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Sleep deprivation results in diverse patterns of synaptic scaling across the Drosophila Mushroom bodies

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SUMMARY

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.

ETOC Blurb

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Author Contributions

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Declaration of Interests

The authors declare no competing interests

Weiss and Donlea find that sleep loss increases pre-synaptic Bruchpilot across the *Drosophila* Mushroom Body (MB) due to plasticity in MB-intrinsic Kenyon cells. Contacts from Kenyon cells to post-synaptic targets show differing changes with sleep loss, indicating that sleep deprivation may differentially alter distinct classes of MB synapses.

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.

RESULTS

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 S1A) 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 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 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 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 1D, 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.

Variable effects of sleep loss on MB abundance of pre-synaptic 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 2A; sleep traces and representative images shown in Figure S1B-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 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 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 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 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 2F), while fluorescent intensity of nSyb^{GFP} was decreased in each MB lobe of sleep deprived flies (Figure 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 2G). 48h of undisturbed recovery after overnight sleep deprivation restored abundance of Rab3^{mCherry} back to control levels (Figure 2E, right), while dSyd-1$^{MI05387\text{-}GFSTF}$ in previously sleep-deprived flies was reduced below the levels observed in undisturbed controls (Figure 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.

Pre-synaptic 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 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 3A-B and S2A). Similar increases in KC expression of BRP were also found using a second broad KC driver, *OK107*-Gal4 (Figures 3C and S2B) [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 3D-F and S2C-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 3G), but no significant increase in STaR signal was detected in starved flies (Figure 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 S3). 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 S2E-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 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 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 3L-M) [44,49]. Based on these data, the broad increase in BRP that we observed in MB lobes (Figures 1A-B) after sleep deprivation can be attributed primarily to KCs, not to other MB cell types.

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 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 S5-6). 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 4B-C) while no effect of sleep loss could be detected on GRASP signal from KCs to PAM DANs (*R58E02*-Gal4) (Figures 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 4F-G), while KC>DPM GRASP was significantly decreased in the γ, α', and β' lobes following overnight sleep loss (Figures 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.

Memories of associative conditioning are encoded within plastic connections between odorcoding 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 5, KC>MBON connections varied across different MB compartments. Sleep deprived flies (Sleep data shown in Figure S5) showed consistently elevated GRASP signal from KCs to MBON-α'1 (Figures 5A-B) and from KCs to MBON-α2 (Figures 5C-D), but decreased GRASP between KCs and MBON-γ5β'2a (Figures 5E-F) and from KCs and MBON-γ2a'1 (Figures 5G-H). Other KC to MBON synapses, including those to MBON-γ4>γ1γ2 (Figure 5I-J), MBON-β'2mp,γ5β'2a (Figure 5K-L), and MBON-γ1pedc (Figure 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 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 5O-P, left panels). While sleep loss increased KC>MBON- a'1 GRASP and decreased KC>MBON-γ5β'2 GRASP (Figures 5B, F), 6h of sleep-promoting THIP treatment reduced GRASP signal in both KC>MBON connections (Figures 5O-P, right panels). Increased sleep, therefore, may not solely drive synaptic changes that are the converse to those that occur during sleep loss.

DISCUSSION

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.

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].

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-γ2α'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 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.

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. 3D-F). Further, recovery sleep or pharmacological sleep enhancement may not simply reverse the effects of sleep loss (Figs 2B, 5O-P) and it is unclear how particular subsets of synaptic proteins or connections may be selected for removal during times of elevated sleep.

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.

STAR Methods

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeffrey Donlea (jdonlea@ucla.edu).

Materials Availability—This study did not generate new unique reagents.

Data and Code Availability—The published article includes all datasets generated during this study. This study did not generate any novel code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

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, *R23E10*-Gal4, UAS-TrpA1, *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 (http://splitGal4.janelia.org/cgi-bin/splitGal4.cgi).

METHOD DETAILS

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.

Immunohistochemistry 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 antimouse 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 labelled lobe to measure mean GFP or anti-V5 intensity in Fiji [110].

