.4.2: Variable effects of sleep loss on MB abundance of presynaptic proteins

While our above results focus on the effects of sleep loss on BRP levels, previous studies have found evidence for coordinated scaling of several synaptic proteins [20]. Other studies, however, suggest that synaptic scaling can change the abundance of some, but not all, pre-synaptic components [21,65]. To examine the effects of sleep loss on additional pre-synaptic machinery, we obtained protein trap reporters for six additional pre-synaptic proteins and measured their abundance in the MB after overnight sleep loss (See schematic in **Figure 2.2A**; sleep traces and representative images shown in **Figure S2.1B-O**). First, we observed the effects of sleep loss on dSyd-1, an active zone component that interacts with BRP and is required for the organization of electron dense T-bars [54]. Fluorescence of dSyd-1$^{MI05387\text{-}GFSTF}$ [56,57], like Brp$^{MI02987\text{-}GFSTF}$, increased after sleep deprivation by ~25-32% across each MB lobe **(Figure 2.2B)**. We next measured the effects of sleep loss on Cacophony (Cac), the primary voltage-gated calcium channel at the pre-synapse [66]. Like reporters for BRP and dSyd-1, abundance of a GFP-tagged reporter for Cac, Cac^{sfGFP} [67], showed a significant increase in MB signal after sleep deprivation **(Figure 2.2C)**. Overnight sleep loss, however, did not broadly increase the abundance of a protein-trap reporter for Rab3 interacting molecule, Rim$^{MI03470\text{-}GFSTF}$ **(Figure 2.2D)**, which influences synaptic accumulation of Cac [68]. We also quantified the effects of sleep loss on Rab3, which exhibits vesicle-like staining at the fly active zone and shares the protein domains that are required for vesicle localization with its mammalian homolog [69–71]. MBs from sleep deprived flies exhibited a significantly reduced amount of Rab3^{mCherry} [70] in each MB lobe **(Figure 2.2E)**. Similar quantifications of reporters for two proteins that localize to synaptic vesicles, Syt1$^{MI02197\text{-}GFSTF}$ [56,57] and nSyb^{GFP} [72]. found that sleep loss elevated Syt1$^{MI02197\text{-}GFSTF}$ [56,57] abundance locally in the γ, β, and β' lobes **(Figure 2.2F)**, while fluorescent intensity of nSyb^{GFP} was decreased in each MB lobe of sleep deprived flies **(Figure 2.2G)**. While the diversity of post-synaptic receptors expressed in MB neurons complicates analysis of post-synaptic plasticity, abundance of the primarily post-synaptic Dlg$^{MI06353\text{-}GFSTF}$ was not changed by

sleep deprivation **(Figure 2.2G)**. 48h of undisturbed recovery after overnight sleep deprivation restored abundance of Rab3^{mCherry} back to control levels **(Figure 2.2E, right)**, while dSyd-1$^{MI05387\text{-}GFSTF}$ in previously sleep-deprived flies was reduced below the levels observed in undisturbed controls **(Figure 2.2B, right)**. Together, these results indicate that sleep loss does not uniformly increase the amount of all pre-synaptic proteins across the MB. Instead, the MBs of sleep-deprived flies show broad increases across all lobes in BRP, dSyd-1, and Cac which physically interact [54], and only local, if any, increases in other synaptic components, including Rim, Rab3, Syt1, and nSyb.

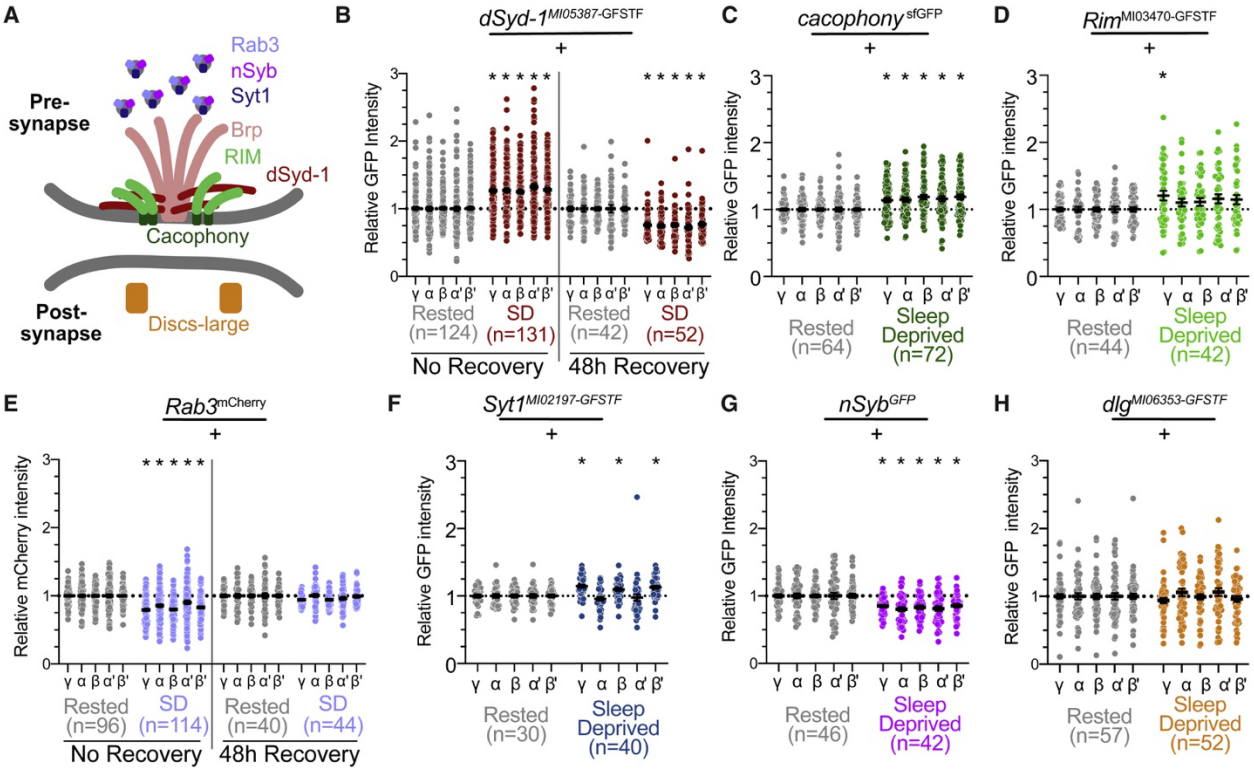

Figure 2.2: Pre-synaptic proteins show variable responses to sleep loss in the MB lobes

(A) Schematic illustration of pre-synaptic active zone, including core protein components observed in these studies. Brp localizes in the electron-dense T-bar, where it physically interacts with Syd-1. Both contribute to the recruitment of other pre-synaptic proteins to the AZ.

RIM is necessary for proper localization of the Ca2+ channel Cacophony in the pre-synaptic plasma membrane. Rab3 regulates priming of vesicles and organization of AZ proteins. Syt-1 is a Ca^{2+} sensor located on synaptic vesicles. Nsyb is localized to synaptic vesicles and mediates vesicle fusion. Dlg is a scaffolding protein that is primarily located at the postsynaptic density.
(B) Abundance of dSyd-1::GFP throughout the MB after overnight sleep deprivation (red) compared to rested controls (grey) when flies were dissected either immediately following sleep deprivation (left) or allowed 48h of *ad libitum* recovery sleep before dissection (right); dSyd-1::GFP intensity in all groups is normalized to rested controls. Two-way ANOVA finds a significant effect of SD ($F_{(3,345)}$=43.12, p<0.0001, n=42-131 hemispheres/group).
(C) Quantification of Cac::sfGFP intensity in MB axonal lobes following overnight sleep deprivation (green) normalized to rested controls (grey). Two-way ANOVA finds a significant effect of SD ($F_{(1,134)}$=18.51, p<0.0001, n=64-72 hemispheres/group).
(D) Quantification of Rim::GFP in the MB lobes of sleep deprived $Rim^{MI03470\text{-}GFSTF}$ flies (green) and rested controls (grey). Two-way ANOVA finds a significant effect of SD ($F_{(1,84)}$=4.871, p=0.03, n=42-44 hemispheres/group), post-hoc comparisons using Sidak's multiple comparisons test finds a significant increase in Rim::GFP abundance in the γ lobes (p=0.048), but not in α (p=0.54), β (p=0.38), α' (p=0.23), or β' (p=0.27).
(E) Fluorescent intensity of endogenous Rab3::mCherry in the MB lobes of sleep deprived *Rab3*^{mCherry}/+ (light blue) compared to rested siblings (grey). Data from brains dissected immediately following sleep-deprivation shown on left; right depicts quantification of brains dissected after 48h of recovery from overnight sleep deprivation. Two-way ANOVA finds a significant lobe x group interaction ($F_{(12,1160)}$=4.472, p<0.0001, n=42-131 hemispheres/group).
(F) Quantification of Syt1::GFP intensity throughout the MB after overnight sleep deprivation (dark blue) compared to rested controls (grey). Two-way ANOVA finds a significant lobe-by-SD interaction ($F_{(4,272)}$=7.94, p<0.0001, n=30-40 hemipsheres/group); post-hoc comparisons using Sidak's multiple comparisons test find a significant increase of Syt1::GFP in the γ (p=0.0005), β

(p=0.029), and β' lobes (p=0.0023). No significant change was detected in the α or α' lobes (p=0.7827 and 0.9937, respectively, by Sidak's multiple comparisons tests).

(G) Abundance of nSyb::GFP in MB lobes of rested (grey) and sleep-deprived flies (magenta). Two-way repeated measures ANOVA finds a significant effect of sleep deprivation on nSyb::GFP abundance ($F_{(1,86)}$=19.33, p<0.0001, n=42-46 hemispheres/group).

(H) Dlg::GFP levels in the MB lobes of rested controls (grey) and sleep-deprived siblings (orange). Two-way repeated measures ANOVA finds no significant effect of sleep deprivation ($F_{(1,107)}$=0.002567, p=0.9597, n=52-57 hemispheres/group).

See also **Figure S2.1** for representative images and sleep traces from each experimental group and genotype.

Scale bars depict 10 μm; error bars represent SEM for all panels.

2.4.3: Presynaptic BRP is elevated in KCs but not in other MB cell types

As described above, the abundance of BRP::GFP is significantly elevated across all MB lobes of sleep deprived *brp*$^{MI02987\text{-}GFSTF}$/+ flies **(Figures 2.1A-B).** The use of a protein-trap reporter, however, does not provide information about which cell types within the MB contribute to the increase in BRP abundance that occurs after extended waking. To better identify the specific neuron classes that show elevated BRP levels after sleep loss, we used Synaptic Tagging with Recombination (STaR), a flp-based reporter to specifically label BRP expression in genetically defined classes of neurons [73]. Like the protein-trap constructs used above, STaR reports the abundance of a BRP-fusion reporter (BRP::V5) expressed under the control of the endogenous *brp* promoter. For these studies, we have used a flp-based reporter that fuses smFP_V5 to BRP in genetically targeted neurons [74,75]. We began testing the effects of sleep loss on specific classes of MB neurons by labeling BRP in odor-encoding, MB-intrinsic Kenyon cells (KCs) using the genetic driver *R13F02*-Gal4 [63]. STaR labelling in KCs using *R13F02*-Gal4 increased significantly in all KC lobes after overnight sleep loss **(Figures 2.3A-B and S2.2A).**

Similar increases in KC expression of BRP were also found using a second broad KC driver, *OK107*-Gal4 **(Figures 2.3C and S2.2B)** [28]. The abundance of smFP_V5-tagged BRP remains elevated in *R13F02*-positive KCs after 24h of recovery after overnight sleep deprivation, but returns to control levels within approximately 48h of recovery **(Figure 2.3D-F and S2.2C-D)**. Because starvation results in sleep loss without the accrual of sleep pressure or learning deficits, we tested whether KC active zones are altered after 24h of starvation [76,77]. *R13F02*-Gal4>STaR flies fed only 2% agar in H_2O slept 70±4.73% less than their fed siblings **(Figure 2.3G)**, but no significant increase in STaR signal was detected in starved flies **(Figure 2.3H-I)**. These results suggest that the increased BRP abundance that we observe in KCs after sleep loss may be correlated with cognitive impairments and increased sleep drive. To understand whether the increased BRP abundance that we observed is uniformly shared across subpopulations of KCs that innervate different regions of the MB lobes, we used more restricted genetic driver lines to label KCs that project into the α/β, α'/β', or γ lobes [29]. We found that not all KC subsets exhibited similar increases in smFP_V5-tagged BRP; KCs with axons targeted to the α/β core, α/β posterior, and γ dorsal regions showed significant increases in pre-synaptic STaR labelling after sleep deprivation, while there was no effect of sleep disruption on BRP abundance in α/β surface or α'/β' anterior/posterior KCs **(Figure S2.3)**. Genetic mosaicism of flp expression using available driver lines prevented consistent measurements of BRP abundance in α'/β' medial and γ main KC subpopulations. Together, these data indicate that increased BRP abundance within subpopulations of KC neurons contributes significantly to the overall elevation of BRP that we observe in MB lobes following sleep deprivation.

Next, we tested whether increased BRP::V5 could also be observed after sleep deprivation **(Figure S2.2E-H)** in other cell types that innervate the MB lobes using STaR. Two populations of dopaminergic neurons project into the MB: PPL1 neurons that project into compartments of the γ, α, and α' lobes, which encode punishment, and PAM neurons that terminate in γ, β, and β' lobe zones, which activate in response to rewarding stimuli [29,35,36,39].

When we quantified pre-synaptic STaR labelling in PPL1 DANs using *TH*-Gal4 [78], a two-way ANOVA detected a significant effect for sleep deprivation. Pairwise comparisons within compartments of the γ, α, and α' lobes, however, found significant increases only in the α3 and α'3 compartments **(Figure 2.3J)**. Similarly, we used *R58E02*-Gal4 to drive STaR expression in PAM DANs projecting into the horizontal lobes of the MB [29,63]. While a Two-way ANOVA found a significant effect for sleep deprivation on STaR fluorescence in PAM DANs, pairwise comparisons detect significant increases in labelled Brp only in the γ lobe and β'2 compartment, and not in the β lobe and β'1 compartment **(Figure 2.3K)**. No increase in BRP::smFP_V5 intensity was detected when we drove STaR in APL (*GH146*-Gal4) or DPM neurons (*C316*-Gal4), which are both large interneurons that project broadly across all MB lobes **(Figures 2.3L-M)** [44,49]. Based on these data, the broad increase in BRP that we observed in MB lobes **(Figures 2.1A-B)** after sleep deprivation can be attributed primarily to KCs, not to other MB cell types.

