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Bidirectional regulation of sleep and synapse pruning after neural injury

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Summary

Following acute neural injury, severed axons undergo programmed Wallerian degeneration over several following days. While sleep has been linked with synaptic reorganization under other conditions, the role of sleep in responses to neural injuries remain poorly understood. To study the relationship between sleep and neural injury responses, we examined *Drosophila melanogaster* following removal of antennae or other sensory tissues. Daytime sleep is elevated after antennal or wing injury, but sleep returns to baseline levels within 24 hours after injury. Similar increases in sleep are not observed when olfactory receptor neurons are silenced or when other sensory organs are severed, suggesting that increased sleep after injury is not attributed to sensory deprivation, nociception, or generalized inflammatory responses. Neuroprotective disruptions of the E3 ubiquitin ligase *highwire* and c-Jun N-terminal kinase *basket* in olfactory receptor neurons weaken the sleep-promoting effects of antennal injury, suggesting that post-injury sleep may be influenced by the clearance of damaged neurons. Finally, we show that pre-synaptic active zones are preferentially removed from severed axons within hours after injury, and that sleep depriving recently injured flies slows the removal of both active zones and damaged axons. These data support a bidirectional interaction between sleep and synapse pruning after antennal injury: locally increasing the need to clear neural debris is associated with increased sleep, which is required for efficient active zone removal after injury.

ETOC Blurb

Singh and Donlea find that severing the antennal nerves of the fruit fly acutely increases sleep. Sleep responses of injured flies can be weakened by manipulations that protect severed axons from removal. Sleep deprivation after injury slows the removal of neural debris from severed axons, indicating a role for sleep in recovery from neural damage.

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Author Contributions

Conceptualization: J.D. & P.S., Methodology: P.S. & J.D., Investigation: P.S. & J.D., Writing – Original Draft: J.D., Writing – Review & Editing: P.S. & J.D., Funding Acquisition – J.D., Supervision – J.D.

Declaration of Interests

The authors declare no competing interests.

Introduction

Sleep is a state that has been broadly conserved across animal species [1,2], but its basic biological functions remain poorly understood. Because deficits in memory and neural plasticity are among the earliest consequences of sleep loss, it is likely that basic functions of sleep promote plasticity [3]. While recent studies support a role for sleep in the homeostatic downscaling and pruning of synapses to prevent circuit saturation and retain the capacity to encode new information [4–9], the contexts during which sleep contributes to synapse remodeling require further examination. Here, we explore the relationship between sleep and the removal of presynapses and axons after axotomy. *Drosophila* antennal transection provides a reliable assay to acutely increase local axonal pruning [10,11], but the role for sleep in clearing injured axons has not been examined in the fly. A growing literature, however, links traumatic brain injury and sleep/wake changes in humans and rodents with several reports describing hypersomnia occurring acutely after neural trauma [12–14]. This increased sleep may facilitate functional recovery after injury; maximizing sleep after injury can reduce neuron loss and limit behavioral impairments in rodents [15,16]. Similarly, disrupted sleep after traumatic brain injury is associated with decreased functional recovery in humans, and treatment for sleep disorders after brain injury can significantly aid cognitive recovery [17]. These studies implicate a role for sleep in acute neural pruning after injury, and *Drosophila* provides a model system with possibilities for precise targeting of both neural damage and genetic manipulations.

To characterize the conditions that promote synaptic pruning during sleep, we have examined *Drosophila* following antennal injury. Primary olfactory receptor neurons (ORNs) send axonal projections from the antennae, where they detect odorants in the external world, to neuropil glomeruli in the antennal lobe [18–20]. In these olfactory glomeruli, ORNs synapse upon dendrites of secondary projection neurons, which convey olfactory signals to circuits in the Mushroom bodies and Lateral horns [18,21,22]. When the antennae are transected, the distal segment of severed ORN axons fragment and are removed over several following days [10]. Similar degeneration has also been observed in chemo- and mechano-sensory neurons after injuries to other appendages, including the maxillary palps, wings, and legs [10,23], indicating that the underlying mechanisms may be generalizable to circuits throughout the nervous system. Interestingly, the fragmentation and clearance of damaged ORNs is controlled by a molecular program that is shared with vertebrate models of degeneration after axonal injury [10,24–27]. Over the past decade, the molecular and genetic programs in *Drosophila* that promote the clearance of damaged axons after neural injury have been carefully dissected [10,28–30]. Removal of injured ORN axons relies upon the neural expression of several genes, including the E3 ubiquitin ligase *highwire* (*hiw*); mutations in *hiw* delay the removal of injured axons from the antennal lobe by weeks [29]. These studies provide an opportunity to test the functional role for sleep in pre-synaptic removal following neural injury. Here, we show that sleep is acutely increased after neural injury. Importantly, injuries that do not sever neurons and injuries in flies expressing neuroprotective manipulations of *highwire* and *basket* do not elicit an increase in sleep, suggesting that post-injury sleep may be linked with the degeneration of damaged neurons. Finally, we measure synaptic pruning and membrane clearance from damaged axons and

find that sleep may promote the removal of both synaptic terminals and axonal membrane after injury.

Results

Sleep is temporarily increased following antennal transection

To measure the effect of neural injury on sleep, 4–7 day old *Canton-S* (*Cs*) females were monitored for two days of baseline sleep, then briefly anesthetized for ~20 seconds with CO_2 while antennae were bilaterally transected at the third segment using forceps (See schematic in Figure 1A). After antennal injury, each fly was placed back into a glass tube containing standard *Drosophila* media and sleep was monitored during recovery. The sleep of injured *Cs* flies was compared to those of siblings that received the same handling and anesthesia exposure, but whose antennae remained intact. Following antennal transection, sleep is strongly increased for ~9–10 hours in injured *Cs* flies compared to uninjured siblings (Figure 1B; the time window of antennal injury is depicted with yellow shading). This temporary sleep increase from injured *Cs* flies dissipates by the following day, when sleep time of injured flies returns to the same levels as in uninjured controls (Figure 1C). Increased sleep after antennal injury can be attributed to a lengthening of sleep bout duration (Figure 1D). Because prolonged sleep bout length is hypothesized to be a marker of sleep intensity in *Drosophila* [31], we also exposed flies to varying durations of mechanical vibration to test arousability in injured flies and uninjured siblings during the day of antennal transection. A smaller portion of injured flies was awakened by 1, 5, or 10 second-long mechanical stimulations using a 0.5g mechanical vibration (Figure S1A, left). A more intense 1.5g stimulus, however, was sufficient to awaken the majority of flies in injured and control groups (Figure S1A, right), suggesting that antennal injury promotes a state of deep sleep with elevated arousal thresholds. To further verify that sleep is increased after antennal injury, we monitored locomotion using multi-beam activity monitors that contain 17 infrared sensors across a 51mm tube length (Trikinetics Inc; Waltham MA, USA). Experiments using these multi-beam monitors confirmed that sleep is increased after antennal transection (Figure S1C). Single beam monitors found a modest but significant decrease in waking activity as measured by counts/waking minute after antennal injury (Figure 1G); measurements using multibeam monitors found no significant main effect of injury on hourly averages of movements/waking minute during the day of injury (Figure S1D). We also tested whether sleep drive is increased after antennal injury by testing the responses of short-sleeping *insomniac* (*inc*) [32,33] and *sleepless* (*sss*) mutants [34]. Neither *inc* nor *sss* mutants had any significant sleep increase following antennal transection (Figure S1E–J), suggesting a link between increased sleep after antennal injury and previously characterized sleep regulatory genes.

Interestingly, sleep during the night following antennal transection is modestly decreased in injured *Cs* flies (Figure 1E), but the length of night-time sleep bouts was not different between injured flies and controls (Figure 1F). We tested whether sleep during the night is increased immediately following injury by observing the responses to antennal transection shortly before the beginning of the night (ZT11–12). As shown in Figure 1H, flies that underwent antennal transection prior to lights-off did not show an increase in sleep at night,

but did increase their sleep for several hours of the subsequent morning. These results indicate that increases in sleep are restricted to the day, possibly due to circadian influence. This response follows the pattern of homeostatic sleep drive that is observed when flies are released into recovery from sleep deprivation at the end of the light phase [35]. Wild-type *Cs* flies housed in constant darkness respond to antennal transection with an increase in sleep after antennal injury (Figure S1B), and mutants for the core circadian genes *clock* (*Clk*^{JRK} [36]) and *cycle* (*cyc*^{01} [37]) show a significant increase in sleep after antennal injury (Figure S2A–D). We also tested responses of null mutants for *timeless* (*tim*^{01} [38]) and *period* (*per*^{01} [39]) in two genetic backgrounds. *tim*^{01} flies slept more after antennal injury in one background (*yw*) (Figure S2E–G), while *per*^{01} mutants did not increase their sleep in either genetic background (Figure S2H–J). Together, these results suggest that an intact circadian clock is not required for increased sleep after antennal injury, but that *period* may influence injury responses via processes independent of circadian timekeeping. Interestingly, mutants for *period*, but not other circadian genes, also exhibit memory deficits [4,40], consistent with non-circadian functions for *per.*