QUANTIFICATION AND STATISTICAL ANALYSIS

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Amount of pre-synaptic BRP in Mushroom bodies is inversely related to recent sleep
- Increased BRP after sleep loss is restricted specifically to Kenyon cells
- Outputs from KCs to different synaptic partners show varied changes with sleep loss

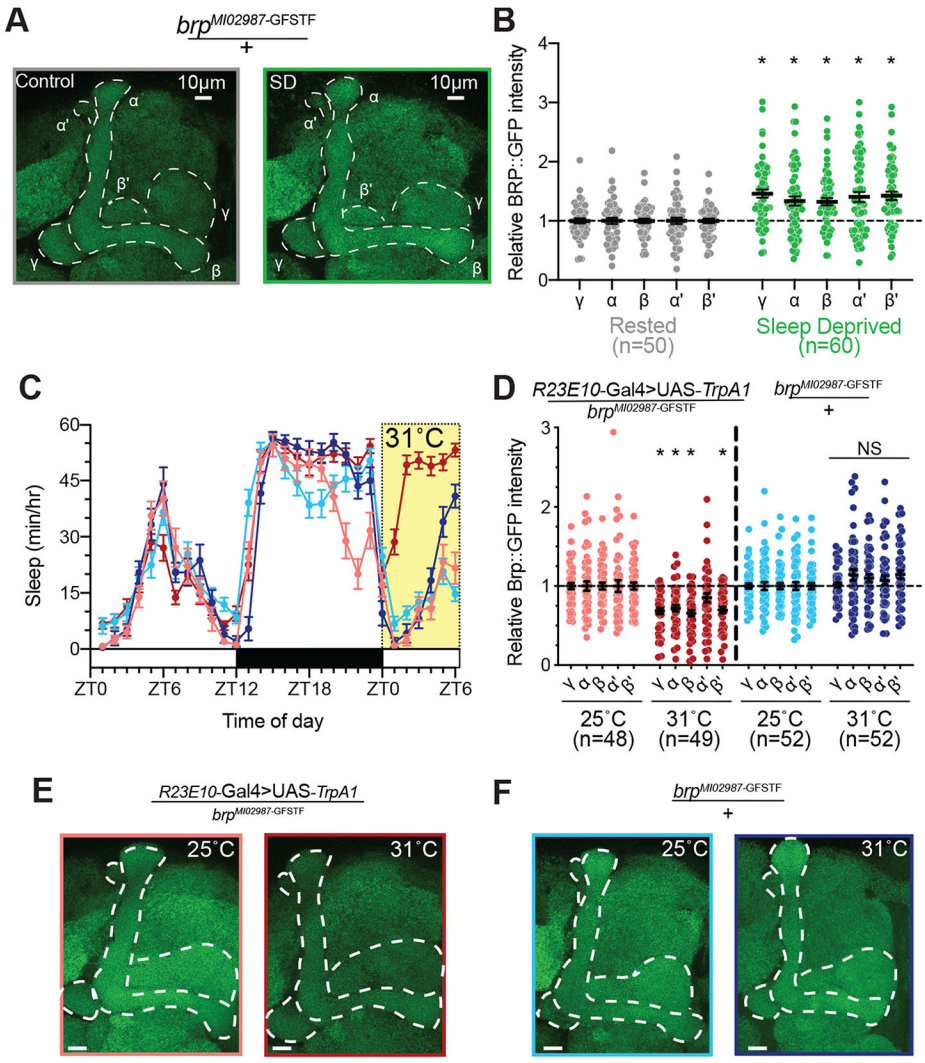

Figure 1 –. Sleep bidirectionally regulates Brp abundance in the mushroom body

(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≤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 S1 for sleep traces from experimental groups shown in Figure 1A-B.

