A bidirectional relationship between sleep and oxidative stress in *Drosophila*

Vanessa M. Hill¹, Reed M. O’Connor¹, Gunter B. Sissoko², Ifeoma S. Irobunda², Stephen Leong², Julie C. Canman³, Nicholas Stavropoulos⁴, Mimi Shirasu-Hiza¹*

¹ Department of Genetics and Development, Columbia University Medical Center, New York, New York, United States of America, ² Columbia University, New York, New York, United States of America, ³ Department of Pathology and Cell Biology, Columbia University Medical Center, New York, New York, United States of America, ⁴ Department of Neuroscience and Physiology, New York University School of Medicine, New York, New York, United States of America

* ms4095@cumc.columbia.edu

Abstract

Although sleep appears to be broadly conserved in animals, the physiological functions of sleep remain unclear. In this study, we sought to identify a physiological defect common to a diverse group of short-sleeping *Drosophila* mutants, which might provide insight into the function and regulation of sleep. We found that these short-sleeping mutants share a common phenotype of sensitivity to acute oxidative stress, exhibiting shorter survival times than controls. We further showed that increasing sleep in wild-type flies using genetic or pharmacological approaches increases survival after oxidative challenge. Moreover, reducing oxidative stress in the neurons of wild-type flies by overexpression of antioxidant genes reduces the amount of sleep. Together, these results support the hypothesis that a key function of sleep is to defend against oxidative stress and also point to a reciprocal role for reactive oxygen species (ROS) in neurons in the regulation of sleep.

Author summary

Most animals sleep; humans sleep nearly a third of their lives. Yet the fundamental functions of sleep remain unknown. Here, we used short-sleeping *Drosophila* mutants to uncover a role for sleep in resistance to oxidative stress. Oxidative stress is an imbalance of reactive oxygen species and antioxidant responses. Although these short-sleeping mutants have defects in diverse pathways, they all exhibit sensitivity to oxidative stress. Moreover, increasing sleep in wild-type flies increased resistance to oxidative stress. This suggests that one function of sleep is to defend against oxidative stress. Finally, reducing oxidative stress in neurons of wild-type flies reduces their sleep, suggesting that oxidative stress also regulates sleep. Taken together, our results support an intriguing hypothesis for a bidirectional relationship between sleep and oxidative stress: oxidative stress triggers sleep, which then acts as an antioxidant for both the body and the brain. These results have implications for human patients suffering from chronic sleep restriction and diseases associated with oxidative stress.
Introduction

A sleeping animal is vulnerable to predators and other dangers in its environment for a large portion of the day. Despite these daily risks, sleep is an evolutionarily conserved behavior throughout the animal kingdom [1–3], suggesting that sleep serves important functions. In support of this, prolonged episodes of acute sleep deprivation in both rodents and invertebrates cause an increased need to sleep [4–7], cognitive impairment [8,9], increased metabolic rate [6,10], and death [6,10,11]. It remains unclear whether these effects are due to loss of sleep or due to the intense stress associated with acute sleep deprivation. Epidemiological studies have revealed that chronic sleep restriction, or shortened sleep duration, in humans is associated with metabolic disorders [12], cardiovascular disease [13], inflammation [14,15], psychiatric disorders [16], and even premature mortality [17,18]. Similar to experimental results involving acute sleep deprivation, it is unclear whether these defects are due to the loss of sleep itself, to associated disruptions in circadian rhythm, or from the very factors that cause sleep loss, such as shift work, aging, or psychological stress. Thus, while current research in both humans and model organisms has demonstrated an important role for sleep in learning and memory [19–22], it has been difficult to identify underlying functions for sleep essential to the organism’s survival or fitness.

Sleep is thought to be regulated by two distinct types of mechanisms: those that control the timing of sleep, such as the circadian system, and those that control the duration of sleep, also called sleep homeostasis mechanisms [23,24]. While the molecular mechanisms underlying circadian regulation have been well characterized, molecular mechanisms regulating sleep homeostasis are less well defined but are thought to be neuronally based [24–29] and context dependent—that is, sleep deprivation or other stress conditions may induce different homeostasis pathways than baseline sleep. Because acute sleep deprivation increases sleep need and results in extended sleep duration at the animal’s next opportunity to sleep, many models of sleep homeostasis propose a feedback mechanism in which the wake state increases sleep-promoting factors, such as adenosine or overall synaptic strength [24,29]. The sleep state then clears or abrogates these factors to allow the wake state.