Increased sleep following injury is dependent upon site of neural damage

After finding that sleep was increased after antennal injury, we next tested whether post-injury sleep was related specifically to neural damage or to generalized inflammatory responses to damage of any tissue. First, we measured whether the amount of post-injury sleep is proportional to the number of damaged axons by measuring the effect of injuring one or two antennae on subsequent sleep. As shown in Figure 2A, severing both antennae produces a larger increase in sleep than removing only one antenna, suggesting that post-injury sleep is correlated with the number of damaged ORNs. To further test the relationship between the magnitude of neural injury and post-injury changes in sleep, we tested whether damage to other sensory tissues also affects sleep. We focused these experiments on the maxillary palps, a second pair of olfactory organs that each contain ~125 ORNs [41], the wings, which contain ~450 sensory neurons per wing in chemosensory sensilla along their anterior edge [42], and the halteres, which each contain ~335 proprioceptive sensory neurons [43]. Our experiments avoided injuring other peripheral areas that contribute to locomotion, such as the legs, or to the entrainment of the circadian clock, including the visual system. A significant increase in post-injury sleep was detected after wing injuries (Figure 2B), but transections of the maxillary palp (Figure 2C) and haltere transections (Figure 2D) had little effect on subsequent sleep. While a modest decrease in sleep was observed during the night after antennal injuries (Figure 1E), wing injury resulted in increased sleep during several night-time hours (Figure 2B). These differing effects on night-time sleep could indicate that the more intense sleep response following antennal injury may decrease homeostatic sleep drive during the following night. Further studies will be required to examine the differential effects of antennal and wing injuries on sleep during the following night.

Next, we tested whether combining injuries at multiple sites might alter sleep during the following hours by subjecting flies to both maxillary palp and haltere injuries (Figure 2E) or both wing and haltere injuries (Figure 2F). These combined injury conditions did not result in additive effects compared to responses after injuries to individual sites, consistent with the

possibilities that sleep is significantly increased only after injuries to the wings or antennae, or that the increase in sleep after neural injury is determined by the largest site of injury. Interestingly, these results include injuries to sensory neurons that project directly into the brain from the antennae and maxillary palps, as well as neurons from the wings and halteres that limit their axons to the ventral nerve cord (VNC). While wing and haltere injuries likely drive degeneration of sensory neuron projections in the VNC, it is not clear whether they also lead to the reorganization of secondary projections into the central brain.

Loss of olfaction does not increase sleep

While the increased sleep after antennal transection is consistent with a role for sleep in neural degradation after injury, it is also possible that the change in sleep might be influenced by nociceptive signaling or loss of sensory perception. To test whether post-injury increases in sleep could be attributed to loss of olfactory perception, we acutely silenced ORNs by expressing the inward rectifying potassium channel Kir2.1 under the control of *orco*-GAL4 and the GAL4 repressor *tubP*-GAL80^{TS} [44–46]. Flies were raised and baseline sleep was measured at 18°C (Figure 3A), then transferred to 29°C for two days to permit Kir2.1 transgene expression in ORNs before sleep was measured again (Figure 3B). No significant difference in daytime sleep was observed between the experimental flies expressing Kir2.1 in ORNs and genetic controls at either 18°C or 29°C (Figure 3C), and a significant reduction in nighttime sleep was detected in experimental flies at 29°C (Figure 3D). We verified that expression of Kir2.1 in ORNs impaired olfactory perception by using an olfactory trap assay. When housed at 18°C, experimental *orco*-GAL4/*tubP*-GAL80^{TS}; UAS-*kir2.1*/+ flies and genetic controls showed similar preferences to traps containing apple juice versus water (Figure S3A, left). After two days at 29°C, however, experimental flies expressing Kir2.1 in ORNs exhibited a significant loss of attraction to apple juice (Figure S3A, right). These results indicate that loss of olfactory perception is not sufficient to increase sleep.

Similarly, if increased sleep after injury can be attributed to clearance of damaged axons and not loss of sensory perception, then mutants with generalized olfactory deficits should exhibit the same post-injury sleep responses as wild-type flies. To test this hypothesis, we performed antennal injuries on *smellblind* (*para*$^{sbl-1}$) mutant flies, which exhibit deficits in discriminating pure odorants [47,48] and socially-relevant pheromonal cues [49,50]. Homozygous *para*$^{sbl-1}$ mutants and heterozygous *para*$^{sbl-1}$/+ controls both showed significant increases in sleep after antennal injury (Figure 3E–G), suggesting that post-injury sleep responses are not primarily caused by sensory loss. Furthermore, flies in which ORNs are silenced by expression of Kir2.1 exhibit a similar increase in sleep after antennal injury as genetic controls (Figure S3B). However, certain *para* mutants that exhibit seizures at elevated temperatures have also been shown to prevent the removal of neurites and synapses from severed axons [51], potentially confounding our hypothesis. We therefore performed antennal transections in *para*$^{sbl-1}$ mutant males and observed robust losses of both ORN axonal membrane, labelled with CD8::GFP, and a genetic reporter for the pre-synaptic protein BRP (Synaptic Tagging with Recombination, STaR [52]) within 24 hours after injury (Figure S3C–D). Taken together, these data are consistent with our hypothesis that increased sleep after injury can be attributed to neural injury, not altered olfactory perception.

Intact ORN axons are required for increased post-injury sleep

If increased sleep after antennal transection is correlated with the degeneration of damaged ORNs, then sleep should not be changed after injury in flies with reduced ORN innervation of the antennal lobes. To test this hypothesis, we measured sleep after injury in flies mutant for the olfactory co-receptor *orco*, which is required for olfactory receptor proteins to form odorantgated ion channels [53,54]. Without any electrical activity, ORNs in *orco* mutants are unable to maintain their axonal projections and withdraw from the antennal lobe [55]. To verify that ORN axons retract in *orco* mutants, we expressed CD8::GFP in ORNs that project their axons into the DM2 glomerulus using *OR22a*-GAL4. DM2 glomeruli in *orco* mutant backgrounds show a 84–93% reduction in GFP-labelled ORN axons compared to heterozygous controls (Figure 4A–B). To test whether intact ORN axons are required for increased sleep after injury, 4–6 day old flies carrying $orco^{1}$ and/or $orco^{2}$ mutations [54] were loaded into activity monitors, then underwent antennal transection after 2–3 days of baseline sleep. While heterozygous controls ($orco^{1}$/+ and $orco^{2}$/+) showed a wild-type increase in sleep after injury, no post-injury changes in sleep could be detected in homozygous $orco^{1}$ or $orco^{2}$ mutants or in $orco^{1}/orco^{2}$ transheterozygotes (Figure 4C–E). Similarly, post-antennal injury sleep bouts were lengthened in heterozygous controls ($orco^{1}$/+ and $orco^{2}$/+), but not in *orco* mutant flies ($orco^{1}$, $orco^{2}$, and $orco^{1}/orco^{2}$) (Figure S4). Because *orco* mutants show significant increases in sleep after wing injury (Figure 4F–H), their lack of sleep responses after antennal transection is likely attributable to ORN loss.

Highwire is required for post-injury sleep induction

Several studies over the past decade have identified a network of genes expressed in *Drosophila* neurons that influence the kinetics of fragmentation and clearance of injured axons [10,27,28,30,56–58]. This network includes the E3 ubiquitin ligase *highwire* (*hiw*), which acts as a core regulator for degeneration of injured axons [29,57]. Mutations in *hiw* prevent the fragmentation and removal of injured ORN axons; severed ORN axons after antennal transection remain largely intact in *hiw* mutants for at least 20 days after injury [29]. To test whether *hiw* is also required to increase sleep after antennal injury, we tested flies with two mutant alleles for *hiw*: $hiw^{\Delta N}$ and hiw^{ND8} [59,60]. *hiw* mutant flies show no change in sleep time after antennal transection, while heterozygous controls increase their sleep comparably to wild-type flies (Figure 5A–C). Because *hiw* mutations alter synaptic connectivity during development [60] and reduce nociceptive responses [61], we observed ORN morphology in $hiw^{\Delta N}$ mutants. We used *OR22a*-GAL4 to drive expression of CD8::GFP and STaR labelling for BRP; while baseline levels of axonal CD8::GFP and BRP::V5 were significantly decreased in $hiw^{\Delta N}$ mutants compared to wild-type controls (Figure S5A–D), we found that $hiw^{\Delta N}$ mutants show no loss in BRP::V5 for at least 33 hours after antennal transection (Figure S5E–F). Because wild-type flies exhibit a robust increase in sleep after transection of 50% of ORNs with the removal of one antenna (Figure 2A), it is unlikely that the post-injury sleep phenotype in *hiw* mutants can be attributed solely to the baseline reduction in ORN pre-synapses. Next, we tested whether *hiw* mutants are capable of increasing their sleep in response to a second physiological stressor, a 1-hr exposure to a 37°C heat stress [62]. Like heterozygous controls, *hiw* mutants exhibit robust increases in sleep after heat stress for 1 hour from ZT0–1 (Figure 5D–F; time of heat exposure depicted with orange shading). This result indicates that the suppressed responses