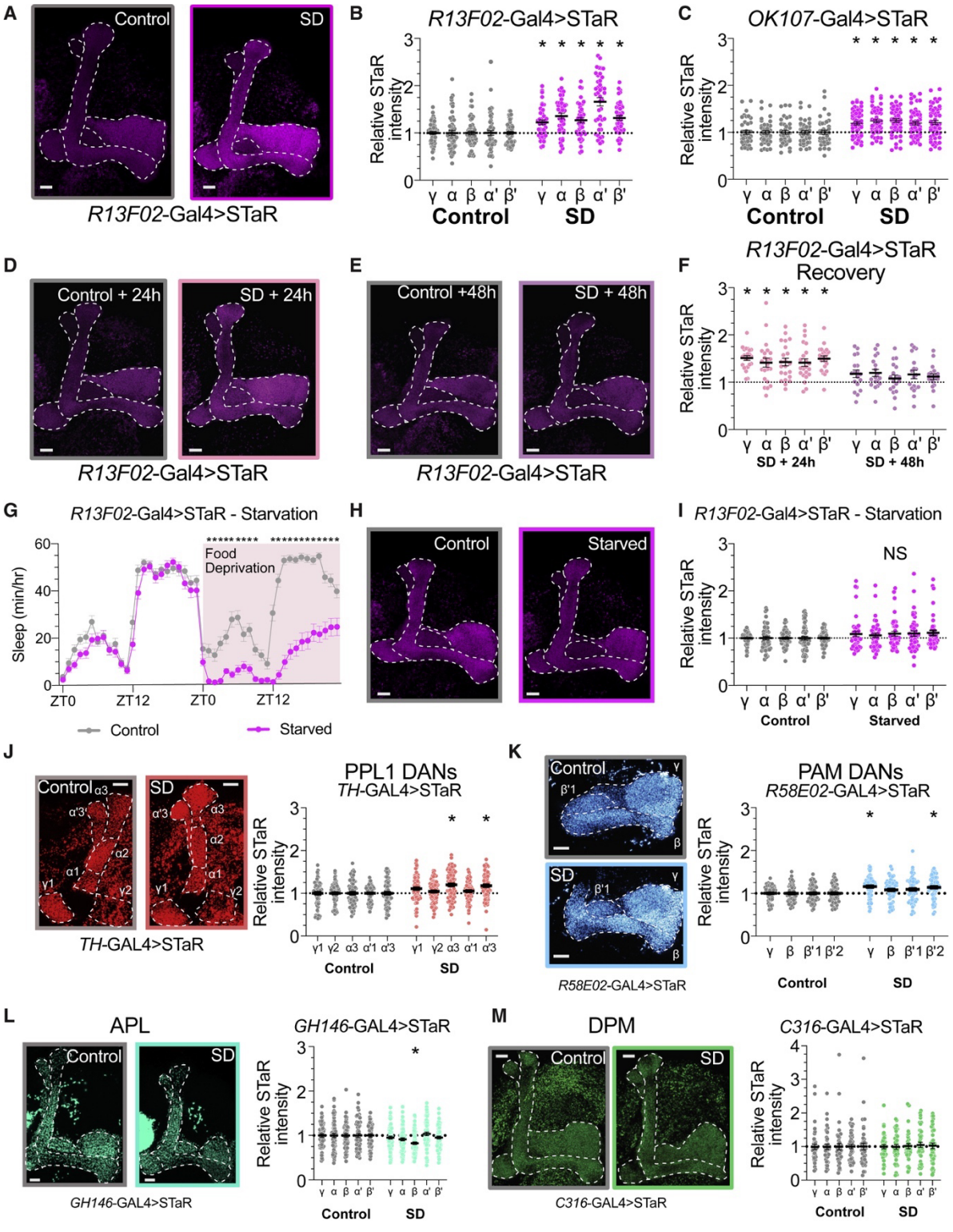
A
Control
SD
R13F02-Gal4>STaR
B
R13F02-Gal4>STaR
Relative STaR intensity
γ α β α' β' γ α β α' β'
Control
SD
C
OK107-Gal4>STaR
Relative STaR intensity
Control
SD
D
Control + 24h
SD + 24h
R13F02-Gal4>STaR
E
Control +48h
SD + 48h
R13F02-Gal4>STaR
F
R13F02-Gal4>STaR
Recovery
Relative STaR intensity
SD + 24h
SD + 48h
G
R13F02-Gal4>STaR - Starvation
Sleep (min/hr)
Food Deprivation
ZT0
ZT12
ZT0
ZT12
Control
Starved
H
Control
Starved
I
R13F02-Gal4>STaR - Starvation
NS
Relative STaR intensity
Control
Starved
J
Control
SD
TH-GAL4>STaR
PPL1 DANs
TH-GAL4>STaR
Relative STaR intensity
γ1 γ2 α3 α'1 α'3 γ1 γ2 α3 α'1 α'3
Control
SD
K
Control
SD
R58E02-GAL4>STaR
PAM DANs
R58E02-GAL4>STaR
Relative STaR intensity
γ β β'1 β'2 γ β β'1 β'2
Control
SD
L
APL
Control
SD
GH146-GAL4>STaR
GH146-GAL4>STaR
Relative STaR intensity
Control
SD
M
DPM
Control
SD
C316-GAL4>STaR
C316-GAL4>STaR
Relative STaR intensity
Control
SD

Figure 2.3: Increased BRP abundance in Kenyon cell axons after sleep deprivation

(A) Representative images from *R13F02*-Gal4>STaR flies after 12 hours of rest (left) or 12 hours of overnight SD (right). Presynapses labelled by STaR (BRP::V5) in magenta.

(B) Quantification of BRP::V5 intensity in rested controls (gray) and after overnight SD (magenta) in KCs labeled by *R13F02*-Gal4. Two-way ANOVA finds a significant effect of SD ($F_{(1,94)}$=43.43, p<0.0001, n=42-54 hemispheres/group).

(C) Quantification of BRP::V5 intensity in rested controls (gray) and after overnight SD (magenta) in KCs labeled by *OK107*-Gal4. Two-way ANOVA finds a significant effect of SD ($F_{(1,94)}$=19.82, p<0.0001, n=42-54 hemispheres/group).

(D-E) Representative images from *R13F02*-Gal4>STaR flies following 24- (D) or 48-hours (E) of recovery sleep from overnight SD.

(F) BRP::smFP_V5 intensity quantification for *R13F02*-Gal4>STaR flies permitted 24- or 48-hours of ad lib recovery sleep following overnight sleep deprivation. Fluorescence intensity is normalized to time-matched rested controls for each SD group. Two-way ANOVA finds a significant effect of group ($F_{(3,82)}$=21.11, p<0.0001, n=18-24 hemispheres/group). * represents p<0.05 by Sidak's pairwise comparisons test for SD vs control at the matched timepoint.

(G) Hourly sleep timecourse from *R13F02*-Gal4>STaR flies that were provided 24h of baseline sleep before either control handling (grey) or food deprivation (magenta). Two-way Repeated Measures ANOVA finds a significant time-by-treatment interaction ($F_{(47,3760)}$=20.51, p<0.0001, n=39-43 flies/group).

(H) Representative images from *R13F02*-Gal4>STaR flies after control handling (left) or 24h of food deprivation (right).

(I) Quantification of BRP::smFP_V5 abundance in MB lobes of *R13F02*-Gal4>STaR flies that have been fed standard fly media (grey) or starved for 24h (magenta). Two-way repeated measures ANOVA finds no significant effect of starvation ($F_{(1,92)}$=3.229, p=0.0756, n=41-53 hemispheres/group).

(J) Left panel depicts representative images from *TH*-Gal4>STaR flies after 12 hours of rest (left) or 12 hours of overnight SD (right). Presynapses labelled by STaR (BRP::V5) in red. Right panel shows quantification of BRP::smFP_V5 intensity in rested controls (gray) and after overnight SD (red) in PPL1 dopaminergic neurons labeled by *TH*-Gal4. Two-way ANOVA finds a significant sleep by MB compartment ($F_{(4,556)}$=6.184, p<0.0001, n=69-72 hemispheres/group).
(K) Left panel: representative images from *R58E02*-Gal4>STaR flies labeling BRP in PAM dopaminergic neurons after 12 hours of rest (left) or 12 hours of overnight SD (right). Pre-synapses labelled by STaR (BRP::V5) in blue. On right, quantification of BRP::smFP_V5 intensity in rested controls (gray) and after overnight SD (blue) in PAM DANs labeled by R58E02-Gal4. Two-way ANOVA finds a significant effect of SD ($F_{(1,104)}$=7.893, p=0.0059, n=50-56 hemispheres/group).
(L) Left, Representative images from *GH146*-Gal4>STaR flies after 12 hours of rest (left) or 12 hours of overnight SD (right). Presynapses labelled by STaR (BRP::V5) in green. Right panel shows quantification of BRP::smFP_V5 intensity in rested controls (gray) and after overnight SD (green) in APL labeled by *GH146*-Gal4. Two-way ANOVA finds a significant lobe by sleep interaction ($F_{(4,480)}$ = 6.672, p<0.0001, n=60-62 hemispheres/group).
(M) On left, representative images from *C316*-Gal4>STaR flies after 12 hours of rest (left) or 12 hours of overnight SD (right). Presynapses labelled by STaR (BRP::smFP_V5) in green. Right panel depicts quantification of BRP::V5 intensity in rested controls (gray) and after overnight SD (green) in DPM labeled by *C316*-Gal4. Two-way ANOVA finds no significant effect of SD ($F_{(1,79)}$=0.04082, p=0.84, n=40-41 hemispheres/group).
See also **Figure S2** for sleep traces from experimental groups shown in **Figure 2.3 A-J**, and **Figure S2.3** for pre-synaptic BRP quantification from subsets of KC neurons.
Scale bars depict 10 μm; error bars represent SEM for all panels.

2.4.4: Divergent consequences of sleep loss on KC output synapse classes

Within the MB lobes, KCs synapse upon several classes of post-synaptic partners, opening the possibility that sleep loss may differentially alter contacts between KCs and each synaptic target. While connections between KCs and MBONs can encode associative memories and odor valence, KCs also provide synaptic inputs to APL and DPM interneurons, and to DANs from both the PAM and PPL1 clusters [79] (See **Figure 2.4A** for MB circuit schematic). Each of these synaptic connections contributes to different aspects of olfactory processing and memory encoding [29,34–36,38,43–46]. To understand how each type of output synapse from KCs might be influenced by sleep loss, we used GFP Reconstitution across Synaptic Partners (GRASP) [80,81] to observe synaptic contacts between KCs and their various synaptic targets in rested and sleep deprived flies (Sleep traces shown in **Figure S2.5-6**). GRASP has previously been used to identify patterns of synaptic contacts in worms [82–84], flies [81,85–88], and mice [89,90] using light microscopy. Here, we expressed an activity-dependent GRASP reporter to label recently active contacts in which KCs release neurotransmitter onto a synaptic partner of interest [81]. First, we observed the effects of sleep deprivation on connectivity from KCs to DANs, which is required for memory formation [40]. Interestingly, GRASP signal from KCs (*MB*-LexA) to PPL1 DANs (*TH*-Gal4) was significantly depressed in the brains of sleep deprived flies **(Figures 2.4B-C)** while no effect of sleep loss could be detected on GRASP signal from KCs to PAM DANs (*R58E02*-Gal4) **(Figures 2.4D-E)**. Next, we used GRASP to measure synaptic contacts from KCs (*MB*-LexA) to the APL (*GH146*-Gal4) and DPM (*C316*-Gal4) interneurons. KC>APL GRASP signal was increased across the MBs of sleep-deprived flies **(Figures 2.4F-G)**, while KC>DPM GRASP was significantly decreased in the γ, α', and β' lobes following overnight sleep loss **(Figures 2.4H-I)**. These results suggest that KC output synapses are not all modulated uniformly during sleep loss, but rather that each KC>interneuron connection may be regulated independently. Interestingly, individual connection types show relatively consistent changes across

compartments and lobes of the KC axons, indicating that subpopulations of KCs may share plasticity rules that influence which connections are altered during prolonged waking.

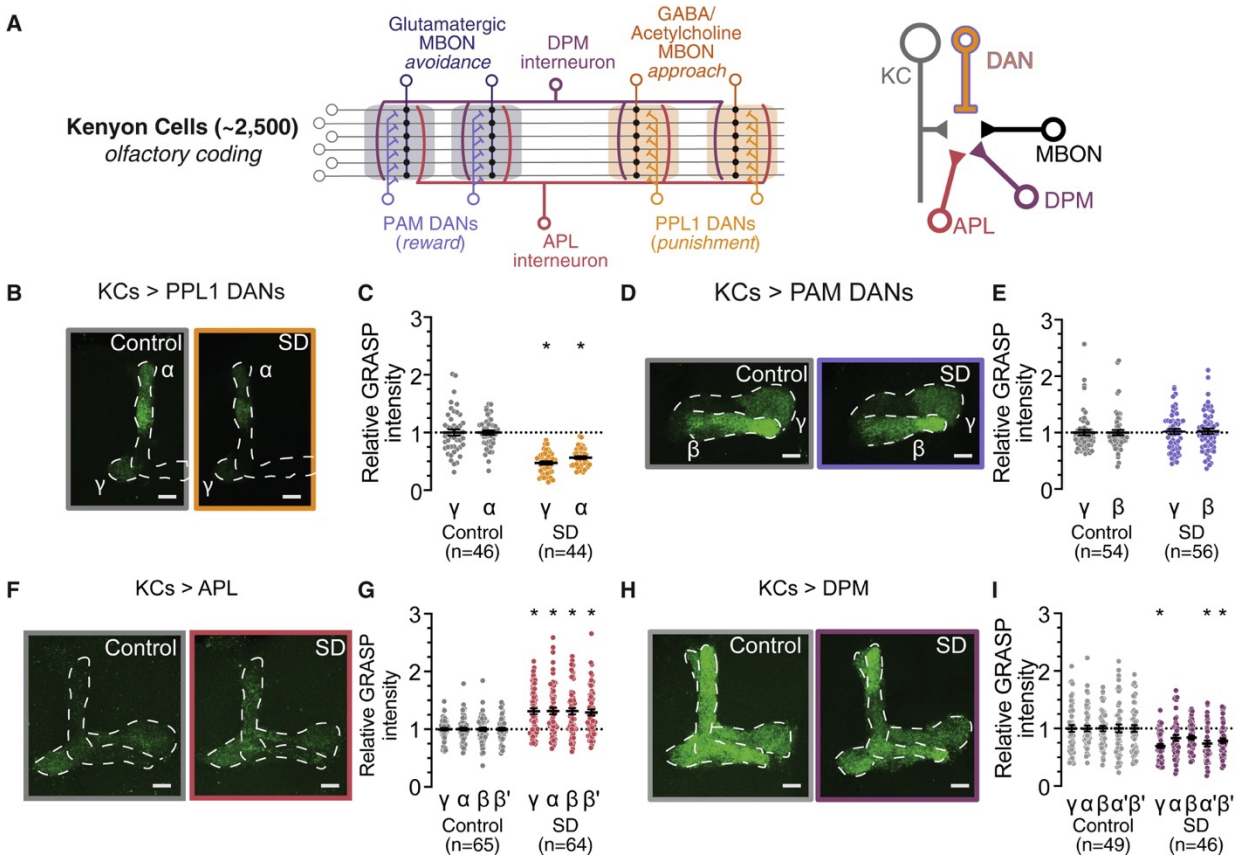

Figure 2.4: Effects of SD on synaptic contacts between KCs and DANs, APL & DPM

(A) Schematic of connectivity between neuronal cell types in the MB. KC axons innervate tiled zones (depicted by shaded regions) that each receive innervation from distinct DANs and provide input to unique MBONs. APL and DPM interneurons receive input from and provide recurrent feedback to KC pre-synapses (Left). KC pre-synapses project onto MBON, DAN, APL, and DPM partners (Right).

(B) Representative images of nsyb GRASP intensity between presynaptic KCs (*MB*-LexA) and postsynaptic PPL1 DANS (*TH*-Gal4) in rested controls (left) and in flies subjected to overnight SD (right).

(C) Quantification of relative KC>PPL1 GRASP intensity after SD (orange) normalized to rested controls (gray). Two-way ANOVA finds a significant effect of SD ($F_{(1,88)}$=91.81, p<0.0001, n=44-46 hemispheres/group).

(D) Representative images of nsyb GRASP intensity between presynaptic KCs (*MB*-LexA) and postsynaptic PAM DANs (*R58E02*-Gal4) in rested controls (left) and in flies subjected to overnight SD (right).

(E) Quantification of relative KC>PAM GRASP intensity in γ and β lobes after SD (purple), normalized to rested controls (gray). Two-way ANOVA finds no significant effect of SD ($F_{(1,108)}$=0.09979, p=0.7527, n=54-56 hemispheres/group).

(F) Representative images of *nsyb* GRASP intensity between presynaptic KCs (*MB*-LexA) and postsynaptic APL (*GH146*-Gal4) in the MB lobes of rested controls (left) and in flies subjected to overnight SD (right).

(G) Quantification of relative KC>APL GRASP intensity after SD (red), normalized to rested controls (gray). Two-way ANOVA finds a significant effect of SD ($F_{(1,127)}$=30.17, p<0.0001, n=64-65 hemispheres/group)

(H) Representative images of *nsyb* GRASP intensity between presynaptic KCs (*MB*-LexA) and postsynaptic DPM (*C316*-Gal4) in rested controls (left) and in flies subjected to overnight SD (right).