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

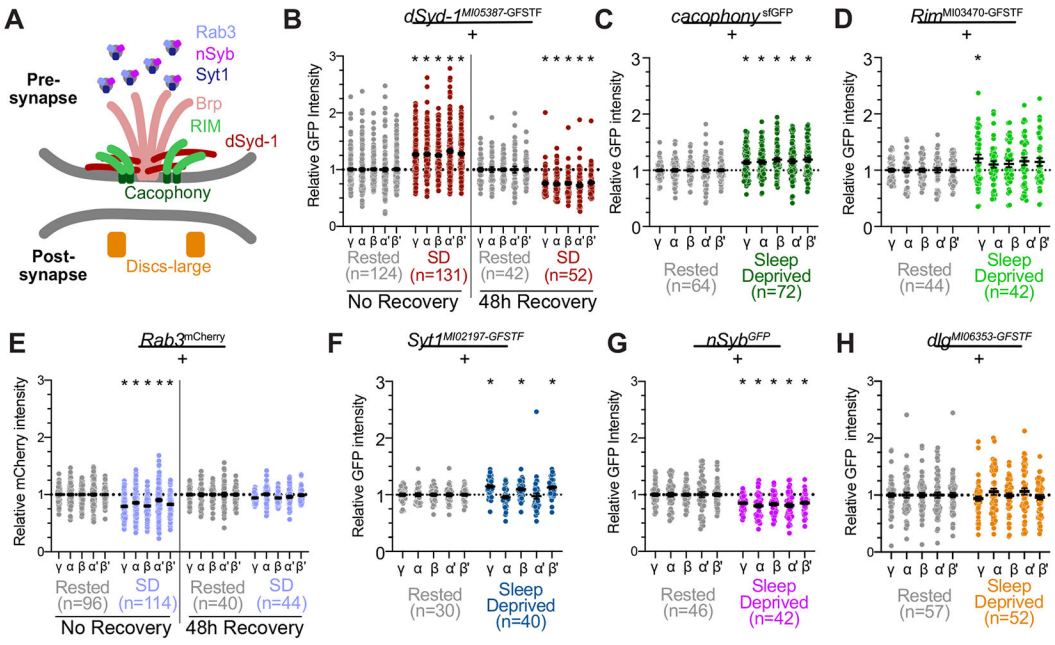

Figure 2 –. Pre-synaptic proteins show variable responses to sleep loss in the MB lobes

(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 S1 for representative images and sleep traces from each experimental group and genotype.

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

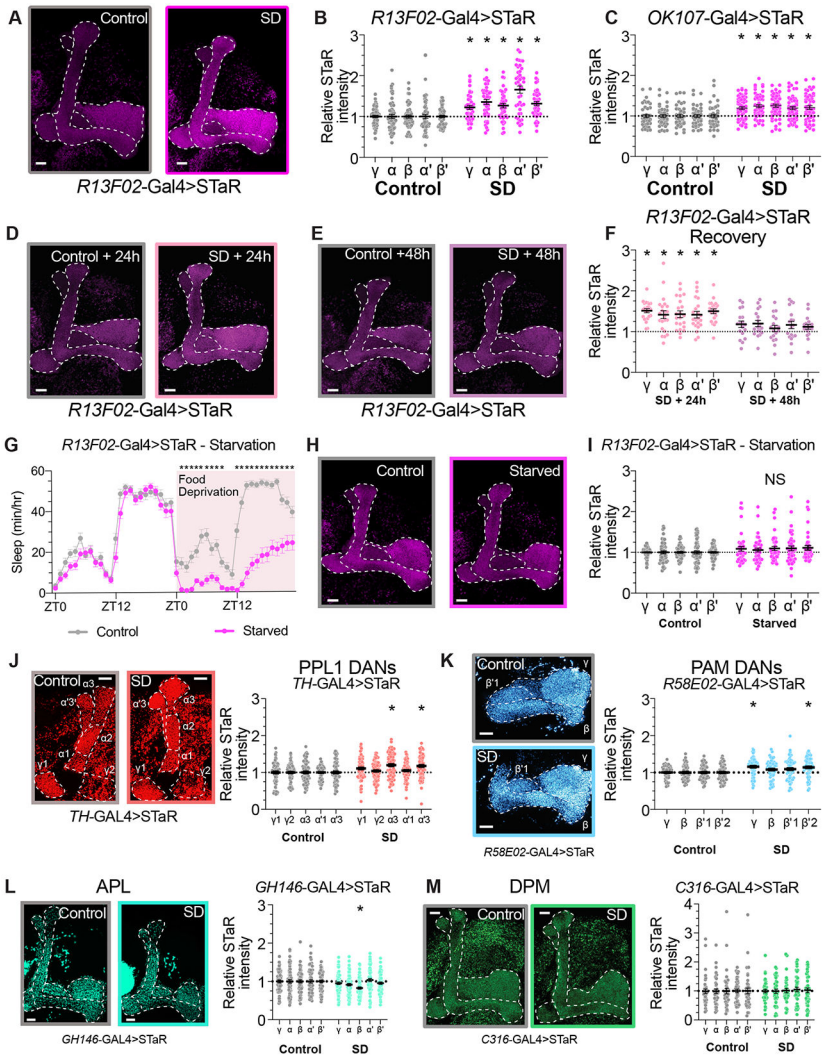

Figure 3 –. Increased BRP abundance in Kenyon cell axons after sleep deprivation

(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). Presynapses 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 3 A-J**, and Figure S3 for pre-synaptic BRP quantification from subsets of KC neurons.