of *hiw* mutants after antennal injury is likely related to the neuroprotective effects of *hiw* loss, not to any developmental changes in sleep regulatory circuits. To test whether the lack of post-injury sleep induction in *hiw* mutants can also be attributed to ORN dysfunction, we expressed an RNAi hairpin targeted against *hiw* transcripts (UAS-hiw^{RNAi}) in ORNs. As shown in Figures 5G–H, flies expressing hiw^{RNAi} show a weakened increase in sleep after antennal injury when compared to genetic controls. A similar effect can be also observed when *orco*-GAL4 drove expression of a partially dominant negative transgene for *hiw*, UAS-$hiw^{\Delta RING}$ [59] (Figure S5G–H). To test whether other genes that influence axonal degeneration after injury are also required for flies to increase their sleep after injury, we also tested the effects of expressing a dominant-negative transgene for the MAPK *basket* (bsk^{DN}) in ORNs [63]. *bsk* is homologous to the mammalian c-Jun N-terminal kinase (JNK) [64], and JNK is activated in response to injury and is required for the degeneration of severed axons in flies and mammals [27,65–68]. Injured *orco*-GAL4/UAS-bsk^{DN} flies showed a dampened increase in sleep after injury compared to genetic controls (Figure 5I–J). While *hiw* has been shown to be an upstream regulator of *bsk* [64], it is unclear whether the two proteins act in a common pathway or independently to promote sleep after antennal injury. Together, these data indicate that both *hiw* and MAPK activity are required in injured neurites to promote sleep, and that the sleep-inducing effects of these molecules are consistent with a cell autonomous mechanism within injured neurons.

Sleep promotes the removal of active zones and plasma membrane from injured ORN axons

Wallerian degeneration of injured ORN axons is an actively regulated process that unfolds over roughly five days after injury [10]. Because the post-injury increase in sleep shown in Figure 1 lasts for only several hours, we became interested in observing the degeneration of injured ORNs within the first day after antennal transection. To detail the morphological changes in damaged ORNs that coincide with post-injury sleep changes, we used *OR22a*-GAL4 to label ORN axons in the DM2 glomerulus with both a membrane-targeted GFP (CD8::GFP) and a flp-based reporter for the active zone protein Bruchpilot (BRP::V5) using STaR [52]. This combination of reporters enabled us to simultaneously image the abundance of remaining axonal plasma membrane (CD8::GFP) and pre-synaptic active zones (BRP::V5) at several timepoints after antennal transection. Over the initial hours post-injury, the amount of GFP-tagged membrane remains unchanged, but a significant loss of STaR-tagged ORN active zones occurs within 6-hours of injury and labelled active zones are nearly entirely removed by 24 hours after injury (Figure 6A–B).

Because post-injury sleep is most strongly elevated during the same time that most active zones were pruned from damaged ORNs, we next tested whether depriving flies of sleep after injury slows the removal of pre-synapses from injured ORNs. Flies were injured in the hour following lights-on (ZT0–1), and either sleep deprived or allowed to sleep *ad libitum* for 9 hours (Figure 6C–D, S6A) or 24 hours (Figure 6E–F, S6B) prior to brain dissection, fixation, and staining. Sleep deprivation was not sufficient to alter either CD8::GFP or STaR labelling in uninjured control flies at either post-injury timepoint (Figure 6C–F). Flies that were allowed *ad libitum* sleep for 9 h after injury removed more STaR-labeled ORN active zones than siblings who were sleep deprived following injury (Figures 6C, D). No

significant effect of sleep on axonal membrane labeled with CD8::GFP was detected 9 h after injury (Figures 6C, D). When flies were dissected 24 hours after injury, STaR labelling was significantly reduced in both rested and sleep deprived groups (Figures 6E, F), but the flies that were sleep deprived after injury showed a significantly weaker reduction in membrane-tethered CD8::GFP (Figures 6E, F). These results suggest that sleep deprivation slows the rate at which both active zone machinery and plasma membrane are removed from severed axons following antennal transection.

Discussion

Sleep contributes to neural remodeling in a variety of neural circuits across several model species. To better understand how sleep contributes to neural remodeling, we have examined the responses of *Drosophila* to injuries targeted to populations of sensory neurons. Injuries delivered to either the antennae or the wings drive an increase in sleep that lasts for several hours. Because no increase in sleep occurred when ORNs were acutely silenced or when antennal injuries were delivered to *orco* mutants with few intact ORNs, we hypothesized that post-injury increases in sleep were associated with the clearance of debris from damaged neurites. Our results are consistent with this hypothesis: sleep deprivation slowed the clearance of STaR-labelled pre-synaptic active zones and GFP-tagged plasma membrane from severed axons.

The fragmentation and clearance of severed axons is controlled by a network of proteins that interact with the E3 ubiquitin ligase *hiw*. Manipulations of these molecules can permit severed axons to remain intact for days or weeks after injury [10,27–30,66]. Immunostaining for active zone proteins have indicated that larval NMJ pre-synapses are removed prior to axonal fragmentation, and can be delayed by manipulations that also slow axonal fragmentation [51,68,69]. The diversity and density of synapses in the antennal lobes, however, have prevented similar characterization of synapse removal in adult ORNs. The recent development of StaR [52], along with other reporters that genetically tag synaptic proteins in targeted neuronal populations [70], now broaden the opportunities to observe active zone clearance in injured ORN neurites in the central brains of adult *Drosophila*. Our data suggest that pre-synapses may be selectively removed from injured axons before the axons are cleared themselves, and that the period of increased sleep after antennal injury closely parallels the timecourse of ORN synapse pruning. Further, sleep after antennal injury plays a functional role in presynaptic elimination: flies allowed to sleep *ad libitum* after injury cleared a significant portion of the pre-synaptic terminals from damaged ORNs, while sleep deprivation slowed post-antennal injury active zone clearance. Although we find that sleep disruption slows both the removal of pre-synapses and of neurites, it is not clear whether sleep promotes these processes via independent or shared functions. It is possible, for instance, that sleep facilitates active zone removal in the first hours after injury and that axonal fragmentation occurs only after synaptic contacts are dismantled. This model is consistent with previous reports of a latent period between axonal injury and membrane fragmentation [71–73], and raises the possibility that active zone removal may be an initial checkpoint of axonal degeneration that must precede axon fragmentation and clearance.

Our results provide evidence for sleep-dependent pruning of synapses from damaged ORNs, but the processes that mediate synapse removal during sleep will require future investigation. One likely mechanism of active zone clearance would be phagocytosis by ensheathing glia. This glial subset shares functional similarities with mammalian microglia, is responsible for engulfing and degrading axonal debris after antennal injury [11, 74], and its activation in response to injury is dampened by sleep loss [75]. Similar effects can also be observed when clathrin-mediated endocytosis is disrupted in glial cells that contribute to the blood-brain barrier [76], suggesting that other types of glia may also influence sleep-dependent clearance of debris from the brain. Because results from the fly parallel recent observations that sleep deprivation can increase activation of and synaptic engulfment by mouse astrocytes and microglia [77], neuro-glial interactions may provide an evolutionarily conserved mechanism for pruning synapses during sleep.

While we find a role for sleep in pruning active zones from damaged axons, the functional consequences of this pruning require extensive investigation. Sleep deprivation after antennal injury, for instance, increases the acute retention of ORN pre-synapses within the antennal lobe. Protecting these synapses may provide the benefit of sustaining maximal circuit stability. It is also possible, however, that pre-synapses and axons from damaged neurons need to be pruned and cleared to permit circuit recovery and the regeneration of functional neural connections. Indeed, Kazama et al., (2011) found that removing one antenna immediately eliminates odor-evoked responses from olfactory projection neurons, the post-synaptic partners of ORNs, on the injured side of the brain. After a recovery period, however, neural projections from the contralateral hemisphere strengthen to the injured side and odor-evoked responses can be partially restored. Importantly, the recovery of olfactory responses in the injured hemisphere requires the clearance of debris from damaged ORNs [78]. Complementary results have also been found after axonal injury in *C. elegans,* where engulfment receptors expressed by phagocytes may also act as adhesion molecules for regenerating motor neuron axons [79]. Sleep has also been linked with increased rates of olfactory granule cell apoptosis in mice following sensory deprivation [80]. It is possible, therefore, that sleep-dependent synapse removal prepares the circuit for subsequent rebalancing and recovery.

Our findings link pre-synapse clearance during sleep with responses to neural injury, but many of the molecules that mediate synapse removal after axonal transection are also shared with the regulation of synapse pruning in other contexts [81,82]. While sleep has also been linked with synapse removal during development or after experience-dependent plasticity [4,8,9,83], it remains unclear whether similar mechanisms may influence the removal of synapses under physiological conditions and following neural trauma. *Hiw* and *bsk* both influence the growth of developing synapses at the larval neuromuscular junction [60,64], indicating that the injury response pathway is likely conserved to other plastic conditions. Hiw protein also localizes to pre-synaptic active zones and regulates evoked neurotransmitter release, suggesting that the molecules that impart injury responses may be required more broadly to maintain synapse structure and physiology [84]. A recent study also found that the sleep regulatory gene *insomniac* is also required for homeostatic plasticity in the larval neuromuscular junction, indicating a link between the molecular pathways that regulate synaptic plasticity and sleep [85]. Interestingly, expression of

hiw$^{\Delta RING}$ in different cell types has opposing effects on the need for sleep: expressing *hiw*$^{\Delta RING}$ in circadian ventral lateral neurons suppresses sleep and causes learning impairments, while expression in mushroom body Kenyon cells enables flies to maintain wild-type learning after sleep loss [86]. These data support the possibility that *hiw* functions during sleep to regulate structural plasticity in a circuit- and/or context-dependent manner. Our results, in aligning with these data and with historical predictions of sleep function, indicate that our assay will enable the identification and dissection of further mechanisms that promote synaptic pruning during sleep.