(I) Quantification of relative KC>DPM GRASP intensity after SD (magenta), normalized to rested controls (gray). Two-way ANOVA finds a significant effect of SD ($F_{(1,93)}$=11.42, p=0.0011, n=46-49 hemispheres/group)

See also **Figure S2.4** for sleep traces from experimental groups shown in **Figure 2.3 B-I.**

Scale bars depict 10 μm; error bars represent SEM for all panels.

Memories of associative conditioning are encoded within plastic connections between odor-coding KCs and MBONs that innervate individual MB lobe compartments [34,38]. To test the effects of sleep loss on KC>MBON connections, we measured GRASP signal to quantify contacts between KCs (*MB*-LexA) and MBONs in several compartments. As shown in **Figure 2.5**, KC>MBON connections varied across different MB compartments. Sleep deprived flies (Sleep data shown in **Figure S2.5)** showed consistently elevated GRASP signal from KCs to MBON-α'1 **(Figures 2.5A-B)** and from KCs to MBON-α2 **(Figures 2.5C-D)**, but decreased GRASP between KCs and MBON-γ5β'2a **(Figures 2.5E-F)** and from KCs and MBON-γ2a'1 **(Figures 2.5G-H)**. Other KC to MBON synapses, including those to MBON-γ4>γ1γ2 **(Figure 2.5I-J)**, MBON-β'2mp,γ5β'2a **(Figure 2.5K-L)**, and MBON-γ1pedc **(Figure 2.5M-N)** are unchanged after overnight sleep deprivation. While discrete MBON subsets produce different neurotransmitters, the neurotransmitter identity of an MBON does not seem to determine pre-synaptic effects of sleep loss (**Figure 2.5**; orange groups denote cholinergic MBONs, dark blue shows glutamatergic, and light blue represents GABAergic [29]). These results suggest that KC>MBON connections are altered during sleep deprivation in a compartment-by-compartment manner. Further studies will be required to understand the rules that govern the variations in plasticity across compartments, as they are not cleanly predicted by the role of an MBON in encoding valence, or by the neurotransmitters produced by individual MBONs. Connections from KCs to MBON-γ5β'2 and to MBON-γ2a'1, for instance, are both reduced after sleep deprivation, but each expresses a different neurotransmitter and activation of each MBON can result in opposing changes in sleep and behavioral valence [34,53]. As shown in our experiments using $Brp^{MI02987\text{-}GFSTF}$ in Figure 1, recent sleep history can bidirectionally influence active zone protein abundance in the MB lobes. To test whether acute sleep induction drives changes that are opposite to those observed after sleep deprivation, we pharmacologically increased sleep using the GABA-A receptor agonist THIP and imaged two pairs of KC>MBON connections. In both genotypes, 6h of THIP administration yielded highly significant increases in sleep **(Figures**

2.5O-P, left panels). While sleep loss increased KC>MBON- a'1 GRASP and decreased KC>MBON-γ5β'2 GRASP **(Figures 2.5B, F)**, 6h of sleep-promoting THIP treatment reduced GRASP signal in both KC>MBON connections **(Figures 2.5O-P, right panels)**. Increased sleep, therefore, may not solely drive synaptic changes that are the converse to those that occur during sleep loss.

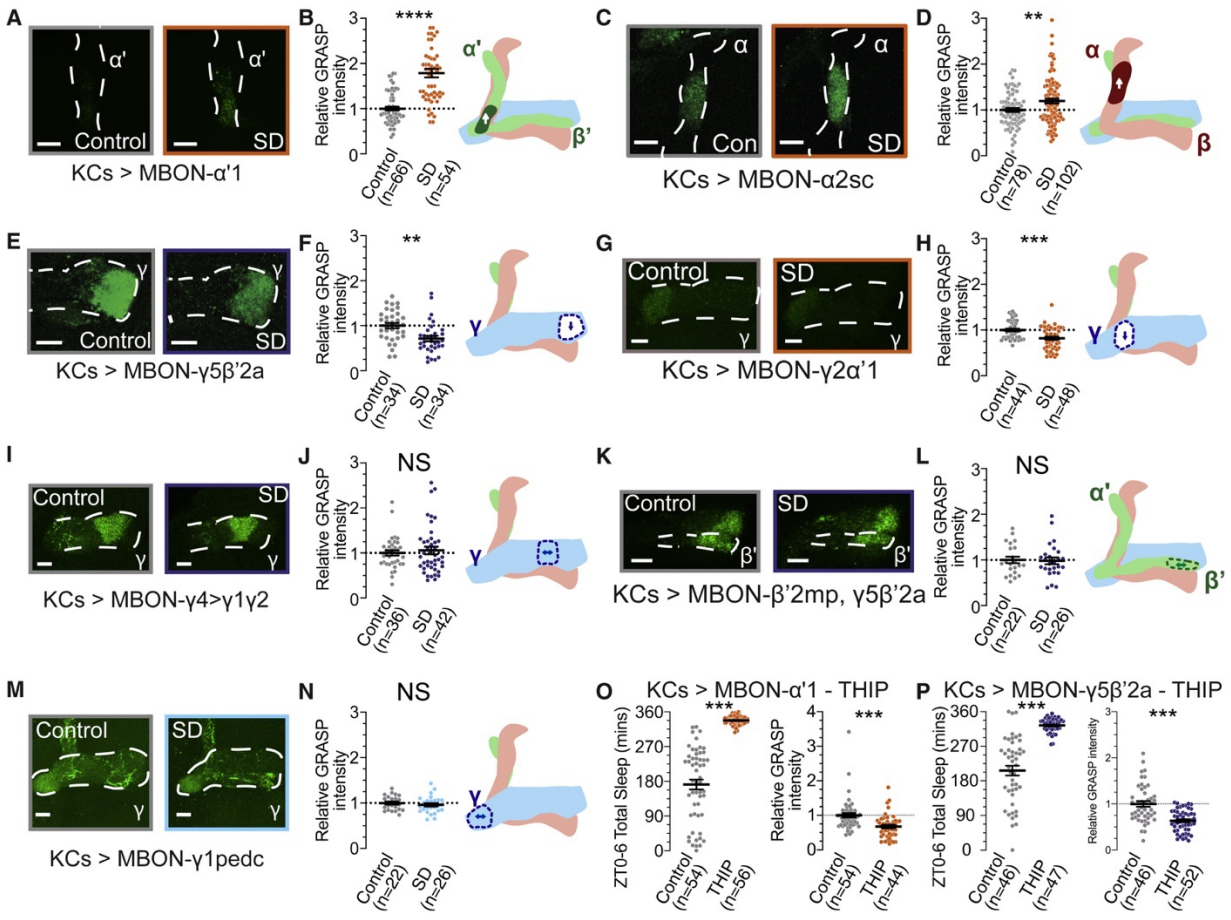

Figure 2.5: KC>MBON connections exhibit compartment-specific changes with SD

(A) Representative images of *nsyb* GRASP intensity between presynaptic KCs (*MB*-LexA) and postsynaptic MBON-α'1 (*MB543B*-Gal4) in rested controls (left) and in flies subjected to overnight SD (right).

(B) Quantification of relative KC>MBON-α'1 GRASP intensity after SD (orange), normalized to rested controls (gray). Two-tailed T-test finds a significant effect of SD (t=8.068, p<0.0001, n=54-66 hemispheres/group).

(C) Representative images of *nsyb* GRASP intensity between presynaptic KCs (*MB*-LexA) and postsynaptic MBON-α2sc (*R71D08*-Gal4) in rested controls (left) and in flies subjected to overnight SD (right).

(D) Quantification of relative KC>MBON-α2sc GRASP intensity after SD (orange), normalized to rested controls (gray). Two-tailed T-test finds a significant effect of SD (t=2.800, p=0.0057, n=78-102 hemispheres/group).

(E) Representative images of *nsyb* GRASP intensity in the γ5 compartment between presynaptic KCs (*MB*-LexA) and postsynaptic MBON-γ5β'2a (*R66C08*-Gal4) in rested controls (left) and in flies subjected to overnight SD (right).

(F) Quantification of relative KC>MBON-γ5β'2a GRASP intensity in the γ5 compartment after SD (blue), normalized to rested controls (gray). Two-tailed T-test finds a significant effect of SD (t=3.411, p=0.0011, n=34 hemispheres/group).

(G) Representative images of *nsyb* GRASP intensity in the γ2 compartment between presynaptic KCs (*MB*-LexA) and postsynaptic MBON-γ2α'1 (*R25D01*-Gal4) in rested controls (left) and in flies subjected to overnight SD (right).

(H) Quantification of relative KC>MBON-γ2α'1 GRASP intensity in the γ2 compartment after SD (orange), normalized to rested controls (gray). Two-tailed T-test finds a significant effect of SD (t=3.793, p=0.0003, n=44-48 hemispheres/group).

(I) Representative images of *nsyb* GRASP intensity in the γ4 compartment between presynaptic KCs (*MB*-LexA) and postsynaptic MBON-γ4>γ1γ2 (*MB434B*-Gal4) in rested controls (left) and in flies subjected to overnight SD (right).

(J) Quantification of relative KC>MBON-γ4>γ1γ2 GRASP intensity in the γ4 compartment after SD (blue), normalized to rested controls (gray). Two-tailed T-test finds no significant effect of SD (t=0.5245, p=0.6015, n=36-42 hemispheres/group).

(K) Representative images of *nsyb* GRASP intensity in the β'2 compartment between presynaptic KCs (*MB*-LexA) and postsynaptic MBON-β'2mp, γ5β'2a (*MB011B*-Gal4) in rested controls (left) and in flies subjected to overnight SD (right).

(L) Quantification of relative KC>MBON-β'2mp, γ5β'2a GRASP intensity in the β'2 compartment after SD (blue), normalized to rested controls (gray). Two-tailed T-test finds no significant effect of SD (t=0.1928, p=0.8480, n=22-26 hemispheres/group).

(M) Representative images of GRASP labelling from presynaptic KCs (*MB*-LexA) and postsynaptic MBON-γ1pedc (*R12G04*-Gal4) in rested controls (left) and flies dissected after overnight sleep loss (right).

(N) Relative quantification of KC>MBON-γ1pedc GRASP intensity in the γ1 compartments of rested (gray) and sleep deprived (light blue) brains. Two-tailed T-test finds no significant effect of SD (t=0.7659, p=0.4476, n=22-26 hemispheres/group).

(O) Left; sleep totals for KC>MBON-α'1 GRASP flies either fed standard fly media (gray) or 0.1 mg/mL THIP (orange). Right; Relative KC>MBON-α'1 GRASP intensity for groups shown in left panel (gray depicts vehicle controls, orange shows 6h treatment with 0.1mg/mL THIP). Two-tailed T-tests find significant effects of THIP treatment on sleep (t=12.95, p<0.0001, n=54-56) and GRASP abundance (t=3.906, p=0.0002, n=44-54 hemispheres/group).

(P) Left; 6h sleep amount for control (gray) and THIP-treated (blue; 0.1mg/mL THIP) KC>MBON-γ5β'2a GRASP flies. Right; Relative intensity of KC>MBON-γ5β'2a GRASP signal in control flies (gray) and flies fed THIP for 6h prior to dissection. Two-tailed T-test finds a significant effect of THIP treatment on sleep (t=10.14, p<0.0001, n=44-47) and on KC>MBON-γ5β'2a GRASP intensity (t=5.492, p<0.0001, n=46-52).

See also **Figure S2.5** for sleep traces from experimental groups in **(A)**-(**L)**.

Scale bars depict 10 μm; error bars represent SEM for all panels.

2.5: Discussion

2.5.1: Effects of sleep loss on abundance of presynaptic proteins in the MB

In this study, we use genetic reporters to quantify the effects of sleep loss on pre-synaptic active zone markers and putative synaptic contacts in the *Drosophila* MB lobes. We find that abundance of Brp, dSyd-1, and Cacophony broadly increase across all MB lobes after overnight sleep deprivation, and that acutely increasing sleep for six hours is sufficient to reduce Brp levels across the α, β, γ, and β' lobes. KCs strongly contribute to the increase in Brp across each MB lobe following sleep loss, while pre-synapses of other MB cell types are less sensitive to sleep disruption. Because release of *Drosophila* neuromodulators likely occurs through a combination of classical neurotransmission and extrasynaptic release [91], our studies do not rule out the possibility that BRP-independent secretion of dopaminergic dense core vesicles might be altered in the MB lobes by sleep loss. The elevated levels of Brp present in KCs of sleep-deprived flies returns to control levels within 48h of *ab libitum* recovery sleep. While associative learning can recover within only a few hours after sleep deprivation[3], our studies indicate that some synaptic consequences of prolonged waking may persist for at least 24h of recovery. These findings parallel those from humans and rodents suggesting that some measures of cognition and neurophysiology recover rapidly after acute sleep loss while others last much longer, even for several days in some cases [92–96]. The tractability of *Drosophila* may provide opportunities for future studies to investigate the processes that mediate recovery from sleep loss and to test whether similar trends in plasticity occur in other neuropil regions across the brain.

Interestingly, sleep deprivation does not seem to increase other active zone components; Rim and Syt1 only show localized changes in some MB lobes, and the primarily post-synaptic marker Dlg shows no significant changes across the MB after sleep loss.

Additionally, we find that the abundance of vesicular proteins Rab3 and nSyb decreases across all MB lobes following overnight sleep deprivation. The varying responses between pre-synaptic components may indicate that sleep-deprivation may alter the abundance of some active zone constituents along differing time courses, or that active zone release machinery may be regulated differently than synaptic vesicle pools. The varied responses of each synaptic reporter that we observe suggests that Brp, dSyd-1, and Cac levels may underlie the consequences of sleep loss on MB functioning, but the precise physiological consequences of these changes on KC neurotransmitter release are unclear. Previous work finds that increasing BRP gene copy number drives changes in other active zone proteins that recapitulate protein levels observed in short sleeping mutants, and also increases sleep in a dose-dependent manner [21]. It is tempting to speculate that increases in Brp with sleep loss may drive concomitant increases in some core active zone scaffolding components, and compensatory decreases in some proteins regulating synaptic vesicle release. Experiments at the *Drosophila* larval NMJ indicate that elevated Brp levels increase the rate of spontaneous release and enhance facilitation with pairs of stimuli, while other markers of synapse strength, including the amplitudes of evoked and spontaneous junction potentials, remained unchanged [21]. It is unclear whether acute changes in Brp with sleep loss induce the same physiological changes at MB-output synapses, and additional studies will be required to understand how plastic mechanisms that contribute to memory formation might be altered by the pre-synaptic changes described above. Recent work finds that pan-neuronal knockdown of dSyd-1 can reduce sleep and dampen homeostatic rebound, even in flies with elevated BRP [21]. Consistent with the idea that dSyd-1 levels may influence sleep pressure, we observed decreased dSyd-1$^{MI05387\text{-}GFSTF}$ abundance in previously sleep-deprived flies after 48h of recovery.

2.5.2: Effects of sleep loss on KC synaptic contacts

While the MB contains several different cell types, pre-synapses in the axons of KCs appear to be uniquely plastic during sleep loss. Our use of an activity-dependent fluorescent GRASP reporter of synaptic contacts observed that sleep loss altered synaptic contacts between KCs and distinct post-synaptic partners in different ways [81]. Among these changes, we found that GRASP fluorescence reporting contacts from KCs to PPL1 DANs is strongly decreased after sleep loss, indicating a weakening of the KC>PPL1 DAN contacts. Interestingly, these connections may be vital for recurrent activation within MB compartments during learning and could contribute to prediction error signals [40]. While further studies will be required to examine the contribution of these particular connections to learning deficits after sleep loss, human subjects have been reported to exhibit impaired error prediction and affective evaluation in learning tasks following sleep loss [97]. Because we observed reduced GRASP signal in KC>PPL1 DAN connections, which mediate aversive reinforcement [39], and not in KC>PAM DAN connections, which influence appetitive reinforcement [36], it is also possible that sleep loss may not equally degrade the encoding of reinforcement signals across all valences or modalities. Recent findings also suggest that not all forms of memory require sleep for consolidation; appetitive olfactory memories can be consolidated without sleep when flies are deprived of food, and sleep-dependent and -independent memory traces in these conditions are stored in separate MB zones [98]. We find that the KC>MBON connections that contribute to sleep-dependent memory (KC> γ2α’1) also show an overall decrease in GRASP signal with sleep loss, while those that are vital for sleep-independent memory (MBON-γ1pedc) show no GRASP change after sleep deprivation. These compartment-specific variations in the effects of sleep on both memory and synaptic distribution further indicate that local MB zones may follow distinct plasticity rules under physiological stressors, including sleep loss.