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

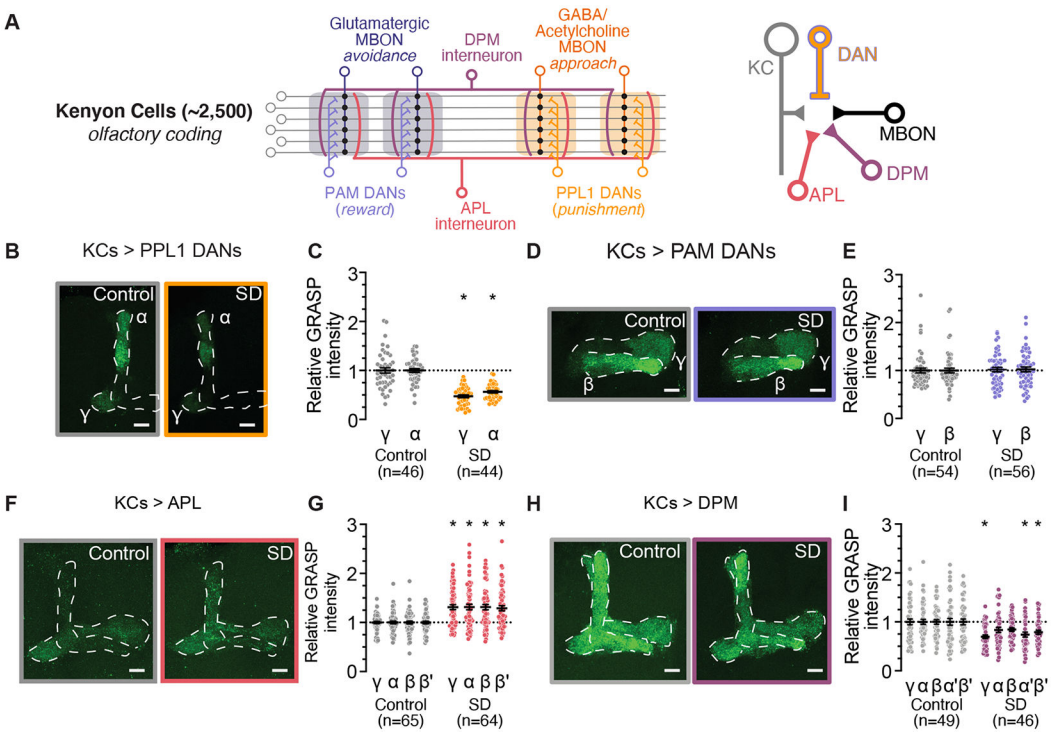

Figure 4 –. Effects of SD on synaptic contacts between KCs and DANs, APL & DPM

(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 S4 for sleep traces from experimental groups shown in Figure 3 B-I.

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

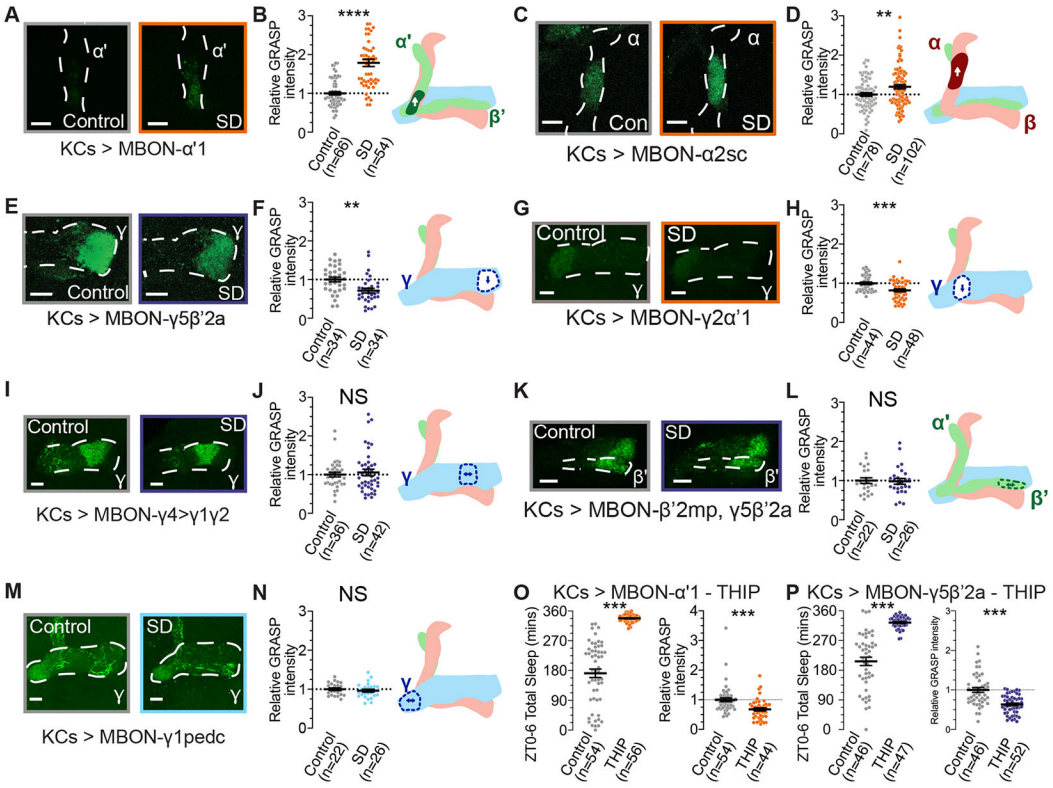

Figure 5 –. KC>MBON connections exhibit compartment-specific changes with SD

(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 S5 for sleep traces from experimental groups in **Figure A-L**.
Scale bars depict 10 μm; error bars represent SEM for all panels.