STAR Methods

Lead contact and materials availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeffrey M. Donlea (jdonlea@ucla.edu). This study did not generate new unique reagents.

Experimental model and subject details

Fly Strains—Fly stocks were reared on standard cornmeal media (per 1L H_20: 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. *Canton-S* flies were from Gero Miesenböck (University of Oxford), w^{1118} flies were from David Krantz (UCLA), LexAOP-CD8::GFP; UAS-flp, PBac{y[+mDint2]w[+mc]=brp(FRT.Stop)V5–2A-LexA-VP16} [52] was from Lawrence Zipursky (UCLA), *yw*,*per*^{01} [39] and *yw*; *tim*^{01} [38] were supplied by Amita Sehgal (University of Pennsylvania), and UAS-*hiw*$^{\Delta RING}$ [59] was from Aaron Diantonio (Washington University in St. Louis). UAS-*hiw*^{RNAi} [87] was ordered from the Vienna *Drosophila* Resource Center. w^+, *per*^{01} [39], w^+;*tim*^{01} [38], *cyc*^{01} [37], *Clk*^{JRK} [36], OR22a-GAL4 [20], UAS-*kir2.1*::GFP [44], *tubP*-GAL80^{TS} [46], UAS-CD8::GFP [88,89], *para*$^{sbl-1}$ and *para*$^{sbl-2}$ [47], *orco*-GAL4 [45], *hiw*$^{\Delta N}$ [60], *hiw*^{ND8} [59], and UAS-*bsk*^{DN} were obtained from the Bloomington *Drosophila* Stock Center.

Method Details

Behavioral Analysis

Sleep—Sleep was measured as previously described [2]. Briefly, 3–7 day old female flies were individually loaded into 65mm-long glass tubes and inserted into *Drosophila* activity monitors (Trikinetics Inc; Waltham MA, USA). Periods of inactivity lasting at least 5 minutes were classified as sleep. Sleep deprivation occurred mechanically via the Sleep Nullifying Apparatus (SNAP) [2]. Trikinetics activity records were analyzed for sleep using custom Visual Basic scripts [2] in Microsoft Excel or the Sleep and Circadian Analysis MATLAB Program (SCAMP) scripts [92] in Matlab R2016B (Mathworks; Natick MA, USA). Antennal, maxillary palp, haltere, and wing transections were completed under CO_2 anesthesia using fine forceps. Control flies received identical handling and CO_2 anesthesia exposure as the injured groups, without any injury. Multibeam monitoring experiments used MB5 monitors (Trikinetics Inc; Waltham MA, USA) to observe fly movements.

Arousability—Arousability was tested by attaching Trikinetics activity monitors to microplate adapters on vortexers (VWR). Vibration force intensities were measured using Vibration 3.83 (Diffraction Limited Design; Southington CT, USA). Arousal tests used a 0.5g or 1.5g stimulation of 1, 5, or 10 second duration, and were delivered during the first 8 hours after injury (ZT1–9).

Olfactory preference—Olfactory preference tests were based on previous descriptions [93,94]. Groups of 10 flies were placed in circular petri dishes (100 mm diameter x 15 mm depth) containing two ~7mm diameter holes on the bottom surface leading to recessed traps. One trap contained 1mL of deionized water while the other contained 1mL unfiltered apple juice. Traps were placed in dark incubators, then the number of flies contained in each trap were counted manually. Odor preference indices were calculated as follows: PI = (# of flies in apple juice trap − # of flies in water trap)/(# of flies in apple juice trap + # of flies in water trap).

Immunohistochemistry and confocal microscopy—*Drosophila* brains were dissected in PBS (1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, 175 mM NaCl; Sigma-Aldrich) and fixed in 4% (w/v) paraformaldehyde (Electron Microscopy; Hatfield PA, USA) in PBS for 30–45 minutes on ice. For GFP immunostaining, brains were incubated in primary antibody (1:1000 chicken anti-GFP, Molecular Probes) overnight followed by secondary antibody (1:1000 anti-chicken antibody conjugated to Alexa488, Molecular Probes) for roughly 24 hours. Immunostaining for V5 used a 48 hour incubation period in mouse anti-V5 conjugated with DyLight550. All brains were mounted in Vectashield (Vector Labs; Burlingame CA, USA) and imaged on a Zeiss LSM880 using a confocal slice thickness of 1 uM. All image processing was completed using Fiji [94]. Quantification of antennal lobe glomerular fluorescent signal intensity used a sum slices projection including all z-slices through DM2 glomeruli, followed by outlining of the labelled glomeruli to measure area and mean GFP or anti-V5 intensity signal.

Quantification and statistical analysis

Statistical Analysis—Data were analyzed in Prism 8 (GraphPad; San Diego CA, USA). Group means were compared using two-tailed T-tests or one- or two-way ANOVAs, with repeated measures where appropriate, followed by planned pairwise comparisons with Holm-Sidak multiple comparisons tests. Mann-Whitney or Kruskal-Wallis tests, followed by Dunn’s multiple comparisons tests, were used in cases where assumptions of normality did not hold. Sample sizes for each experiment are depicted in each figure panel or in the appropriate figure legend. All group averages shown in data panels depict mean ± SEM.

Data and code availability

All software used during this study is described in the Key Resources Table. Further information about analysis or data are available upon reasonable request from the Lead Contact, Jeffrey M. Donlea (jdonlea@ucla.edu).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sleep in *Drosophila* is temporarily increased following antennal removal
- Increased sleep after injury can be attributed to neural damage
- Sleep after injury promotes the removal of pre-synaptic proteins and axonal debris

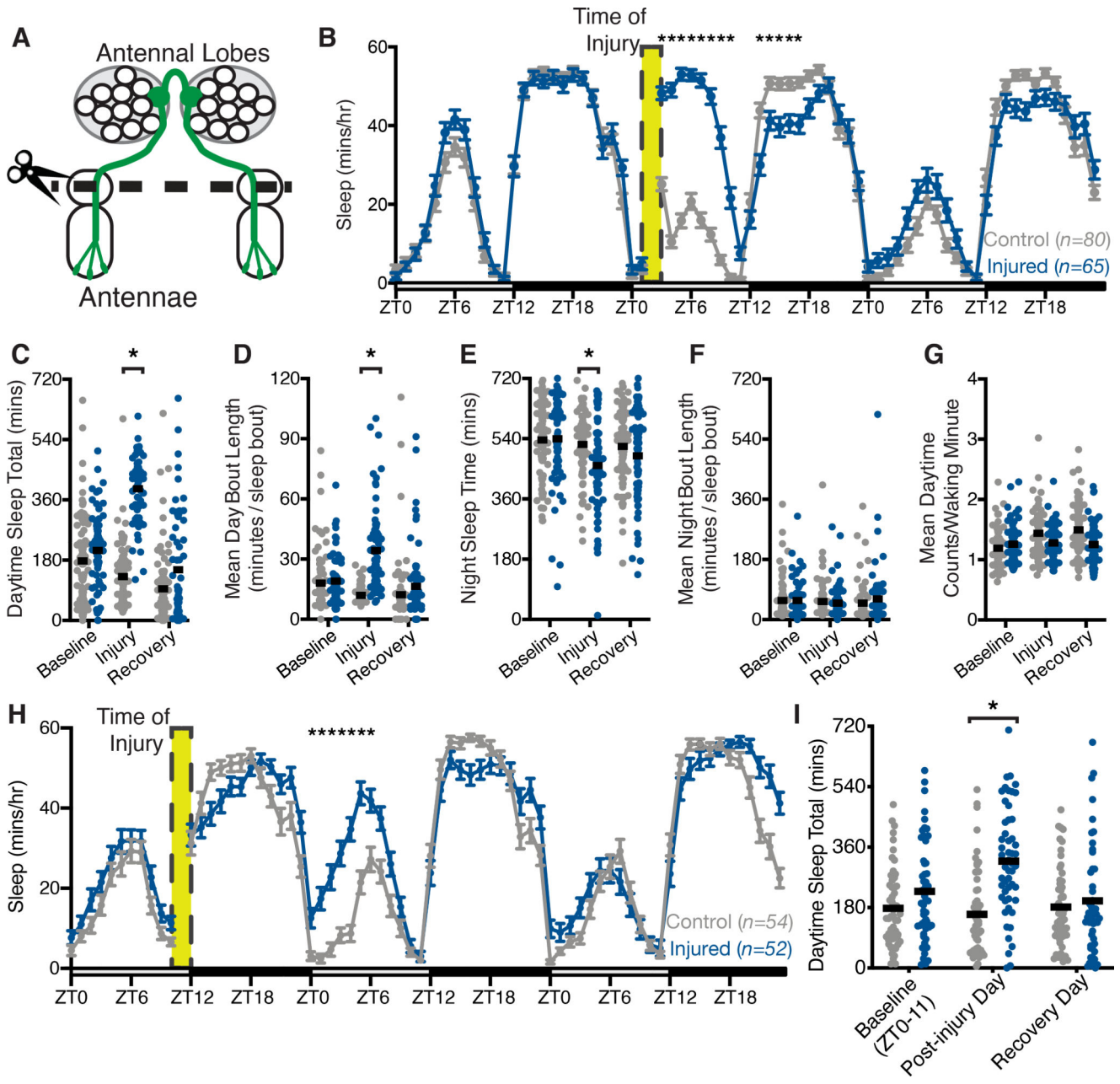

Figure 1 –. Sleep is temporarily increased following antennal transection

(A) Schematic of primary olfactory receptor neurons (ORNs) in *Drosophila melanogaster*. Antennae are bilaterally transected at the 3rd segment to sever ORN axons.