Additionally, GRASP signal from KCs to APL is significantly elevated following sleep loss, suggesting a strengthening of KC>APL connections. KCs and APL form a negative

feedback circuit, where KCs activate APL, and APL inhibits KCs; this feedback inhibition maintains sparseness of odor coding and odor specificity of memories [42]. It is possible that KCs compensate for increased synaptic abundance accumulated during sleep loss by recruiting inhibition from APL. While further experimentation is needed to examine the role of these connections in the regulation of net synaptic strength during sleep loss, sleep deprivation results in increased cortical excitability in humans and rodents [99,100], and hyperexcitability is often counteracted by increased synaptic inhibition [101,102]. Conversely, sleep loss reduces connectivity between KCs and DPM, a second large interneuron that may facilitate recurrent activity in the MB lobes [46,103].

2.5.3: MB compartment-specific plasticity rules after sleep loss

Our results also indicate that KC>MBON synaptic contacts exhibit a variety of changes in response to sleep deprivation. The specific KC>MBON connections that show significantly elevated or reduced GRASP signal here are not clearly assorted based on valence encoding, contribution to specific associative memory assays, or influence on sleep/wake regulation [34,53]. Activity in several MB cell types, including α'/β' KCs, MBON-γ5β'2, MBON-γ2 α'1, DPM, and PAM DANs regulates sleep [34,48,50–53]. The observation that KC>MBON-γ5β'2a labelling is reduced with sleep loss complements previous observations of reduced electrical activity in MBON-γ5β'2 following sleep deprivation [53]. Other sleep-promoting MB neurons, however, such as DPM[48], do not show an overall increase in BRP abundance, suggesting either that other changes in excitability, synaptic drive, or post-synaptic adaptations might drive homeostatic sleep regulation in these cells, or that distinct subsets of connections within the populations that we label here might be sleep-regulatory. The compartment-to-compartment variance in KC>MBON responses to sleep loss also parallels previous findings that plasticity rules can vary between MBONs during heterosynaptic plasticity [37]. While our GRASP results suggest diverse changes in putative synaptic contacts with sleep loss, the functional effects of these changes

require further study. It is important to note that a significant portion of MB synapses are comprised of connections between either pairs or groups of KCs [79,104]. The genetic strategies that we have used in this study have prevented reliable visualization and quantification of these connections. As a result, the effect of sleep loss on KC>KC synapses has not been examined here but may comprise a portion of the increase in KC pre-synaptic abundance that we observe in **Figure 2.3**. While our studies identify synaptic classes that exhibit altered GRASP labelling across sleep loss, future studies using super resolution imaging and/or physiology could examine the structural and molecular changes that underlie this plasticity. Connections between neurons in the MB may be also influenced by non-neuronal cell types, including astrocytes. Astrocytic contact with KCs can be reduced by sleep loss [105] and astrocytic calcium levels correlate with sleep pressure [106], which both suggest that astrocytic processes could be positioned to mediate sleep-dependent plasticity in the MB.

2.5.4: Potential roles of postsynaptic plasticity and neuronal excitability

The broad conservation of release machinery across active zones within and between cell types has simplified our examination of pre-synaptic plasticity during sleep loss. Assays of both Hebbian and homeostatic plasticity have also identified a variety of post-synaptic adaptations. Interestingly, postsynaptic densities isolated from rodent cortex show significant reorganization of post-synaptic GluR5 receptors, which depends upon the activity of Homer1 [107], and sleep-dependent phosphorylation of CaMKII and GluR1 contribute to consolidation of visual cortex plasticity [23]. Because MBONs exhibit post-synaptic plasticity during other contexts, including the formation of associative memories [37], sleep deprivation may also alter post-synaptic organization of MBONs or other cell types in the MB. Although the distribution of Dlg is not significantly changed by sleep loss, the rich variety of post-synaptic receptors for acetylcholine, dopamine, GABA, and other signals in the MB requires development of additional reporters to examine these post-synaptic consequences of insufficient sleep in MB neurons.

Additionally, while our data outline changes in pre-synaptic protein abundance and pre-synaptic KC contacts that result from sleep loss, the possibility that these synaptic changes may be accompanied by homeostatic compensation in neuronal excitability or firing patterns remains to be tested. Because sleep-deprived flies can recover the capacity to learn after only a brief nap [3], homeostatic adjustments in post-synaptic strength and/or excitability may permit MBs to compensate for pre-synaptic changes that appear to persist for at least 24 hours after sleep deprivation **(Figs. 2.3D-F)**. Further, recovery sleep or pharmacological sleep enhancement may not simply reverse the effects of sleep loss **(Figs 2.2B, 2.5O-P)** and it is unclear how particular subsets of synaptic proteins or connections may be selected for removal during times of elevated sleep.

2.5.5: Concluding remarks

The consequences of sleep loss on synaptic connectivity are not clearly understood, but previous work has found net changes in synaptic abundance or size across brain regions [16,17,20,107]. We characterize a diverse array of synaptic responses to sleep loss among different cell types within the same circuit. Our findings may suggest that distinct cell types and connections within the MB are governed by heterogeneous plasticity rules during sleep disruption. While previous studies have characterized the synaptic effects of sleep history on individual cell types within plastic circuits, our data provide a more comprehensive understanding of the consequences of sleep loss on MB circuits. While this project outlines the local effects of sleep loss on MB connectivity, it is unclear whether specific neural subsets also drive BRP increases within other neuropil compartments of sleep-deprived brains[20]. Here, we find an overall increase in the abundance of reporters for some, but not all, pre-synaptic proteins. These pre-synaptic changes are not distributed equally across all cell types; they are most pronounced in MB-intrinsic KCs. Further, output connections from KCs to different classes of synaptic partners show varying patterns of plasticity in MB sub-circuits that contribute to

encoding odor valence, comprise recurrent feedback loops, or relay reinforcement signals. Our results indicate that sleep loss may degrade MB-dependent memory by altering several different classes of synapses, but future studies will be required to test the specific roles of changes at individual synapse types and the mechanisms by which prolonged waking reorganizes MB connectivity.

2.6: Supplemental data

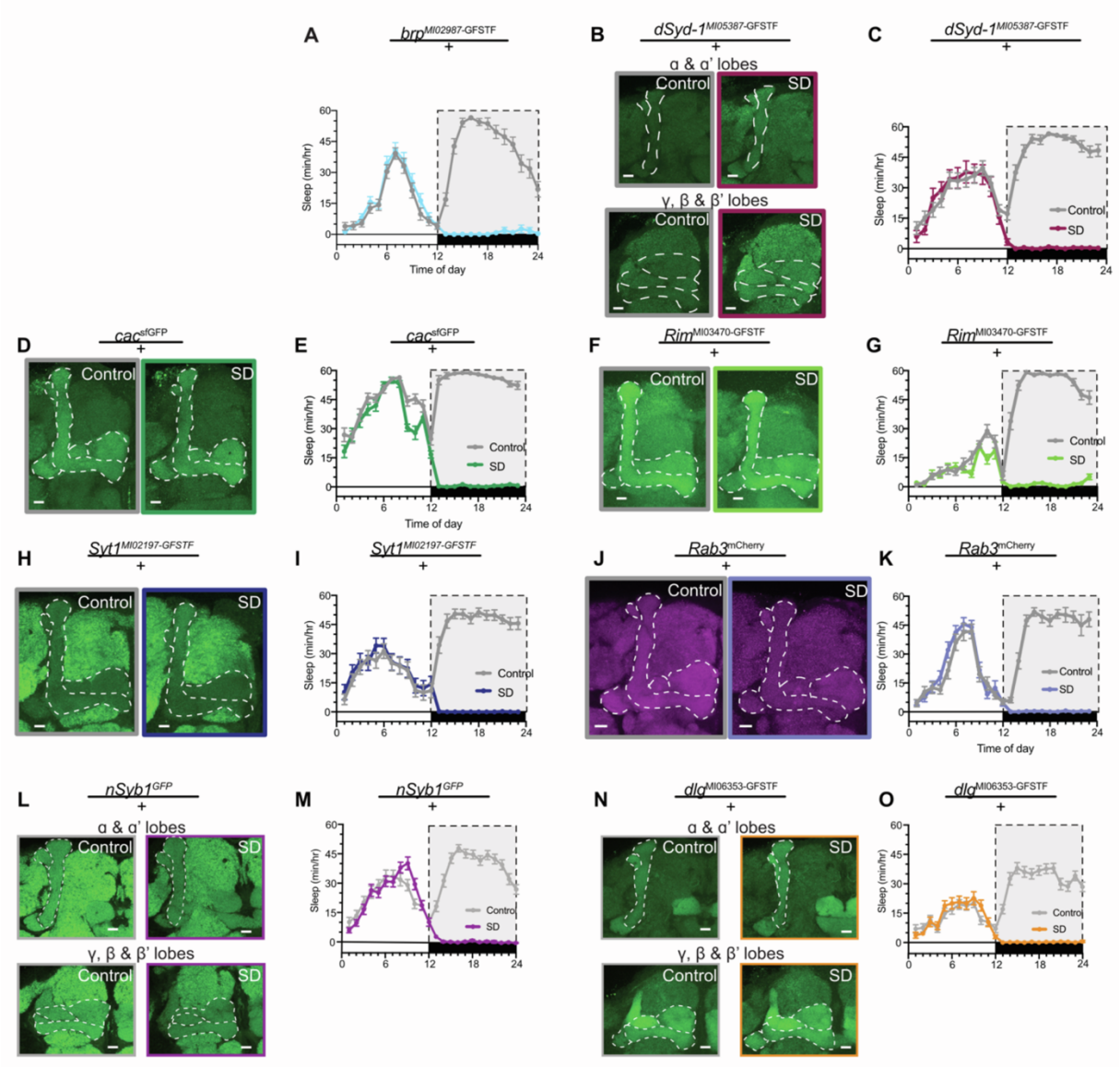

Figure S2.1: Representative images and sleep patterns of synaptic protein reporters, related to Figures 2.1-2.2

(A) Hourly sleep traces of *brp*$^{MI02987\text{-}GFSTF}$/+ flies. Flies were either allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (blue) before dissection. Two-way repeated- measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,3013)}=133.5$, $p<0.0001$, n=59 control, 74 SD).

(B-C) Example images of dSyd-1::GFP (A) and sleep traces (B) from both sleep deprived and control *dSyd-1*$^{MI05387\text{-}GFSTF}$/+ flies. Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,2553)}=100.2$, $p<0.0001$, n=64 control, 49 SD).

(D-E) Confocal images of Cac::sfGFP (C) and hourly sleep measurements (D) from *cac*^{sfGFP}/+ flies that were allowed either *ad libitum* sleep or 12-h overnight sleep deprivation before dissection. Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,2093)}=122.2$, $p<0.0001$, n=48 control, 45 SD).

(F-G) Rim::GFP images (E) and hourly sleep traces (F) from sleep deprived and control *Rim*$^{MI03470\text{-}GFSTF}$/+ flies before dissection. Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,2392)}=97.51$, $p<0.0001$, n=58 control, 48 SD).

(H-I) Example images of Syt1::GFP (G) and pre-dissection sleep traces (H) from both sleep deprived and rested control *Syt1*$^{MI02197\text{-}GFSTF}$/+ flies. Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,1932)}=84.32$, $p<0.0001$, n=37 control, 49 SD).

(J-K) Representative Rab3::mCherry images (I) and hourly sleep patterns prior to dissection (J) from *Rab3*^{mCherry}/+ flies. Two-way repeated-measures ANOVA of hourly sleep time course

finds a significant time-by-SD interaction $F_{(23,1587)}=76.46$, $p<0.0001$, n=24 control, 28 SD).

(L-M) Representative Nsyb::GFP images (I) and hourly sleep patterns before dissection (M) from *nSyb*^{GFP}/+ flies. Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,2070)}=63.05$, $p<0.0001$, n=44 control, 48 SD).

(N-O) Dlg::GFP images (N) and sleep timecourses before dissection (O) from *dlg*$^{MI06353\text{-}GFSTF}$/+ flies. Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time- by-SD interaction $F_{(23,1748)}=44.06$, $p<0.0001$, n=40 control, 38 SD).

Scale bars depict 10 μm; error bars represent SEM for all panels.

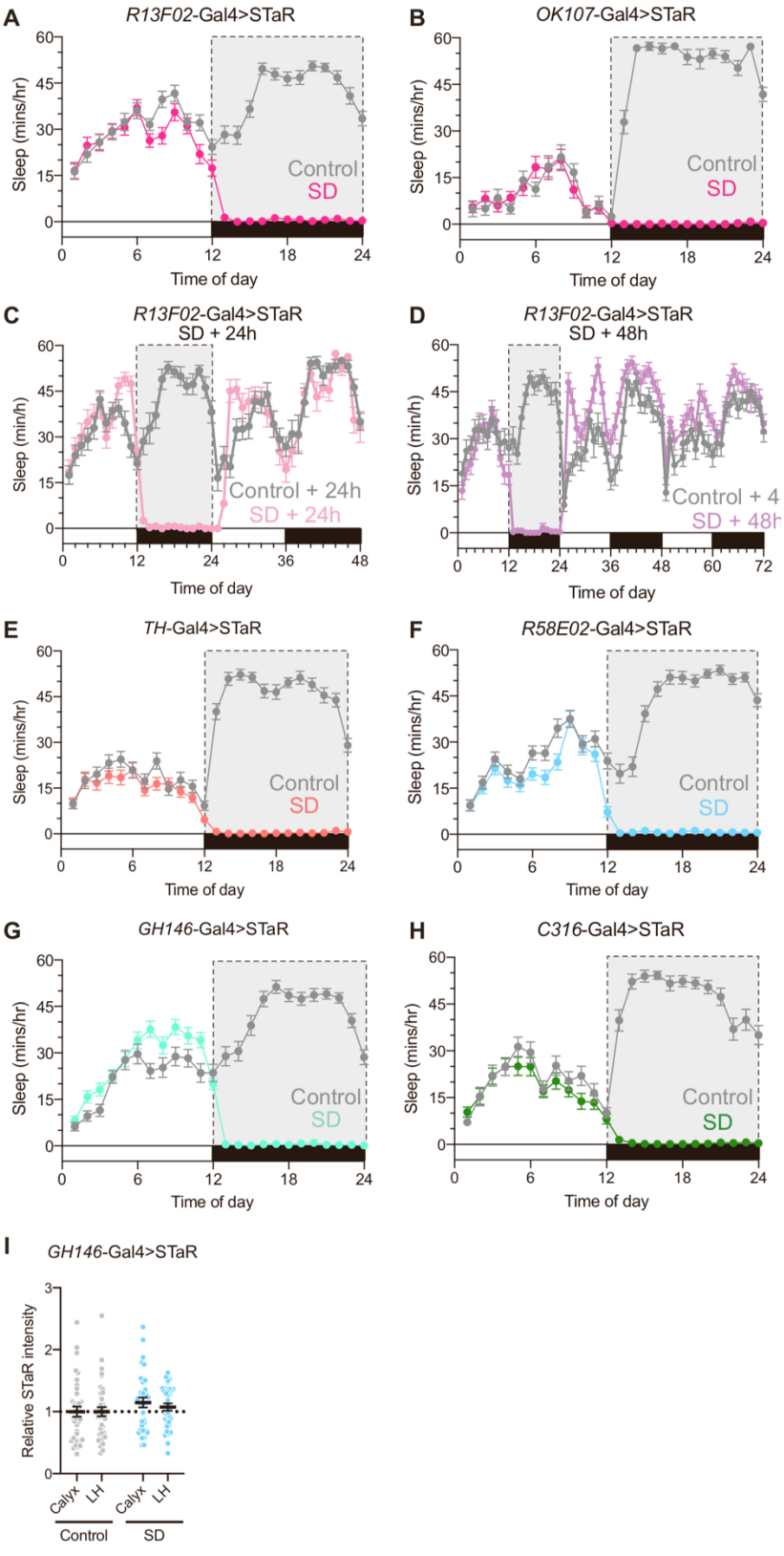
A
R13F02-Gal4>STaR
Sleep (mins/hr)
Time of day
Control
SD
B
OK107-Gal4>STaR
C
R13F02-Gal4>STaR
SD + 24h
Sleep (min/h)
Control + 24h
SD + 24h
D
R13F02-Gal4>STaR
SD + 48h
E
TH-Gal4>STaR
F
R58E02-Gal4>STaR
G
GH146-Gal4>STaR
H
C316-Gal4>STaR
I
GH146-Gal4>STaR
Relative STaR intensity
Calyx
LH
Control
SD

Figure S2.2: Sleep patterns of flies expressing STaR reporter in different MB cell types prior to dissection, related to Figure 2.3

(A) Hourly sleep traces of *R13F02*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (magenta). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction ($F_{(23,1564)}=54.88$, $p<0.0001$, n=36 control, 34 SD).