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: *Drosophila* Strains		
Canton-S	Gero Miesenböck (University of Oxford)	
brp $^{MI02987\text{-}GFSTF}$	Bloomington *Drosophila* Stock Center	RRID:BDSC_59292
R23E10-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_49032
UAS-*TrpA1* (II)	Bloomington *Drosophila* Stock Center	RRID:BDSC_26263
dSyd-1 $^{MI05387\text{-}GFSTF}$	Bloomington *Drosophila* Stock Center	RRID:BDSC_59414
cacophony ^{sfGFP}	Kate O'Connor-Giles (Brown University)	
Rim $^{MI03470\text{-}GFSTF}$	Bloomington *Drosophila* Stock Center	RRID:BDSC_60200
Syt1 $^{MI02197\text{-}GFSTF}$	Bloomington *Drosophila* Stock Center	RRID:BDSC_59788
Rab3 ^{mCherry}	Bloomington *Drosophila* Stock Center	RRID:BDSC_81509
nSyb ^{GFP}	Troy Littleton (MIT)	
dlg $^{MI06353\text{-}GFSTF}$	Bloomington *Drosophila* Stock Center	RRID:BDSC_59417
STaR effector: w^{-}; 20xUAS-RSR.PEST, 79C23S-RSRT-STOP-RSRT-smGFP_V5-2A-LexA/cyo	Orkun Akin (UCLA)	
R13F02-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_48571
OK107-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_854
TH-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_8848
R58E02-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_41347
GH146-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_30026
C316-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_30830
MB594B split-Gal4	HHMI Janelia Research Campus	RRID:BDSC_68255
MB185B split-Gal4	HHMI Janelia Research Campus	RRID:BDSC_68267
MB371B split-Gal4	HHMI Janelia Research Campus	RRID:BDSC_68383
MB463B split-Gal4	HHMI Janelia Research Campus	RRID:BDSC_68370
MB607B split-Gal4	HHMI Janelia Research Campus	RRID:BDSC_68256
GRASP Effector: *w**; P{w[+mC]=lexAop-*nSyb*-spGFP$_{1\text{-}10}$}2, P{w[+mC]=UAS-CD4-spGFP$_{11}$}2; MKRS/TM6B	Bloomington *Drosophila* Stock Center	RRID:BDSC_64315
MB-LexA	Scott Waddell (University of Oxford)	
MB543B split-Gal4	HHMI Janelia Research Campus	RRID:BDSC_68335
R71D08-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_61645
R66C08-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_49412
R25D01-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_49122
R12G04-Gal4	Bloomington *Drosophila* Stock Center	RRID:BDSC_48523
MB434B split-Gal4	HHMI Janelia Research Campus	RRID:BDSC_68325
MB011B split-Gal4	HHMI Janelia Research Campus	RRID:BDSC_68294
Antibodies		
Chicken anti-GFP	ThermoFisher	A10262 RRID:AB_2534023

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-chicken Alexa488	ThermoFisher	A11039 RRID:AB_142924
Mouse anti-V5 DyLight550	BioRad	MCA1360GA RRID:AB_567249
Mouse anti-GFP	Sigma	G6539–100UL RRID:AB_259941
Anti-mouse Alexa488	ThermoFisher	A11001 RRID:AB_2534069
Software		
Visual Basic sleep analysis macros	Paul Shaw (Washington University in St. Louis)	
Prism 8	Graphpad (www.graphpad.com)	
FIJI	FIJI (Imagej.net/FIJI)	
Chemicals		
THIP hydrochloride (4,5,6,7-Tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol hydrochloride)	Sigma-Aldrich	T101-100MG
Phosphate buffered saline tablets	Sigma-Aldrich	P4417-100TAB
Triton X-100	Simga-Aldrich	X100-100ML
VECTASHIELD Mounting Medium	Vector Laboratories	H-1000
Glyoxal 3% Fixative	Electron Microscopy Sciences	16525
Paraformaldehyde 16% Solution	Electron Microscopy Sciences	15710-S