(B) Sleep in minutes/hour in *Canton-S* (*Cs*) flies from either control (grey) or antennal transection (blue) groups. Sleep is acutely increased during the hours after antennal injury, but returns to control levels by 24-hours later. Two-way repeated measures ANOVA of hourly sleep timecourse finds a significant time by treatment interaction ($F_{(10,10010)}$=22.37, p<0.0001; n=65 injured, 80 control).

(C-D) Sleep time **(C)** and mean sleep bout length **(D)** during the day is increased immediately following injury, but decreases back to baseline on the following day of

recovery. Two-way repeated measures ANOVA finds a significant day by treatment interaction for day sleep time ($F_{(2,286)}$=63.98, p<0.0001) and day bout length ($F_{(2,286)}$=26.24, p<0.0001).

(E) Sleep time is decreased in *Cs* flies during the night immediately following antennal transection. Two-way repeated measures ANOVA finds a significant day by treatment interaction ($F_{(2,290)}$=5.497, p=0.0045). * signifies pairwise p<0.05.

(F) Antennal transection does not alter the length of sleep bouts during the night. Two-way repeated measures ANOVA finds no day by treatment interaction ($F_{(2,290)}$=1.167, p=0.3128).

(G) Activity counts/waking minute during baseline, injury day, and recovery day in controls in injured flies. Two-way repeated measures ANOVA finds a significant day by treatment interaction ($F_{(2,290)}$=18.06, p<0.0001).

(H) Antennal injury at ZT11–12 is followed by an increase in sleep during the subsequent day. Two-way repeated-measures ANOVA finds a significant time-bytreatment interaction ($F_{(70,7280)}$ = 7.617, p < 0.0001; mean ± SEM).

(I) Daytime sleep is increased for one day following antennal transection at ZT11–12 and returns to control levels by the following day of recovery. Two-way repeated-measures ANOVA finds a significant day-by-treatment interaction ($F_{(2,208)}$ = 12.40, p < 0.0001).

See also Figures S1 and S2.

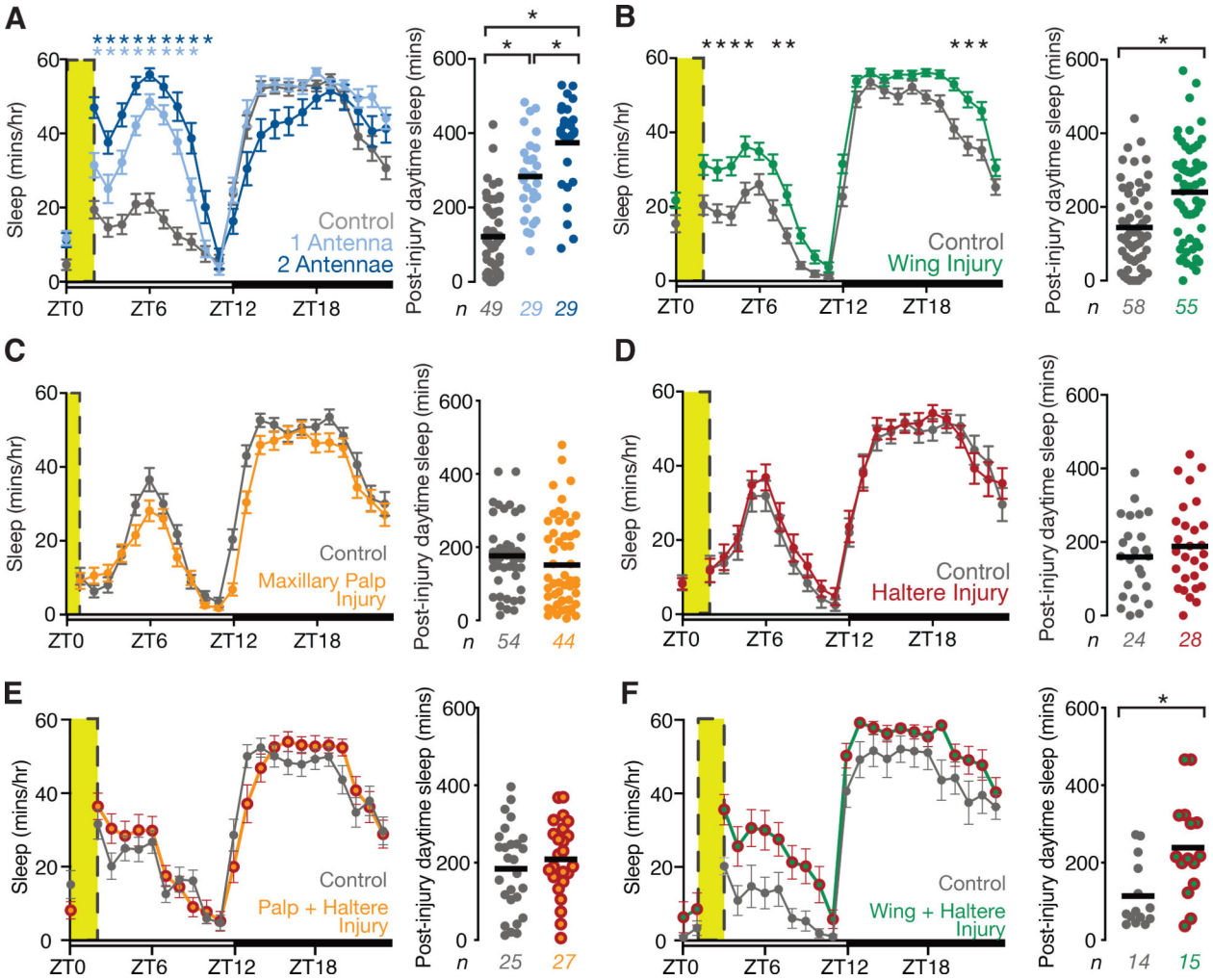

Figure 2 –. Post-injury increases in sleep vary with site of injury

(A) Hourly sleep in wild-type *Cs* flies after transection of 2 antennae (dark blue) or 1 antenna (light blue) compared to uninjured controls (gray). Post-injury daytime sleep totals for each group is shown on right. One-way ANOVA finds a significant effect of condition on post-injury daytime sleep ($F_{(2,104)}$=54.05, p<0.0001, n=29 in each injured group, 49 controls). * signifies pairwise p<0.05.

(B) Hourly sleep totals (left) and post-injury daytime sleep totals (right) from *Cs* flies that underwent wing injury (green) and uninjured controls (gray). Two-way unpaired t-test for post-injury sleep totals finds a significant effect of wing injury (t= 4.078; p< 0.0001; n=58 injured, 55 controls).

(C) Sleep timecourse (left) and post-injury daytime sleep totals (right) for *Cs* flies after maxillary palp removal (orange) compared to uninjured siblings (gray). Two-way unpaired t-test for post-injury sleep totals finds no significant effect of palp injury (t= 1.078; p= 0.2834; n=54 injured, 49 controls).

(D) Hourly sleep totals (left) and post-injury daytime sleep totals (right) from *Cs* flies that underwent haltere removal (red) and uninjured controls (gray). Two-way unpaired t-test for

post-injury sleep totals finds no significant effect of haltere injury (t= 0.9145; p= 0.3648; n=28 injured, 24 controls).

(E) Sleep time course (left) and post-injury sleep totals (right) for flies that underwent injuries to both the maxillary palps and halteres (red with orange outline) and uninjured controls (gray). Two-way unpaired t test finds no significant difference in post-injury sleep time (t = 0.8543; p = 0.3970; n = 25 injured, 27 controls).

(F) Hourly sleep timecourse (left) and post-injury sleep totals (right) for flies that underwent injuries to both the wings and halteres (red with green outline) and uninjured controls (gray). Two-way unpaired t-test finds a significant effect of injury on post-injury sleep time (t=3.060, p=0.005, n=15 injured, 14 controls).