(B) Hourly sleep traces of *OK107*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (magenta). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,1012)}=95.18$, $p<0.0001$, n=24 control, 22 SD).

(C) Hourly sleep traces of *R13F02*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (pink), and 24 hours of recovery. Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(47,1222)}=27.94$, $p<0.0001$, n=15 control, 13 SD).

(D) Hourly sleep traces of *R13F02*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (pink), and 48 hours of recovery. Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(71,2414)}=21.46$, $p<0.0001$, n=20 control, 16 SD).

(E) Hourly sleep traces of *TH*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (salmon). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,2208)}=90.49$, $p<0.0001$, n=45 control, 53 SD).

(F) Hourly sleep traces of *R58E02*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (blue). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,2070)}=84.49$, $p<0.0001$,

n=44 control, 48 SD).

(G) Hourly sleep traces of *GH146*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (light green). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,2093)}=97.84$, $p<0.0001$, n=42 control, 51 SD).

(H) Hourly sleep traces of *C316*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (green). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,1886)}=68.95$, $p<0.0001$, n=35 control, 49 SD).

(I) Relative *GH146*-Gal4>STaR intensity in olfactory projection neuron axons in the MB calyces (Calyx) and lateral horn (LH). Data from rested controls shown in gray; flies dissected after overnight sleep deprivation depicted in light blue. Two-way repeated measures ANOVA finds no significant main effect for sleep deprivation ($F_{(1,70)}=1.202$, $p=0.2767$, n=34-38).

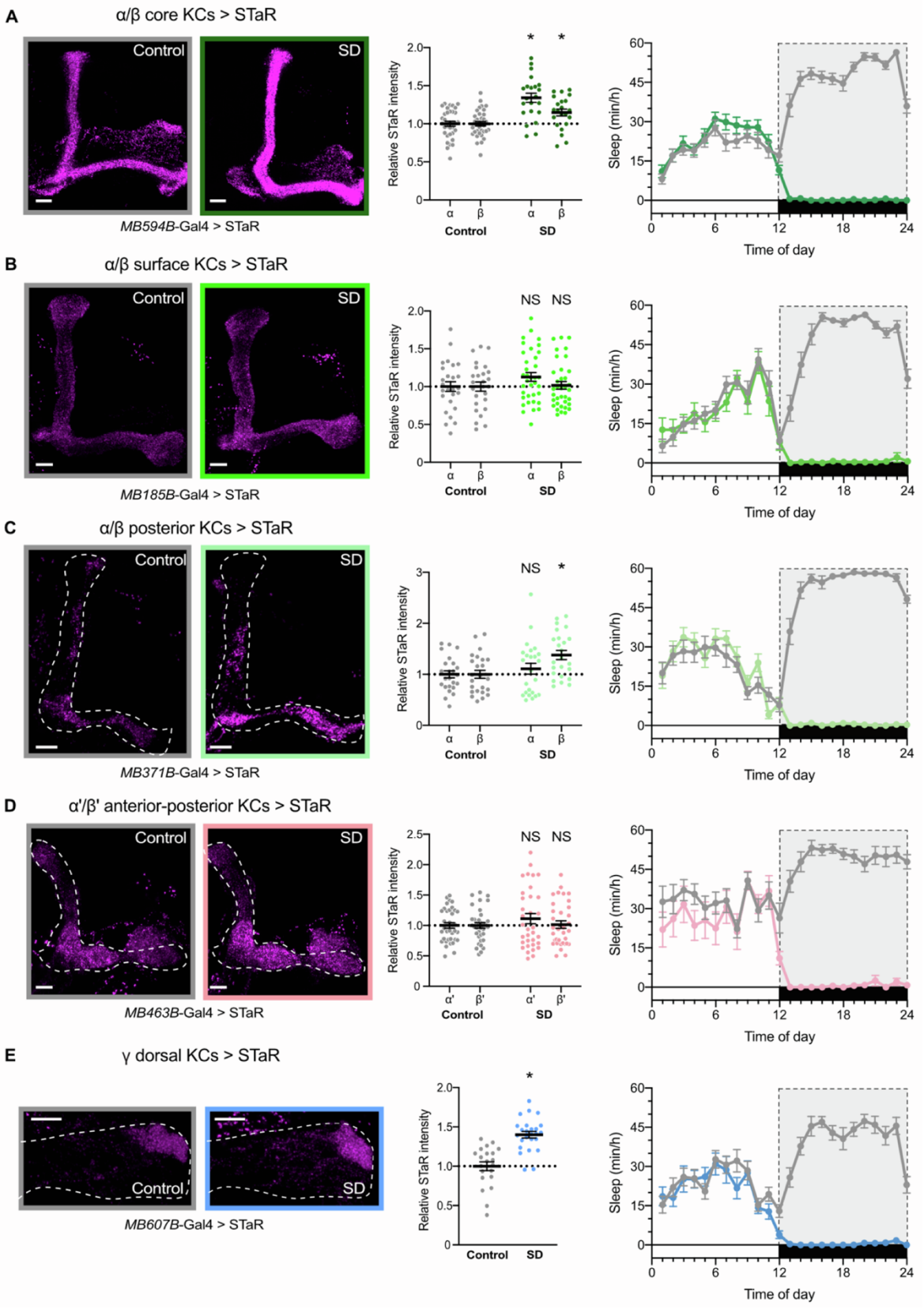
A
α/β core KCs > STaR
Control
SD
MB594B-Gal4 > STaR
Relative STaR intensity
Control
SD
Sleep (min/h)
Time of day
B
α/β surface KCs > STaR
Control
SD
MB185B-Gal4 > STaR
NS
NS
Relative STaR intensity
Control
SD
Sleep (min/h)
Time of day
C
α/β posterior KCs > STaR
Control
SD
MB371B-Gal4 > STaR
NS
*
Relative STaR intensity
Control
SD
Sleep (min/h)
Time of day
D
α'/β' anterior-posterior KCs > STaR
Control
SD
MB463B-Gal4 > STaR
NS
NS
Relative STaR intensity
Control
SD
Sleep (min/h)
Time of day
E
γ dorsal KCs > STaR
Control
SD
MB607B-Gal4 > STaR
*
Relative STaR intensity
Control
SD
Sleep (min/h)
Time of day

Figure S2.3: Effect of SD on BRP-positive punctae in KC subtypes, related to Figure 2.3

(A) Representative images (left) and quantification of BRP::V5 intensity (center) from α/β core KCs in *MB594B*>STaR flies after *ad libitum* sleep (gray) or 12 hours of overnight SD (green). Panel on right shows sleep traces for rested and sleep-deprived *MB594*>STaR flies during the 24-h prior to dissection. Two-way ANOVA finds a significant effect of SD on BRP::V5 ($F_{(1,54)}=21.52$, $p<0.0001$, n=22-34 hemispheres/group).

(B) Representative images (left) and quantification of BRP::V5 intensity (center from α/β surface KCs in *MB185B*>STaR flies after 12 hours of rest (gray) or 12 hours of overnight SD (green). Right hand panel depicts sleep patterns for *MB185B*>STaR flies during the 24-h before dissection. Two-way ANOVA finds no significant effect of SD on BRP::V5 ($F_{(1,60)}=0.9211$, $p=0.341$, n=26-36 hemispheres/group)

(C) Representative images (left), and quantification of BRP::V5 intensity (center) from α/β posterior KCs *MB371B*>STaR flies after 12 hours of rest (gray) or 12 hours of overnight SD (green). Panel on right shows sleep traces for rested and sleep-deprived *MB371B*>STaR flies during the 24-h prior to dissection. Two-way ANOVA finds a significant effect of SD ($F_{(1,46)}=4.497$, $p=0.0394$, n=24 hemispheres/group).

(D) Representative images (left) and quantification of BRP::V5 intensity (centerfrom α'/β' anterior-posterior KCs of *MB463B*>STaR flies after 12 hours of rest (gray) or 12 hours of overnight SD (pink). Sleep traces for *MB463B*>STaR from both experimental groups is shown in the right panel. Two-way ANOVA finds no significant effect of SD on BRP::V5 ($F_{(1,68)}=0.6134$, $p=0.4362$, n=34-36 hemispheres/group).

(E) Representative images (left) and BRP::V5 quantification (center) from γ dorsal neurons of *MB607B*>STaR flies after 12 hours of rest (gray) or 12 hours of overnight SD (blue). Presynapses labelled by STaR (BRP::V5) in magenta. Sleep traces for both experimental

groups are shown in right-hand panel. Two-tailed T-test finds a significant effect of SD on BRP::V5 in γ dorsal neurons (t=5.818, p<0.0001, n=21-25 hemispheres/group).

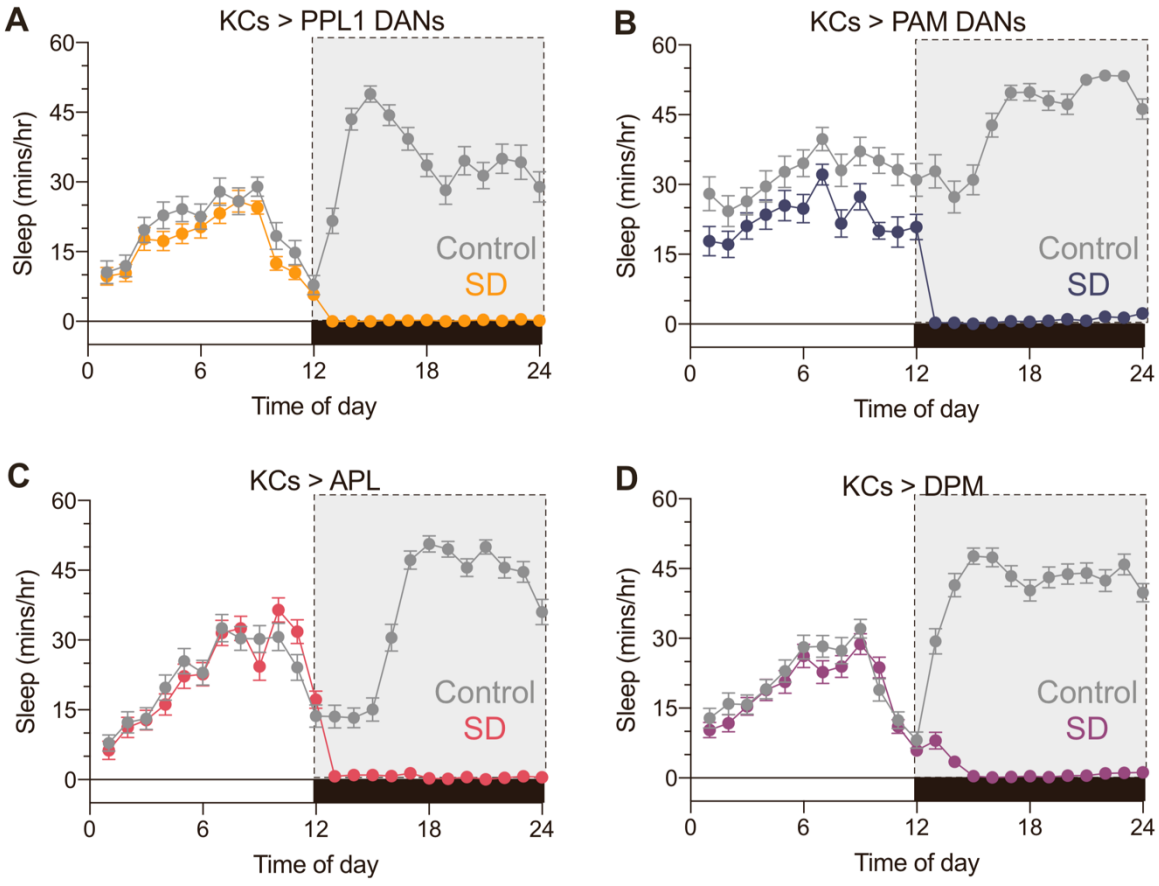

Figure S2.4: Sleep patterns of flies expressing GRASP between KCs and non-MBON cell types prior to dissection, related to Figure 2.4

(A) Hourly sleep traces of GRASP/+; *TH*-Gal4/*MB*-LexA flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (orange). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,1748)}=48.89$, p<0.0001, n=34 control, 44 SD).

(B) Hourly sleep traces of GRASP/+; *R58E02*-Gal4/*MB*-LexA flies that were allowed ad libitum sleep (light gray) or were sleep deprived overnight for 12 hours (dark gray). Two-way repeated-

measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,1978)}=49.86$, $p<0.0001$, n=42 control, 46 SD).

(C) Hourly sleep traces of *GH146*-Gal4/GRASP; *MB*-LexA/+ flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (pink). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,2208)}=69.97$, $p<0.0001$, n=42 control, 56 SD).

(D) Hourly sleep traces of GRASP/+; *C316*-Gal4/*MB*-LexA flies that were allowed ad libitum sleep (light gray) or were sleep deprived overnight for 12 hours (maroon). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(23,2806)}=77.93$, $p<0.0001$, n=69 control, 55 SD).

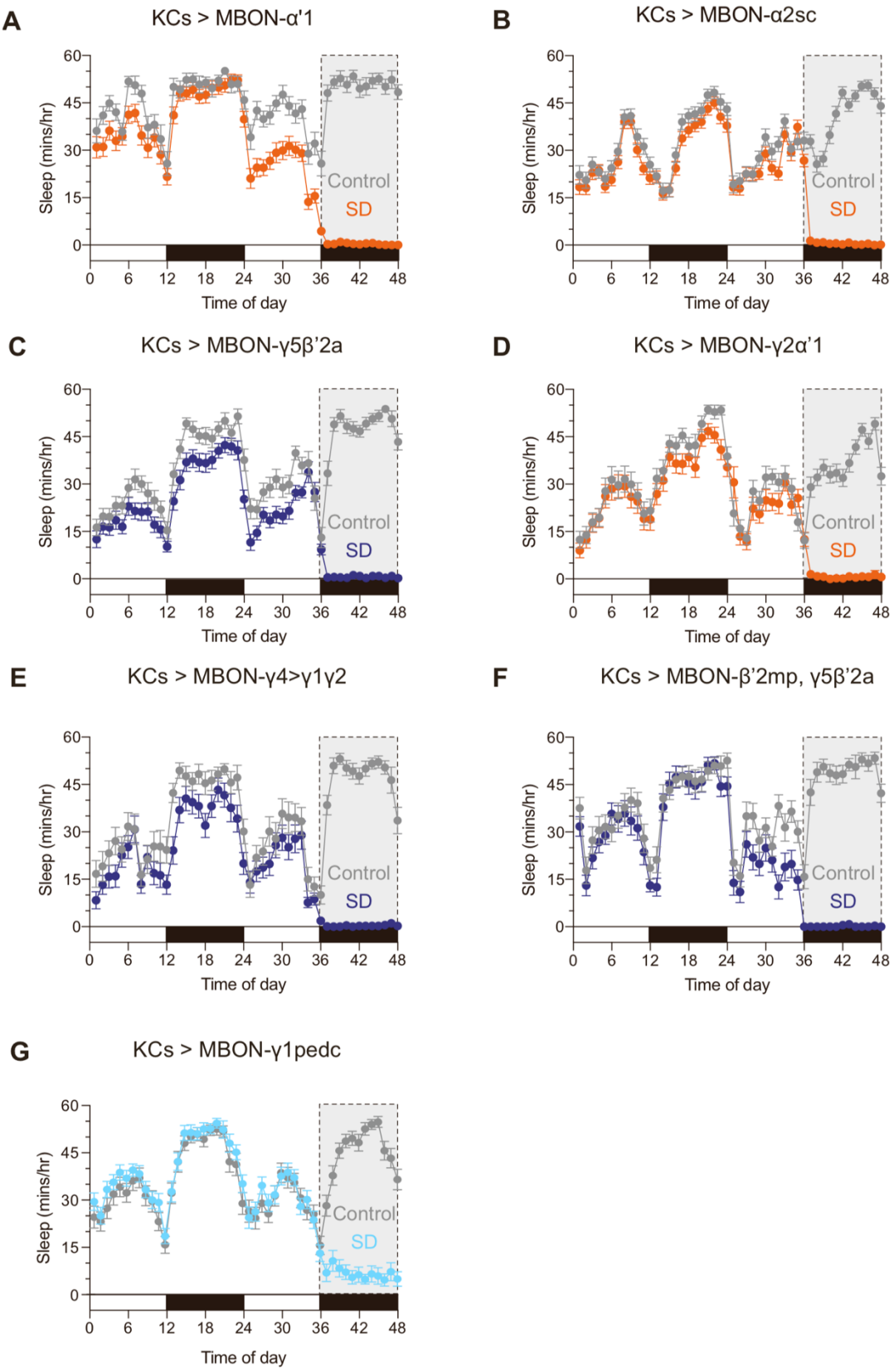
A
KCs > MBON-α'1
B
KCs > MBON-α2sc
C
KCs > MBON-γ5β'2a
D
KCs > MBON-γ2α'1
E
KCs > MBON-γ4>γ1γ2
F
KCs > MBON-β'2mp, γ5β'2a
G
KCs > MBON-γ1pedc
Sleep (mins/hr)
Time of day
Control
SD

Figure S2.5: Sleep patterns of flies expressing KC>MBON GRASP prior to dissection, related to Figure 5

(A) Hourly sleep traces of GRASP/+; *MB543B*-Gal4*/MB*-LexA flies that were allowed 24 hours of baseline sleep, followed by either ad libitum sleep (gray) or sleep deprivation overnight for 12 hours (orange). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(47,2726)}=36.6$, $p<0.0001$, n=25 control, 35 SD).

(B) Hourly sleep traces of GRASP/+; *R71D08*-Gal4*/MB*-LexA flies that were allowed 24 hours of baseline sleep, followed by either ad libitum sleep (gray) or sleep deprivation overnight for 12 hours (orange). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(47,4982)}=42.01$, $p<0.0001$, n=46 control, 64 SD).

(C) Hourly sleep traces of GRASP/+; *R66C08*-Gal4*/MB*-LexA flies that were allowed 24 hours of baseline sleep, followed by either ad libitum sleep (gray) or sleep deprivation overnight for 12 hours (dark blue). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(47,3008)}=32.57$, $p<0.0001$, n=26 control, 40 SD).

(D) Hourly sleep traces of GRASP/+; *R25D01*-Gal4*/MB*-LexA flies that were allowed 24 hours of baseline sleep, followed by either ad libitum sleep (gray) or sleep deprivation overnight for 12 hours (orange). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(47,3572)}=26.24$, $p<0.0001$, n=37 control, 41 SD).

(E) Hourly sleep traces of GRASP/+; *MB434B*-Gal4*/MB*-LexA flies that were allowed 24 hours of baseline sleep, followed by either ad libitum sleep (gray) or sleep deprivation overnight for 12 hours (dark blue). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(47,1974)}=20.94$, $p<0.0001$, n=20 control, 24 SD).

F) Hourly sleep traces of GRASP/+; *MB011B*-Gal4*/MB*-LexA flies that were allowed 24 hours of baseline sleep, followed by either ad libitum sleep (gray) or sleep deprivation overnight for 12 hours (dark blue). Two-way repeated-measures ANOVA of hourly sleep time course finds a

significant time-by-SD interaction $F_{(47,1598)}=25.25$, $p<0.0001$, n=17 control, 19 SD).

(G) Hourly sleep traces of GRASP/+; *R12G04*-Gal4/*MB*-LexA flies allowed 24h of baseline sleep followed by either ad libitum sleep (gray) or overnight sleep deprivation for 12h (light blue). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F_{(47,2538)}=34.38$, $p<0.0001$, n=27-29 flies/group).

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CHAPTER THREE

Sleep Deprivation Drives Brain-Wide Changes in Cholinergic Presynapse Abundance in *Drosophila melanogaster*

3.1: Abstract

Sleep is an evolutionarily conserved behavioral state that is essential for normal cognitive and physiological functions. While there is abundant evidence that absence of sleep results in profound deficits, sleep's function in the brain remains elusive. Previous studies suggest that sleep may benefit the brain by renormalizing synaptic strength through depression downscaling of synapses that are potentiated during wake. We have previously found that sleep loss drives an accumulation of the presynaptic protein Bruchpilot (BRP) within cholinergic Kenyon cells (KCs) within the mushroom body (MB), but not in other classes of MB neurons. While previous work has observed a net BRP increase across the whole brain after sleep loss, it is unclear whether the same cell-type specific trends in plasticity that we observe in the memory encoding MBs can be generalized to other neuropil regions of the central brain. Here, we investigate how sleep loss affects abundance of genetically labeled endogenous BRP across each neuropil region of the central brain. Because sleep deprivation can differentially affect excitatory versus inhibitory synapses, we examine the effects of mechanical sleep loss on synaptic abundance of different neurotransmitter types within each neuropil region. Our results indicate that sleep loss broadly increases cholinergic synapse abundance across many neuropil regions, while synapses of other neurotransmitter types are less sensitive to sleep loss.

3.2: Introduction

While the primary function or functions of sleep have yet to be elucidated, evidence suggests that the processes of sleep and synaptic plasticity are intertwined (Abel et al., 2013; Rasch and Born, 2013; Tononi and Cirelli, 2014). Sleep is elevated during periods of heightened synaptic organization, such as during early development (Roffwarg et al., 1966; Shaw et al., 2000; Kayser et al., 2014), recovery from neural injury (Singh and Donlea, 2020; Stanhope et al., 2020),

and memory consolidation (Walker et al., 2002; Ganguly-Fitzgerald et al., 2006). One hypothesis suggests that sleep serves to renormalize synaptic strength through the downscaling of synapses that are potentiated during wake (Tononi and Cirelli, 2014), yet other studies have reported synaptic potentiation during sleep (Frank et al., 2001; Aton et al., 2013, 2014). It is likely that sleep has varying effects on plasticity, depending on developmental stage (Roffwarg et al., 1966; Vienne et al., 2016; Mander et al., 2017), waking experience (Ganguly-Fitzgerald et al., 2006; Huber et al., 2007; Keene et al., 2010; Beckwith et al., 2017; Kirszenblat et al., 2019), and cell type (Delorme et al., 2021; Puentes-Mestril et al., 2021; Weiss and Donlea, 2021). Our recent study found that overnight sleep deprivation (SD) leads to diverse patterns of synaptic scaling in the *Drosophila* mushroom bodies (MB) depending on cell type (Weiss and Donlea, 2021). We found that cholinergic memory-encoding Kenyon cells (KCs), but not other MB cell types, exhibit increased presynaptic Bruchpilot (BRP) throughout the axonal lobes following SD. Complementary studies have found that sleep deprivation enhances cholinergic signaling onto GABAergic interneurons in learning/memory-related circuits, which likely increases inhibition onto memory-encoding cells (Delorme et al., 2021). However, it remains unclear whether the cell-type specific trends in plasticity seen in the MB can be generalized to other neuropil regions. specific trends in plasticity seen in the MB can be generalized to other neuropil regions.

While previous work has described plastic changes with sleep loss in discrete brain regions or cell types (Frank et al., 2001; McDermott et al., 2003; Modirrousta et al., 2004; Basheer et al., 2005; Aton et al., 2014; Yamaguchi, 2017; Wong et al., 2020; Delorme et al., 2021; Puentes-Mestril et al., 2021; Weiss and Donlea, 2021), or whole brain homogenates (Gilestro et al., 2009; Dissel et al., 2015; Huang et al., 2020), genetic tools available in Drosophila allow us to visualize changes in synapse abundance in each neuropil region throughout the central brain. Because SD can differentially affect excitatory vs. inhibitory synapses (Aton et al., 2013; Diering et al., 2017; Delorme et al., 2021; Puentes-Mestril et al., 2021), here we quantify the effects of sleep loss on abundance of BRP of different neurotransmitter types across each neuropil of the central brain.

Using cell-type-specific genetic reporters of endogenous BRP, we find that cholinergic neurons exhibit a net increase in synaptic abundance across each neuropil region in the central brain, whereas other cell types are less sensitive to sleep loss. These results support our previous findings in the MB that cholinergic neurons are particularly sensitive to sleep loss, and that different cell types across the central brain can be differentially affected by SD.

3.3: Materials and Methods

3.3.1: Fly strains and environment

Fly stocks were fed standard cornmeal media (per 1L H20: 12 g agar, 29 g Red Star yeast, 71 g cornmeal, 92 g molasses, 16mL methyl paraben 10% in EtOH, 10mL propionic acid 50% in H20) at 25°C with 60% relative humidity and entrained to a daily 12hr light, 12hr dark schedule.

All flies were reared in environmentally-controlled chambers at 25°C and 60% relative humidity on a 12hr light:12hr dark schedule. Chat2A-Gal4, vGlut-Gal4, and Gad1-Gal4 were ordered from the Bloomington Drosophila Stock Center. TH-Gal4 was provided by Dr. David Krantz (UCLA), and STaR effector flies (w-; 20xUAS-RSR.PEST, 79C23S-RSRT-STOP-RSRT-smGFP_V5-2A-LexA/cyo) were provided by Dr. Orkun Akin (UCLA).

Fly stocks were generated by the labs of Drs. Matt Pecot (STaR effector) (Chen et al., 2014), Serge Birman (TH-GAL4) (Friggi-Grelin et al., 2003), Aaron Diantonio (vGlut-Gal4) (Daniels et al., 2008), Yi Rao (Chat2A-Gal4) (Deng et al., 2019), and Gero Miesenböck (Gad1-Gal4) (Ng et al., 2002).

3.3.2: Behavior

Sleep was measured as previously described (Shaw et al., 2002). 3-7 day old adult female flies were housed individually in 65mm borosilicate glass tubes (5mm diameter) containing fly food coated with paraffin wax on one end and a foam plug in the other. Locomotor activity was measured using Drosophila Activity Monitors from Trikinetics (Waltham MA, USA) and sleep was

analyzed using Visual Basic macros in Microsoft Excel (Shaw et al., 2002) or SCAMP analysis scripts in MATLAB (Donelson et al., 2012).Baseline sleep was monitored in all groups, and sleep deprivation was performed for 12 hours during the dark phase using mechanical stimulation via the SNAP method (Shaw et al., 2002).

3.3.3: Immunochemistry and Confocal Imaging

Flies were anesthetized on ice, then brains were dissected in PBS and fixed in 3% glyoxal for 25 minutes. After fixation, brains were washed in PBS and PBTX (PBS + 0.3% Triton-x100) and incubated in 3% Normal Goat Serum in PBTX for one hour. Immunostaining for V5 used a 48-hour incubation period in 1:300 mouse anti-V5 conjugated with DyLight550 in PBTX (Bio-Rad). After antibody incubation, brains were washed in PBS and mounted on slides using Vectashield fluorescence mounting medium from Electron Microscopy Services (Burlingame CA, USA). All specimens were imaged on a Zeiss 880 laser scanning confocal microscope using a 20x objective with an optical slice thickness of 0.98 μm. Matching image acquisition settings were used for each brain within individual experiments.

3.3.4: Image Registration

Whole brain confocal stacks were registered to an adult brain template (JFRC2010) from Janelia Research Campus by using the Cachero, Ostrovsky 2010 registration algorithm to perform affine and warp transformations within the CMTK Registration plugin for Fiji (Cachero et al., 2010; Schindelin et al., 2012; Ostrovsky et al., 2013). Registered images were excluded from further analysis when we observed visible distortion or misalignment with the template stack. Each experiment was replicated using at least three independent batches of flies. Mean intensity for any neuropil compartment was excluded for an individual brain when at least 30% of pixels were not successfully registered within a region.

3.3.5: Statistical Analyses

To control for variability between experimental batches, Brp signal intensity at each pixel location in the registered images was divided by the mean intensity of control brains from the respective batch. Cumulative distributions and Kolmogorov-Smirnov tests for pixel intensities across whole confocal stacks were calculated using Numpy 1.20.3 (Harris et al., 2020) and Scipy 1.6.2 (Virtanen et al., 2020) with Python 3.7.6. Localized mean fluorescence within each neuropil were calculated in Python and compared using Two-way Repeated Measures ANOVAs followed by pairwise Holm-Sidak comparisons using GraphPad Prism 9.3.1 (San Diego, CA, USA).

3.4: Results

3.4.1: Sleep deprivation increases overall brain-wide BRP abundance in cholinergic neurons

To examine the consequences of sleep loss on synaptic abundance throughout the central brain, we used synaptic tagging with recombination (STaR), a flp-based reporter to specifically label BRP expression in genetically defined neuronal populations (Chen et al., 2014). STaR reports the abundance of a BRP-fusion reporter (BRP::V5) expressed under the control of the endogenous *brp* promoter. Brp is a core component of pre-synaptic active zones (Kittel et al., 2006; Wagh et al., 2006), and pre-synaptic Brp protein levels correlate closely with active zone size and release probability (Matkovic et al., 2013; Akbergenova et al., 2018; Hong et al., 2020). For these studies, sleep in each fly was monitored for 2-4 baseline days, then flies were either mechanically deprived of sleep for 12h overnight or allowed *ad libitum* rest (**Figure S3.1**). The next morning, we dissected brains from individuals in each group, stained for BRP::V5 and acquired confocal stacks to cover the whole central brain. To quantitatively compare BRP::V5 intensity across brains, we registered each brain image to a common template (Cachero et al., 2010; Ostrovsky et al., 2013).

We began testing the effects of sleep loss on overall brainwide BRP abundance in different neurotransmitter cell types. In cholinergic neurons labeled by the genetic driver using the genetic driver *Chat*^{2A}-Gal4 (Deng et al., 2019), brainwide BRP::V5 intensity increased by 27% after a night of sleep loss compared to rested controls (**Figure 3.1A**). Next, we quantified BRP::V5 intensity in dopaminergic neurons labeled by *TH*-Gal4 (Friggi-Grelin et al., 2003) (**Figure 3.1B**). BRP::V5 intensity was largely unaffected by sleep loss, showing a 2% decrease in intensity after SD. Glutamatergic neurons labeled by *vGlut*-Gal4 (Daniels et al., 2008) (**Figure 3.1C**) were similarly unaffected by SD, with a 4% decrease in BRP::V5 intensity in sleep deprived animals compared to controls. Finally, following sleep deprivation, GABAergic neurons labeled by *Gad1*-Gal4 (Ng et al., 2002) (**Figure 3.1D**) exhibited a modest 9% increase in BRP::V5 intensity. We then performed Kolmogorov-Smirnov tests (K-S tests) to compare the cumulative probability distributions of pixel intensities of rested and sleep deprived brains within each neurotransmitter class (**Figure 3.1A'-D'**). A K-S test in cholinergic neurons found D=0.134 (**Figure 3.1A'**), a D value more than double that of any other neurotransmitter we tested (**Figure 3.1B'-D'**). These data indicate that prolonged waking results in net brainwide synaptic expansion in cholinergic neurons, but not in dopaminergic, glutamatergic, or GABAergic neurons.