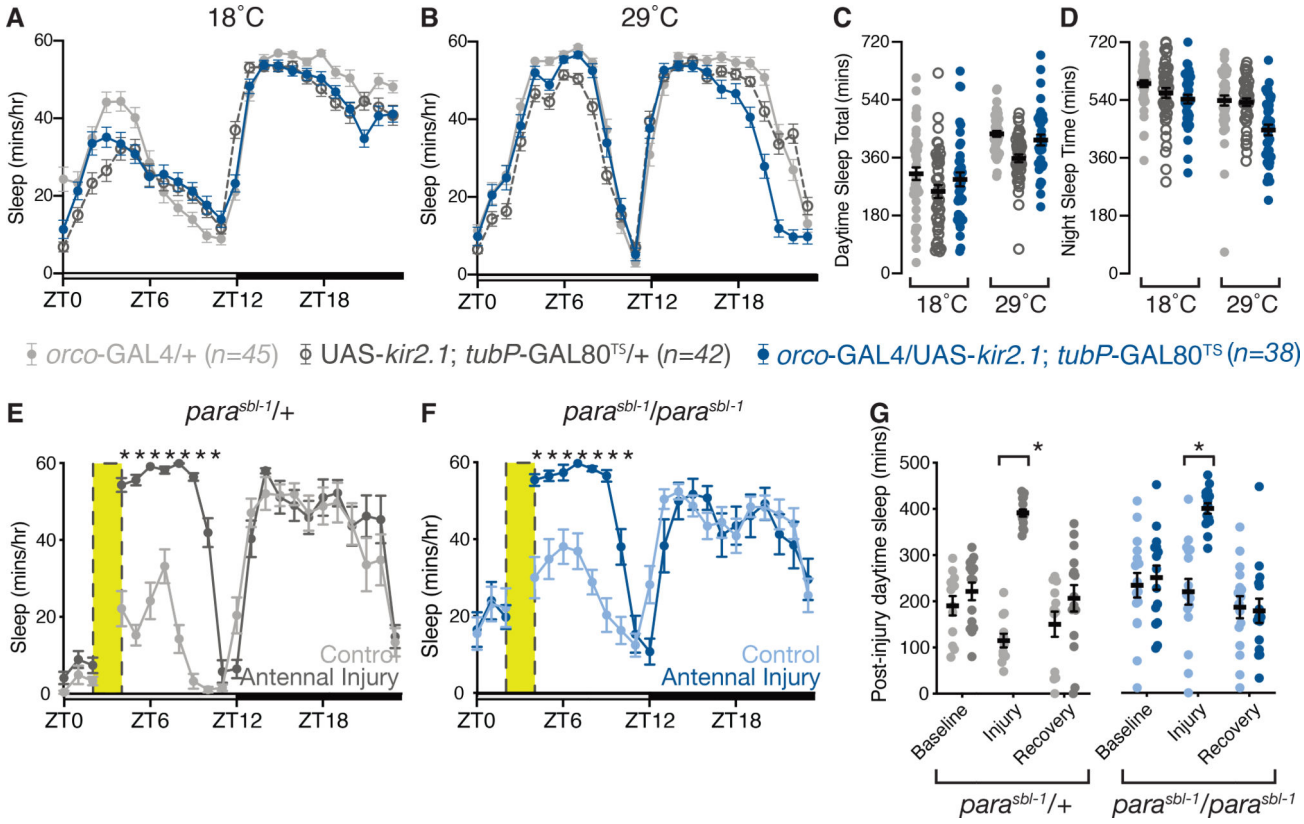

Figure 3 –. Loss of olfactory perception does not contribute to post-injury increases in sleep

(A-B) Silencing ORNs by expressing UAS-*kir2.1* under the control of *orco*-GAL4 does not alter sleep. When housed at 18°C to repress Kir2.1 expression in *orco*-GAL4/UAS-*kir2.1*; *tubP*-GAL80^{TS}/+ flies (A) or at 29°C to increase ORN expression of Kir2.1 (B).

(C) Day sleep totals at 18°C and 29°C for flies shown in (A-B). Two-way repeated measures ANOVA finds a significant main effect for temperature ($F_{(1,122)}$=105.7, p<0.001) and for genotype ($F_{(2,122)}$=6.159, p=0.0028).

(D) Total night sleep amounts for flies shown in (A-B) at 18°C and 29°C. Two-way repeated measures ANOVA finds a genotype x temperature interaction ($F_{(1,122)}$=4.468, p=0.0134).

(E-F) Hourly sleep for *para*$^{sbl-1}$/+ controls (E) and *para*$^{sbl-1}$ homozygous mutants (F) during the day of antennal injury. Injured flies increased their sleep for several hours after injury compared to uninjured siblings. Two-way repeated measures ANOVA finds a significant time x injury interaction for *para*$^{sbl-1}$/+ controls ($F_{(22,550)}$=15.36, p<0.0001, n=12–15 flies/group) and for *para*$^{sbl-1}$ mutants ($F_{(22,638)}$=8.113, p<0.0001, n=15–16 flies/group).

(G) Daytime sleep for *para*$^{sbl-1}$/+ heterozygotes (left; injured flies in dark gray, controls in light gray) and *para*$^{sbl-1}$ homozygous mutants (right; injured flies in dark blue, controls in light blue) during baseline, injury, and recovery days. Both *para*$^{sbl-1}$/+ heterozygotes and *para*$^{sbl-1}$ mutants exhibit similar increases in sleep after antennal transection. Two-way ANOVA finds a significant day x group interaction ($F_{(6,108)}$=15.71, p<0.0001, n=12–16 flies/group).

See also Figure S3.

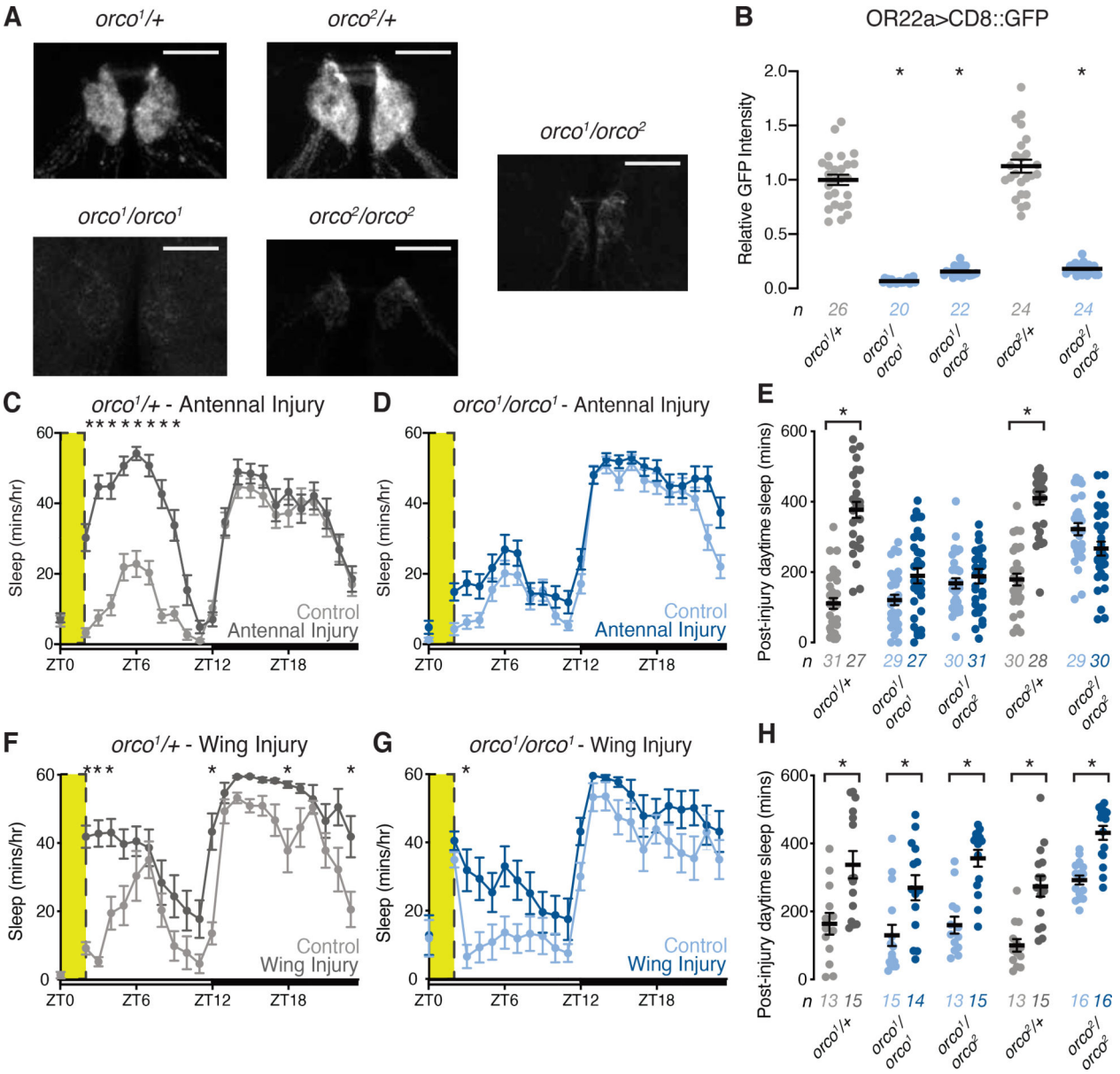

Figure 4 –. *orco* mutations drive ORN axon degeneration during early adulthood and prevent later changes in sleep after injury

(A) Representative images of membrane-bound CD8::GFP in ORNs under the control of *OR22a*-GAL4 in a heterozygous background (*orco*^{1}/+ or *orco*^{2}/+; top) or in *orco* mutants (*orco*^{1}, *orco*^{2}, and *orco*^{1}/*orco*^{2}). Scale bars represent 20μm.

(B) Quantification of OR22a-GAL4 driven UAS-CD8::GFP in *orco* mutants (*orco*^{1}, *orco*^{2}, and *orco*^{1}/*orco*^{2}) and heterozygous controls (*orco*^{1}/+ and *orco*^{2}/+). One-way ANOVA finds a significant effect of genotype ($F_{(4,111)}$=191.3, p<0.0001, n=20–26 hemispheres/group).

(C) Hourly sleep timecourse during the day of antennal injury for *orco*^{1}/+ flies. Sleep amounts for injured flies shown in dark gray and for uninjured controls in light gray. Two-way repeated measures ANOVA finds a significant injury x time interaction ($F_{(23,1288)}$=11.92, p<0.0001; n=27 injured, 31 control).

(D) Hourly sleep timecourse for injured *orco*^{1} homozygous mutant flies (dark blue) and uninjured sibling controls (light blue). Injured *orco*^{1} mutants show a dampened increase in sleep relative to heterozygous *orco*^{1}/+ flies. Two-way repeated measures ANOVA finds a significant injury x time interaction ($F_{(23,1334)}$=1.629, p=0.031; n=27 injured, 29 control).
(E) Post-injury daytime sleep totals for *orco* heterozygous controls (*orco*^{1}/+ and *orco*^{2}/+; grays) and *orco* mutant genotypes (*orco*^{1}, *orco*^{2}, and *orco*^{1}/*orco*^{2}; blues). Injured are depicted in dark shades, uninjured siblings in light shades. Each dot represents an individual fly, group means depicted by black bars. Two-way ANOVA finds a significant genotype x injury interaction ($F_{(4,286)}$=27.71, p<0.0001, n=27–31 flies/group).
(F) Hourly sleep timecourse for *orco*^{1}/+ flies during the day of wing injury. Sleep amounts for injured flies shown in dark grey and for uninjured controls in light grey. Two-way repeated measures ANOVA finds a significant time x injury interaction ($F_{(23,598)}$=3.562, p<0.0001, n=13–15 flies/group).
(G) Hourly sleep timecourse on the day of wing injury for *orco*1 homozygous mutants. Sleep amounts for injured mutants in dark blue and for uninjured mutants in light blue. Two-way repeated measures ANOVA finds a significant effect of injury ($F_{(1,27)}$=9.087, p=0.0055, n=14–15 flies/group).
(H) Daytime sleep totals on the day of wing injury for *orco* mutants (*orco*^{1}, *orco*^{2}, and *orco*^{1}/*orco*^{2}) and heterozygous controls (*orco*^{1}/+ and *orco*^{2}/+). For each genotype, injured flies showed an increase in daytime sleep after injury compared to uninjured siblings. Two-way ANOVA finds a significant effect of injury ($F_{(1,135)}$=84.21, p<0.0001) and genotype ($F_{(4,135)}$=12.55, p<0.0001).
See also Figure S4.

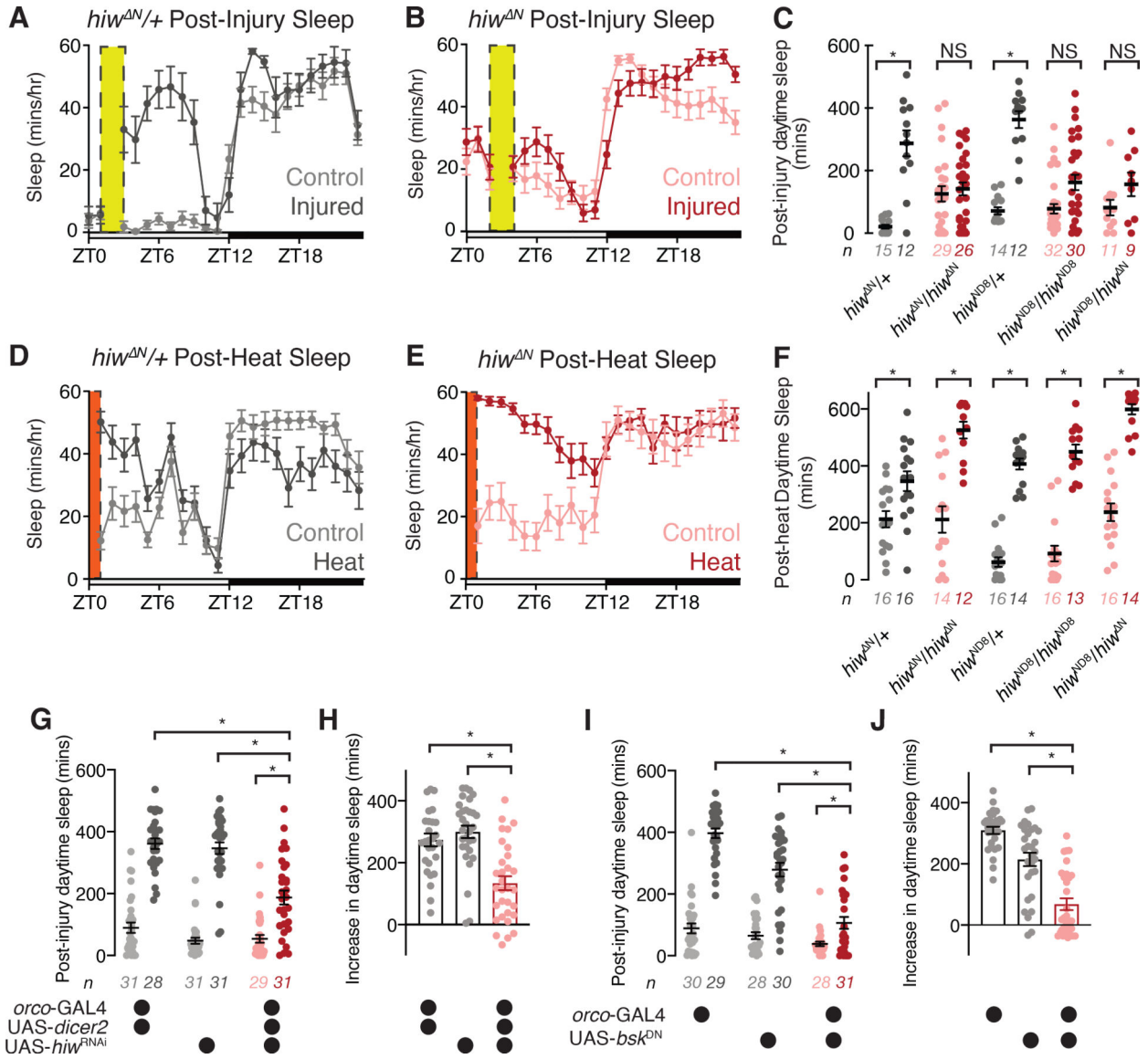

Figure 5 –. Expression of *hiw* is required for increased post-injury sleep

(A) Hourly sleep timecourse for $hiw^{\Delta N}$/+ heterozygous flies during the day of antennal injury. Sleep is significantly elevated in injured heterozygotes (dark gray) compared to uninjured controls (light gray) in the hours after injury (time of injury marked with yellow shading). Two-way repeated measures ANOVA shows a significant injury x time interaction ($F_{(23,575)}$=9.519, p<0.0001; n = 12 injured, 15 controls).

(B) Injury day sleep timecourse for homozygous $hiw^{\Delta N}$ mutants. No significant change in sleep was detected between injured mutants (dark red) and control siblings (light red). Two-way repeated measures ANOVA finds no significant effect of injury ($F_{(1,53)}$=1.019, p=0.3173; n = 26 injured, 29 controls).

(C) Total daytime sleep during the hours between injury and lights off for *hiw* heterozygotes ($hiw^{\Delta N}$/+ and hiw^{ND8}/+; injured shown in dark gray, controls in light gray) and mutants ($hiw^{\Delta N}/hiw^{\Delta N}$, hiw^{ND8}/hiw^{ND8}, and $hiw^{\Delta N}/hiw^{ND8}$; injured shown in dark red, controls in

light red). Two-way ANOVA shows a significant injury x genotype interaction ($F_{(4,180)}$=11.43, p<0.001)

(D) Hourly sleep timecourse for *hiw*$^{\Delta N}$/+ heterozygotes following 1-hour of housing at 37°C (heated flies shown in dark gray, controls in light gray). Sleep was increased in heated flies for several hours compared to siblings that were maintained at 25°C. Two-way repeated measures ANOVA finds a significant heat x time interaction ($F_{(23,690)}$=8.545, p<0.0001; n=16 each group).

(E) Hourly sleep timecourse after heat stress for *hiw*$^{\Delta N}$ homozygous mutants (heated flies in dark red, controls in light red). Heated flies show a robust sleep increase throughout the daytime relative to unheated siblings. Two-way repeated measures ANOVA finds a significant heat x time interaction ($F_{(23,552)}$=8.468, p<0.0001; n=12 heated, 14 control).