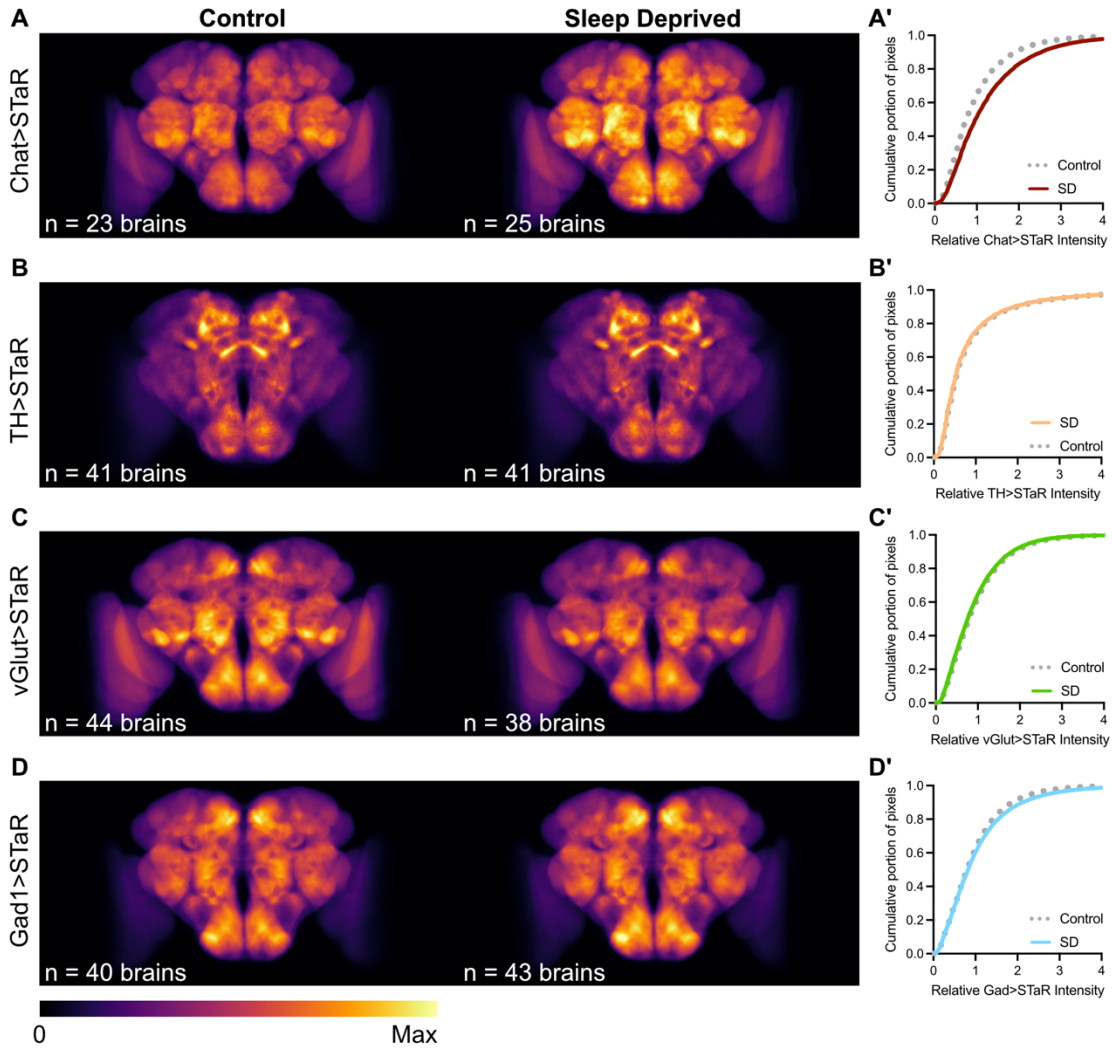

Figure 3.1: Sleep deprivation increases overall brain-wide BRP abundance in cholinergic neurons, but not in dopaminergic, glutamatergic, or GABAergic neurons

(A) Average brains for control (left) and sleep deprived (right) *Chat*^{2A}-Gal4>STaR flies.

(A') Cumulative distribution plots of total pixel intensities of *Chat*^{2A}-Gal4>STaR brains for control (gray) and sleep deprived (maroon) flies. Kolmogorov-Smirnov test finds D=0.134, n=4.63x10^{8}-5.03x10^{8} pixels/group.

(B) Average brains for control (left) and sleep deprived (right) *TH*-Gal4>STaR flies.

(B') Cumulative distribution plots of total pixel intensities of *TH*-Gal4>STaR brains for control (gray) and sleep deprived (orange) flies. Kolmogorov-Smirnov test finds D=0.047, n=$8.25x10^8$-$8.65x10^8$ pixels/group.

(C) Average brains for control (left) and sleep deprived (right) *vGlut*-Gal4>STaR flies.

(C') Cumulative distribution plots of total pixel intensities of *vGlut*-Gal4>STaR brains for control (gray) and sleep deprived (green) flies. Kolmogorov-Smirnov test finds D=0.029, n=$7.85x10^8$-$8.85x10^8$ pixels/group.

(D) Average brains for control (left) and sleep deprived (right) *Gad1*-Gal4>STaR flies.

(D') Cumulative distribution plots of total pixel intensities of *Gad1*-Gal4>STaR brains for control (gray) and sleep deprived (blue) flies. Kolmogorov-Smirnov test finds D=0.056, n=$8.05x10^8$-$8.65x10^8$ pixels/group.

3.4.2: Neuropil regions within each neurotransmitter group are similarly affected by sleep loss

Next, we performed a region by region analysis of changes in BRP::V5 intensity after sleep loss for each neurotransmitter. Following overnight SD, STaR labeling in cholinergic neurons using *Chat^{2A}-Gal4* increased significantly in every neuropil region examined, by ~21%-39% (**Figure 3.2A, B, Table S3.1**). Regional BRP::V5 intensity was largely unaffected by sleep loss in dopaminergic neurons, with no significant changes in intensity observed (**Figure 3.2C, Table S3.2**). Similarly, there were no significant changes in regional BRP::V5 intensity in glutamatergic neurons (**Figure 3.2D, Table S3.3).** In GABAergic neurons, we observed no significant changes in BRP::V5 in every neuropil region except the prow (PRW), which saw an 18% increase in intensity following a night of sleep loss (**Figure 3.2E, Table S3.4**). The prow is a sparsely studied neuropil region that receives input from gustatory receptor neurons (GRNs) (Scheffer et al., 2020). Interestingly, trends in BRP::V5 intensity with sleep loss remained consistent in every neuropil region for each neurotransmitter studied. Together, these results suggest that sleep deprivation uniformly affects synapse abundance throughout the entire brain

within a neurotransmitter class. However, region-specific analyses may reveal more nuanced cell-type-specific trends in synaptic scaling, as we previously observed in the MB (Weiss and Donlea, 2021).

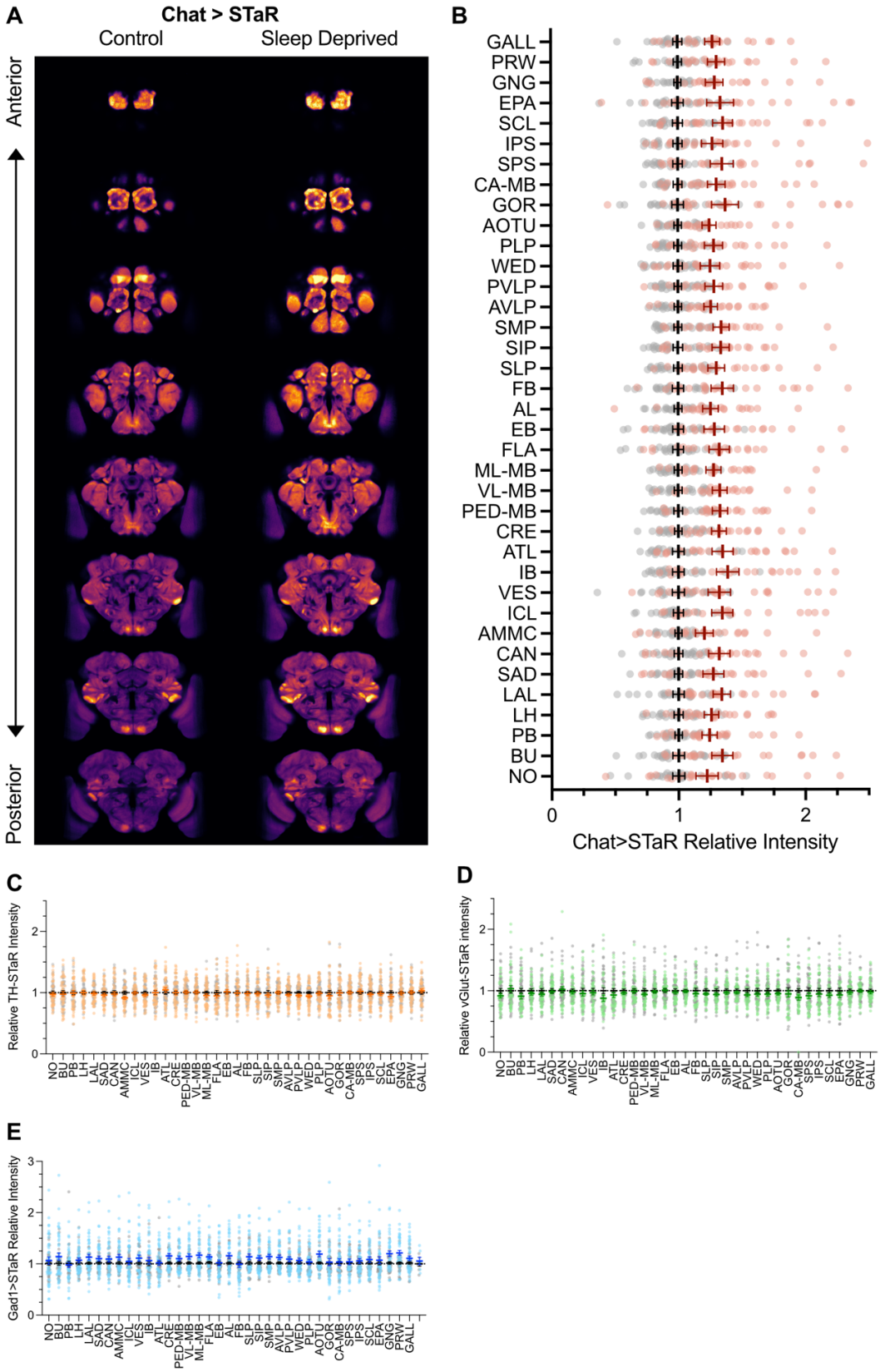
A
Chat > STaR
Control
Sleep Deprived
Anterior
Posterior
B
GALL
PRW
GNG
EPA
SCL
IPS
SPS
CA-MB
GOR
AOTU
PLP
WED
PVLP
AVLP
SMP
SIP
SLP
FB
AL
EB
FLA
ML-MB
VL-MB
PED-MB
CRE
ATL
IB
VES
ICL
AMMC
CAN
SAD
LAL
LH
PB
BU
NO
0
1
2
Chat>STaR Relative Intensity
C
Relative TH-STaR Intensity
D
Relative vGlut-STaR Intensity
E
Gad1>STaR Relative Intensity

Figure 3.2: Neuropil regions within each neurotransmitter group are similarly affected by sleep loss

(A) Sections along the z-axis through average brains for control (left) and sleep deprived (right) *Chat*^{2A}-Gal4>STaR flies.

(B) Regional analysis of BRP intensity *Chat*^{2A}-Gal4>STaR flies after 12-hr of overnight sleep loss (maroon) normalized to rested controls (gray). Two-way ANOVA finds a significant effect of SD ($F_{(1,46)}$=19.98, p<0.0001, n=24-25 brains/group). Pairwise comparisons using Sidak's multiple comparisons test found significant increases in Brp::V5 intensity in each neuropil region after sleep deprivation relative to rested siblings (p<0.05 for each test, see Table S3.1 for regional statistics).

(C) Regional analysis of BRP intensity *TH*-Gal4>STaR flies after 12-hr of overnight sleep loss (orange) normalized to rested controls (gray). Two-way ANOVA finds no significant effect of SD ($F_{(1,80)}$=0.2679, p=0.6061, n=41 brains/group). See Table S3.2 for regional statistics).

(D) Regional analysis of BRP intensity *vGlut*-Gal4>STaR flies after 12-hr of overnight sleep loss (green) normalized to rested controls (gray). Pairwise comparisons using Sidak's multiple comparisons test found no significant changes in Brp::V5 intensity in each neuropil region after sleep deprivation relative to rested siblings (p>0.805 for each test, see Table S3.3 for regional statistics).

(E) Regional analysis of BRP intensity *Gad1*-Gal4>STaR flies after 12-hr of overnight sleep loss (blue) normalized to rested controls (gray). Two-way ANOVA finds a significant group-by-region interaction ($F_{(36,2875)}$=2.713, p<0.001, n=40-43 brains/group). Pairwise comparisons using Sidak's multiple comparisons test found a significant increase in BRP:V5 intensity in the prow after sleep deprivation relative to rested siblings (p=0.030), but no significant changes in Brp::V5 intensity in other neuropil regions (p>0.2199 for each test, see Table S3.4 for regional statistics).

3.5: Discussion

3.5.1: Effects of sleep loss on cholinergic neurons

In this study, we used a genetic reporter to quantify the effects of sleep deprivation on the presynaptic active zone marker BRP in different neurotransmitter cell types throughout the *Drosophila* central brain. We find that BRP abundance in cholinergic neurons increases consistently throughout each neuropil region examined, whereas BRP abundance of dopaminergic, glutamatergic, and GABAergic cells remains largely unchanged following a night of sleep loss. These findings are consistent with our previous study, which found that cholinergic Kenyon cells, but not other MB cell types, exhibit increased synapse abundance after SD (Weiss and Donlea, 2021). Both studies find ~30% increase in BRP::V5 intensity in cholinergic neurons with SD. While we previously observed increased BRP throughout the MB lobes when using a non-cell-type-specific reporter, we found more refined patterns of synaptic scaling when we examined individual MB cell types (Weiss and Donlea, 2021). Similarly, although we did not see any net changes in BRP abundance in dopaminergic, glutamatergic, and GABAergic cells, it is possible that smaller subpopulations of these cells undergo synaptic scaling that we are unable to detect when examining large groups of neurons. While we examine the accumulation of presynaptic BRP in this study, the development of additional genetic reporters will allow the investigation of the consequences of sleep deprivation on postsynaptic receptors in *Drosophila*.

Interestingly, thermogenetic activation of all cholinergic neurons in *Drosophila* almost completely suppresses sleep, leading to homeostatic recovery sleep following stimulation (Seidner et al., 2015). While thermogenetic activation of dopaminergic and octopaminergic neurons, stimulation does not elicit subsequent recovery sleep. Some cholinergic neurons may therefore play a unique role in maintaining sleep homeostasis (Seidner et al., 2015). Previous work in flies and rodents suggests that cholinergic neurons may become increasingly active with extended wake (Modirrousta et al., 2004; Bushey et al., 2015; Delorme et al., 2021; Ge et al., 2021). It is possible that elevated activity of cholinergic cells drives the increase in BRP

abundance we observe after sleep loss. As acetylcholine is the primary excitatory neurotransmitter in the *Drosophila* central brain (Breer and Sattelle, 1987; Restifo and White, 1990; Lee and O'Dowd, 1999), our findings of elevated BRP abundance in cholinergic neurons after SD may be consistent with reports of increased cortical excitability seen in humans and rodents (Vyazovskiy et al., 2009; Huber et al., 2013). After sleep loss, excitatory neurons often increase their drive onto inhibitory neurons (Delorme et al., 2021; Weiss and Donlea, 2021), perhaps in a compensatory effort to prevent runaway excitation (Peng et al., 2010; Xue et al., 2014).

3.5.2: Potential roles for sleep in maintenance of E/I balance

Recent studies suggest that sleep is associated with the maintenance of excitatory/inhibitory (E/I) balance in the brain (Chellappa et al., 2016; Bridi et al., 2019; Delorme et al., 2021; Puentes-Mestril et al., 2021). Previous work has demonstrated that global cortical excitability increases during waking (Vyazovskiy et al., 2009; Liu et al., 2010; Huber et al., 2013; Chia et al., 2021), whereas cortical excitability decreases over the course of sleep. Consistent with these findings, our data show that sleep deprivation increases presynaptic abundance in excitatory cholinergic neurons. However, sleep's effect on E/I balance may vary based on time of day (Chellappa et al., 2016; Bridi et al., 2019; Pacheco et al., 2021), sleep stage (Niethard et al., 2016; Tamaki et al., 2020), and brain region (Puentes-Mestril et al., 2021). Notably, disrupted E/I balance is implicated in the pathogenesis of neurological and psychiatric disorders (Rubenstein and Merzenich, 2003; Kehrer et al., 2008; Gogolla et al., 2009; Yizhar et al., 2011; Bi et al., 2020; Lauterborn et al., 2021). Disrupted E/I balance may therefore contribute to behavioral and cognitive consequences of sleep loss.

3.5.3: Potential roles for sleep stages in regulation of synaptic plasticity

While cholinergic tone is high during wake and low during SWS, cholinergic neurons are also active during REM sleep (Baghdoyan and Lydic, 1999; Nissen et al., 2006; Platt and Riedel, 2011; Inayat et al., 2019). Currently, there are no widely defined sleep stages in flies as in vertebrates, although recent studies have begun to identify discrete stages of *Drosophila* sleep (Alphen et al., 2013, 2021; Yap et al., 2017; Tainton-Heap et al., 2021), future work may elucidate the potential role of *Drosophila* sleep stages in synaptic plasticity.

3.5.4: Concluding remarks

The consequences of sleep loss on synaptic plasticity are not yet well understood, but previous work has found net changes in synaptic abundance or size across brain regions (Bushey et al., 2011; Diering et al., 2017; Vivo et al., 2017; Weiss and Donlea, 2021). Here, we find that cholinergic neurons in the *Drosophila* central brain are uniquely sensitive to sleep loss. Our results indicate that sleep deprivation results in elevated BRP abundance in cholinergic neurons across neuropil regions, but future studies will be required to test the mechanisms by which prolonged waking drives increased synapse abundance.

3.6: Supplemental data

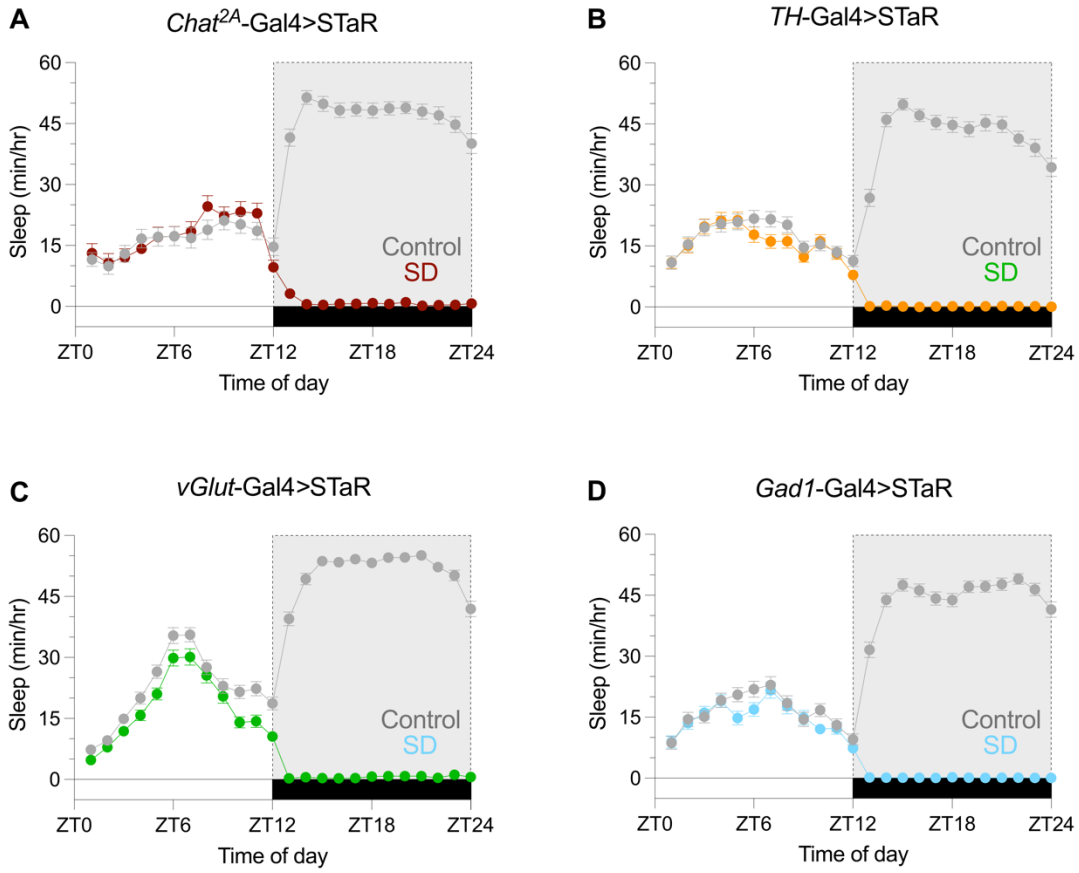

Figure S3.1: Sleep patterns of flies expressing STaR reporter in different neurotransmitter cell types, related to Figures 3.1-3.2

(A) Hourly sleep traces of *Chat*^{2A}-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (maroon). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F(23,2530)=103.9$, $p<0.0001$, n=56 control, 56 SD).

(B) Hourly sleep traces of *TH*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (orange). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F(23,4255)=148.2$, $p<0.0001$,

n=86 control, 102 SD).

(C) Hourly sleep traces of *vGlut*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (green). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F(23,4761)=231.2$, $p<0.0001$, n=56 control, 56 SD).

(D) Hourly sleep traces of *Gad1*-Gal4>STaR flies that were allowed ad libitum sleep (gray) or were sleep deprived overnight for 12 hours (blue). Two-way repeated-measures ANOVA of hourly sleep time course finds a significant time-by-SD interaction $F(23,4508)=165.8$, $p<0.0001$, n=104 control, 105 SD).

Region	SD - Control Mean Difference	P value
Nodulus (NO)	0.2234	0.0126
Bulb (BU)	0.3444	0.0008
Protocerebral bridge (PB)	0.2444	0.0121
Lateral horn	0.2592	0.0121
Lateral accessory lobe (LAL)	0.3404	0.0009
Saddle (SAD)	0.2749	0.0086
Cantle (CAN)	0.3216	0.0019
Antennal mechanosensory and motor center (AMMC)	0.205	0.0126
Inferior clamp (ICL)	0.3457	0.0008
Vest (VES)	0.3218	0.0019
Inferior bridge (IB)	0.3892	<0.0001
Antler (ATL)	0.3486	0.0007
Crepine (CRE)	0.321	0.0019
Peduncle – Mushroom body (PED-MB)	0.3288	0.0015
Vertical lobe – Mushroom body (VL-MB)	0.3274	0.0015
Medial lobe – Mushroom body (ML-MB)	0.2784	0.008
Flange (FLA)	0.3247	0.0017
Ellipsoid body (EB)	0.285	0.0074
Antennal lobe (AL)	0.2561	0.0121
Fan-shaped body (FB)	0.3491	0.0007
Superior lateral protocerebrum (SLP)	0.302	0.0038
Superior intermediate protocerebrum (SIP)	0.337	0.0011
Superior medial protocerebrum (SMP)	0.3411	0.0009
Anterior ventrolateral protocerebrum (AVLP)	0.2594	0.0121
Posterior ventrolateral protocerebrum (PVLP)	0.2845	0.0074
Wedge (WED)	0.254	0.0121
Posterior lateral protocerebrum (PLP)	0.2829	0.0074
Anterior optic tubercle (AOTU)	0.2478	0.0121
Gorget (GOR)	0.3736	0.0002
Calyx – Mushroom body (CA-MB)	0.3049	0.0037
Superior posterior slope (SPS)	0.349	0.0007
Inferior posterior slope(IPS)	0.2732	0.0086
Superior clamp (SCL)	0.3553	0.0005
Epaulette (EPA)	0.336	0.0011
Gnathal ganglion (GNG)	0.2903	0.0062
Prow (PRW)	0.305	0.0037
Gall (GALL)	0.2748	0.0086

Table S3.1: Two-way repeated measures ANOVA results for *Chat*^{2A}-Gal4>STaR flies

Region	SD - Control Mean Difference	P value
Nodulus (NO)	-0.02449	>0.9999
Bulb (BU)	-0.02435	>0.9999
Protocerebral bridge (PB)	-0.006491	>0.9999
Lateral horn	0.00008455	>0.9999
Lateral accessory lobe (LAL)	-0.01382	>0.9999
Saddle (SAD)	-0.03777	>0.9999
Cantle (CAN)	-0.02223	>0.9999
Antennal mechanosensory and motor center (AMMC)	-0.0849	0.5286
Inferior clamp (ICL)	-0.006094	>0.9999
Vest (VES)	-0.03618	>0.9999
Inferior bridge (IB)	0.001393	>0.9999
Antler (ATL)	0.04542	>0.9999
Crepine (CRE)	0.001595	>0.9999
Peduncle – Mushroom body (PED-MB)	0.006719	>0.9999
Vertical lobe – Mushroom body (VL-MB)	0.007082	>0.9999
Medial lobe – Mushroom body (ML-MB)	-0.04784	0.9997
Flange (FLA)	-0.05377	>0.9999
Ellipsoid body (EB)	0.01375	>0.9999
Antennal lobe (AL)	-0.006819	>0.9999
Fan-shaped body (FB)	-0.01338	>0.9999
Superior lateral protocerebrum (SLP)	-0.01621	>0.9999
Superior intermediate protocerebrum (SIP)	-0.03986	>0.9999
Superior medial protocerebrum (SMP)	0.0008823	>0.9999
Anterior ventrolateral protocerebrum (AVLP)	-0.03542	>0.9999
Posterior ventrolateral protocerebrum (PVLP)	-0.04789	0.9982
Wedge (WED)	-0.05638	0.9772
Posterior lateral protocerebrum (PLP)	-0.01246	>0.9999
Anterior optic tubercle (AOTU)	-0.05101	>0.9999
Gorget (GOR)	-0.004868	>0.9999
Calyx – Mushroom body (CA-MB)	0.002938	>0.9999
Superior posterior slope (SPS)	0.02102	>0.9999
Inferior posterior slope(IPS)	-0.02906	>0.9999
Superior clamp (SCL)	-0.01273	>0.9999
Epaulette (EPA)	-0.06279	0.9994
Gnathal ganglion (GNG)	-0.006786	>0.9999
Prow (PRW)	0.006631	>0.9999
Gall (GALL)	0.03022	>0.9999

Table S3.2: Two-way repeated measures ANOVA results for *TH*-Gal4>STaR flies

Region	SD - Control Mean Difference	P value
Nodulus (NO)	-0.08201	0.9757
Bulb (BU)	0.03274	0.9999
Protocerebral bridge (PB)	-0.08922	0.9871
Lateral horn	-0.04294	0.9997
Lateral accessory lobe (LAL)	-0.0565	0.9986
Saddle (SAD)	-0.01593	0.9999
Cantle (CAN)	0.02177	0.9999
Antennal mechanosensory and motor center (AMMC)	-0.02312	0.9999
Inferior clamp (ICL)	-0.04489	0.9999
Vest (VES)	-0.03294	0.9999
Inferior bridge (IB)	-0.1219	0.9499
Antler (ATL)	-0.0697	0.998
Crepine (CRE)	-0.0362	0.9998
Peduncle – Mushroom body (PED-MB)	-0.01063	0.9999
Vertical lobe – Mushroom body (VL-MB)	-0.06081	0.9956
Medial lobe – Mushroom body (ML-MB)	-0.01832	0.9999
Flange (FLA)	0.01027	0.9999
Ellipsoid body (EB)	-0.02553	0.9999
Antennal lobe (AL)	-0.01136	0.9999
Fan-shaped body (FB)	-0.04923	0.9998
Superior lateral protocerebrum (SLP)	-0.053	0.9963
Superior intermediate protocerebrum (SIP)	-0.06153	0.9871
Superior medial protocerebrum (SMP)	-0.03328	0.9999
Anterior ventrolateral protocerebrum (AVLP)	-0.02946	0.9999
Posterior ventrolateral protocerebrum (PVLP)	-0.06802	0.9826
Wedge (WED)	-0.05423	0.9993
Posterior lateral protocerebrum (PLP)	-0.05498	0.9993
Anterior optic tubercle (AOTU)	-0.04592	0.9968
Gorget (GOR)	-0.05033	0.9999
Calyx – Mushroom body (CA-MB)	-0.1098	0.805
Superior posterior slope (SPS)	-0.0825	0.9968
Inferior posterior slope(IPS)	-0.05241	0.9998
Superior clamp (SCL)	-0.0749	0.9812
Epaulette (EPA)	-0.06788	0.9993
Gnathal ganglion (GNG)	-0.02878	0.9999
Prow (PRW)	-0.004215	0.9999
Gall (GALL)	-0.02187	0.9999

Table S3.3: Two-way repeated measures ANOVA results for *vGlut*-Gal4>STaR flies

Region	SD - Control Mean Difference	P value
Nodulus (NO)	0.1028	0.9815
Bulb (BU)	0.1077	0.9657
Protocerebral bridge (PB)	-0.05466	0.9943
Lateral horn	0.0605	0.992
Lateral accessory lobe (LAL)	0.1243	0.6887
Saddle (SAD)	0.0853	0.9557
Cantle (CAN)	0.1015	0.9557
Antennal mechanosensory and motor center (AMMC)	0.06443	0.9738
Inferior clamp (ICL)	0.05533	0.9943
Vest (VES)	0.1316	0.8375
Inferior bridge (IB)	0.02938	0.9993
Antler (ATL)	-0.003271	0.9993
Crepine (CRE)	0.1308	0.4674
Peduncle – Mushroom body (PED-MB)	0.09388	0.8749
Vertical lobe – Mushroom body (VL-MB)	0.1178	0.6555
Medial lobe – Mushroom body (ML-MB)	0.1461	0.2391
Flange (FLA)	0.1167	0.6169
Ellipsoid body (EB)	0.02277	0.9993
Antennal lobe (AL)	0.1167	0.6555
Fan-shaped body (FB)	0.02553	0.9993
Superior lateral protocerebrum (SLP)	0.1103	0.7765
Superior intermediate protocerebrum (SIP)	0.09573	0.8756
Superior medial protocerebrum (SMP)	0.1166	0.5235
Anterior ventrolateral protocerebrum (AVLP)	0.1081	0.5897
Posterior ventrolateral protocerebrum (PVLP)	0.07538	0.9738
Wedge (WED)	0.04932	0.9943
Posterior lateral protocerebrum (PLP)	0.01857	0.9993
Anterior optic tubercle (AOTU)	0.1623	0.2199
Gorget (GOR)	0.07378	0.9943
Calyx – Mushroom body (CA-MB)	-0.0201	0.9993
Superior posterior slope (SPS)	0.04704	0.9943
Inferior posterior slope(IPS)	0.02357	0.9993
Superior clamp (SCL)	0.06462	0.992
Epaulette (EPA)	0.0966	0.9815
Gnathal ganglion (GNG)	0.1323	0.3561
Prow (PRW)	0.1821	0.03
Gall (GALL)	0.1156	0.4674

Table S3.4: Two-way repeated measures ANOVA results for *Gad1*-Gal4>STaR flies

3.7: References

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