(F) Daytime sleep after heat stress in *hiw* heterozygotes (*hiw*$^{\Delta N}$/+ and *hiw*^{ND8}/+; heated flies shown in dark gray, controls in light gray) and mutants (*hiw*$^{\Delta N}$/*hiw*$^{\Delta N}$, *hiw*^{ND8}/*hiw*^{ND8}, and *hiw*$^{\Delta N}$/*hiw*^{ND8}; heated shown in dark red, controls in light red). All genotypes exhibited a significant increase in sleep after heat stress. Two way ANOVA finds a significant heat x genotype interaction ($F_{(4,137)}$=5.867, p=0.0002).

(G) Post-injury daytime sleep responses are weakened in flies expressing *hiw*^{RNAi} in ORNs (injured flies in dark red, controls in light red) compared to parental controls (UAS-*dcr2*/+; *orco*-gal4/+ and UAS-*hiw*^{RNAi}/+; injured in dark gray, controls in light gray). Two-way ANOVA finds a significant genotype x injury interaction ($F_{(2,175)}$=14.00, p<0.0001).

(H) The magnitude of increased sleep after injury is reduced by expression of *hiw*^{RNAi} in ORNs (red) compared to genetic controls (UAS-*dcr2*/+; *orco*-gal4/+ and UAS-*hiw*^{RNAi}/+; gray). Data for this panel were calculated using the same flies that were included in Figure 5G. One-way ANOVA finds a significant effect for genotype ($F_{(2,87)}$=18.15, p<0.0001).

(I) Post-injury daytime sleep in flies expressing a dominant-negative transgene for *basket* within ORNS (*orco*-GAL4/UAS-*bsk*^{DN}; injured in dark red, controls in light red) compared to genetic controls (*orco*-GAL4/+ and UAS-*bsk*^{DN}/+; injured in dark gray, controls in light gray). The amount of increased sleep after injury is reduced in *orco*-GAL4/UAS-*bsk*^{DN} relative to both genetic controls. Two-way ANOVA shows a significant genotype x injury interaction ($F_{(2,170)}$=27.5, p<0.0001); * signifies p<0.05 using pairwise Tukey comparisons.

(J) Increased sleep after injury is dampened in *orco*-GAL4/UAS-*bsk*^{DN} flies (red) compared to *orco*-GAL4/+ and UAS-*bsk*^{DN}/+ controls. Data for this panel were calculated using the same flies that were included in Figure 5I. One-way ANOVA finds a significant main effect of genotype ($F_{(2,87)}$=45.12, p<0.0001, n=29–31 flies/group).

See also Figure S5.

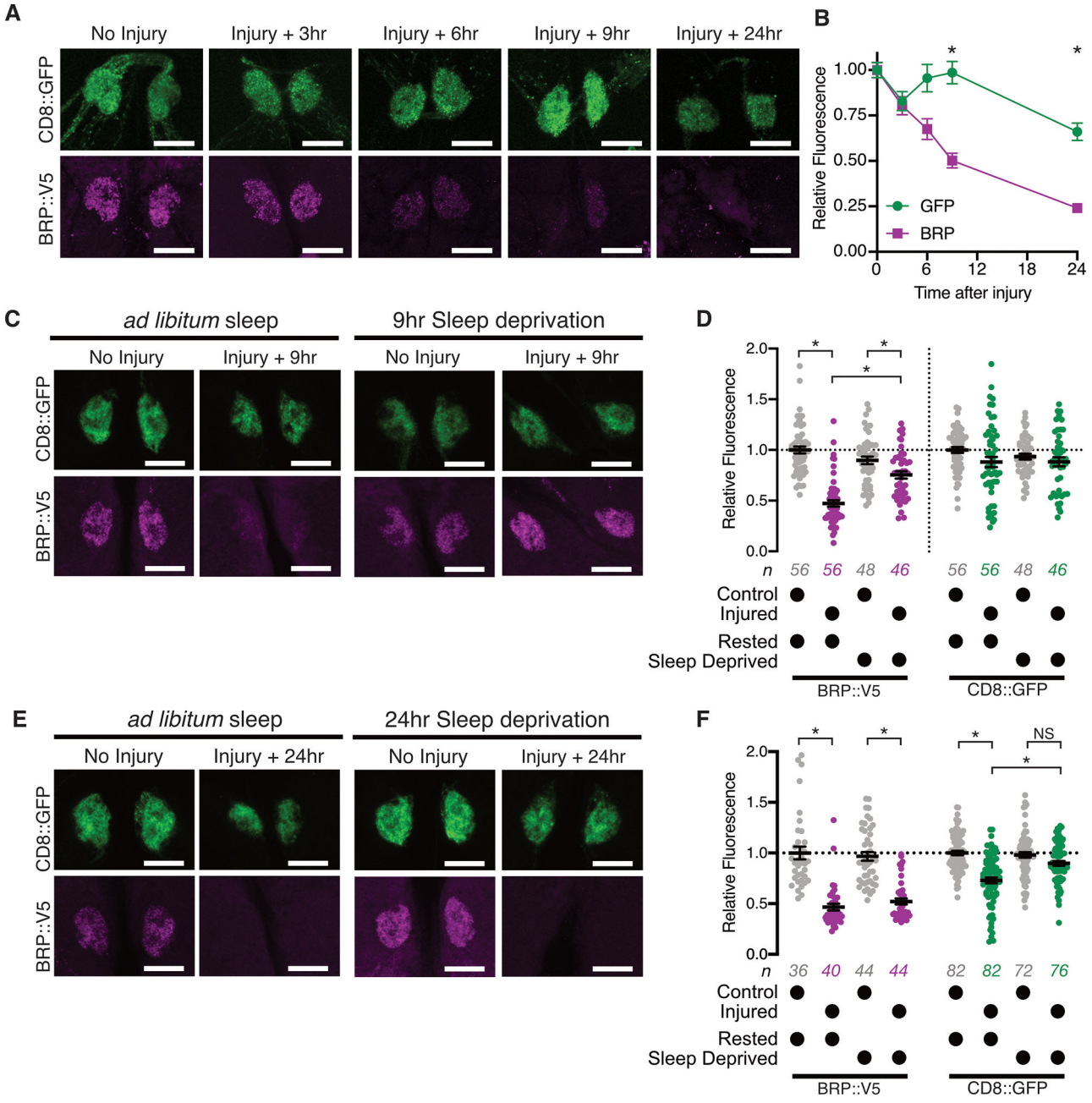

Figure 6 –. Sleep deprivation reduces axon and synapse clearance following antennal transection

(A) Representative images from *OR22a*-GAL4>STaR flies (*OR22a*-GAL4/LexAOP-CD8::GFP; UAS-*flp*, *brp*(FRT.Stop)V5–2A-LexA-VP16/+) either without antennal injury or 3, 6, 9, or 24 hours post-antennal transection. OR22a cells labelled with membrane-targeted CD8::GFP in green, and pre-synapses from the same cells labelled by STaR (BRP::V5) in magenta. Scale bars represent 20μm.

(B) Quantification of BRP::V5 (Magenta) and CD8::GFP (Green) in OR22a-expressing ORNs after injury. BRP::V5 labelling is significantly more reduced over the 24 hours following injury than membrane-bound CD8::GFP. Two-way ANOVA finds a significant time x reporter interaction ($F_{(4,542)}$=11.79, p<0.0001, n=26–70 hemispheres per group).

(C) Representative images from injured flies that were either allowed sleep (left) or sleep deprived (right) for 9 hours after antennal transection. StaR-labelled ORN synapses were largely removed when flies were allowed to rest after injury, but StaR signal remained high when animals were sleep deprived after injury. Scale bars represent 20μm.
(D) Relative BRP::V5 intensity (left; uninjured controls shown in gray, injured in magenta), magenta shows injured hemispheres) or CD8::GFP (right; uninjured controls shown in gray, injured in green) in OR22a-positive antennal lobe glomeruli in flies that were allowed 9 h of *ad libitum* recovery sleep after antennal transection or control handling. Two-way ANOVA finds a significant sleep condition-by-injury interaction for BRP::V5 ($F_{(1,202)} = 31.54$, $p < 0.0001$; n = 48–56 hemispheres/group) and a significant effect of injury for CD8::GFP ($F_{(1,202)} = 4.863$, $p = 0.0316$; n = 48–56 hemispheres/group).
(E) Representative images from flies that were dissected 24 hours after antennal injury or control handling. Images from flies that were allowed *ad libitum* sleep shown on left and from sleep deprived flies on right. STaR signal, magenta, is reduced in both rested and sleep deprived flies at 24 hours after injury, but CD8::GFP shows a stronger reduction after injury in flies that were allowed to sleep.
(F) Relative fluorescence signal for BRP::V5 (left; uninjured controls in gray, injured flies in magenta) and CD8::GFP (right; uninjured controls in gray, injured flies in green) under the control of *OR22a*-GAL4 at 24 h after injury or control handling. Two-way ANOVA finds a significant effect of injury for BRP::V5 ($F_{(1,160)} = 136.0$, $p < 0.0001$; n = 36–44 hemispheres/group) and an injury-by-sleep interaction for CD8::GFP ($F_{(1,308)} = 14.24$, $p = 0.0002$; n = 72–82 hemispheres/group).
See also Figure S6.