A controversial hypothesis for the function of sleep is the free radical flux theory of sleep, proposed in a theoretical paper by Reimund in 1994. Reimund proposed that reactive oxygen species (ROS) accumulate in neurons during the wake state and that sleep allows for the clearance of ROS in the brain [30]. ROS are chemically reactive by-products of metabolism, which, when not properly neutralized, cause damaging covalent modifications that inhibit the function of proteins, lipids, and DNA and can lead to cell death. Thus, the free radical flux hypothesis proposed that the core function of sleep is to act as an antioxidant for the brain. Despite the appeal of this hypothesis, data to support it are conflicting. While some groups have reported decreased antioxidant capacity and oxidative damage in the brains of sleep-deprived rats and mice [31–34], other reports have contradicted these findings [35–37]. As a result, the Reimund hypothesis has fallen out of favor as a model for sleep function. Notably, all studies testing the Reimund hypothesis focused on the effects of acute sleep deprivation. In contrast to acute sleep deprivation, the relationship between chronic sleep restriction and oxidative stress has not been thoroughly investigated, despite the physiological relevance of chronic sleep restriction widespread in modern society [38].

In recent years, the fruit fly has become a powerful, genetically tractable model system for the study of sleep [39,40]. Forward genetic screens have identified a number of Drosophila mutants that are short sleeping and retain intact circadian rhythms. Loss-of-function mutations in ion channels and ion-channel regulators, including sleepless, which regulates the potassium channel Shaker and nicotinic acetylcholine receptors (nAChRs), have been shown to...
reduce sleep [20,26,41,42]. Other short sleep–causing mutations include the redeye allele of the nAChRα4 subunit [43], the fumin allele of the dopamine transporter (DAT) [44], and loss of function of the putative ubiquitin ligase adaptor encoded by insomniac (inc) [45,46]. It has been hypothesized that these mutations cause short sleep by increasing neuronal excitability [24]. These mutants allow researchers to investigate the effects of chronic short sleep independent of circadian defects. While the specific genes affected vary widely and it is not clear whether these mutants sleep less than controls because of reduced sleep need or an inability to sleep, the common phenotype of these diverse mutants is chronic short sleep. Thus, together these mutants provide a system for identifying a “core” or essential function of sleep; we hypothesized that if chronic short sleep has negative effects on health, these diverse short-sleeping Drosophila mutants might share a common physiological defect independent of the specific mechanism driving their short sleep.

In this study, we sought to identify a physiological defect common to short-sleeping flies that might provide insight into the function and regulation of sleep. We found that diverse short-sleeping mutants are sensitive to acute oxidative stress, exhibiting shorter survival times than controls, and that increasing total sleep duration of wild-type flies promotes survival after oxidative challenge. We further showed that neuronal overexpression of antioxidant genes in wild-type flies reduces sleep. Our data demonstrate that one function of sleep is to increase the organism’s resistance to oxidative stress and support the hypothesis that sleep abrogates neuronal oxidative stress; these results also point to a role for neuronal ROS in the homeostatic regulation of sleep.

**Results**

**Neuronal knockdown of inc does not compromise lifespan, metabolism, or immunity**

To identify specific physiological functions of sleep (Fig 1A), we first focused on neuron-specific RNA interference (RNAi) of the inc gene, which has been shown to cause short sleep [45,46]. inc encodes a putative adaptor protein for Cullin-3 (Cul3), an E3 ubiquitin ligase expressed in both the brain and the body. Cul3 is involved in a number of crucial biological processes, and inc null mutants have reduced lifespan [45]. In contrast, neuron-specific RNAi of inc was reported to cause short sleep without affecting lifespan [45], suggesting that reduction of Inc activity in nonneuronal tissues affects lifespan in a sleep-independent manner. For this reason, we used flies expressing neuron-specific inc-RNAi as our initial model of short sleep.

We verified that animals expressing an upstream activation sequence (UAS)-inc-RNAi construct via the pan-neuronal driver elav-GAL4, hereafter referred to as neuronal inc-RNAi flies, exhibited a 30% reduction in total sleep time relative to isogenic controls carrying one copy of either the inc-RNAi construct or elav driver alone (Fig 1B, p < 0.0001 relative to either control; S1A Fig). We further confirmed that neuronal inc-RNAi flies exhibit normal lifespan compared to controls (Fig 1C, p > 0.5 compared to either control), consistent with a previous report [45] and with recent findings on inbred short-sleeping Drosophila lines that have normal lifespan [47]. This result confirms earlier findings that chronic short sleep does not itself shorten lifespan.

Changes in sleep are often associated with altered metabolic energy storage. In humans and mice, sleep loss is associated with metabolic dysfunction such as obesity [48,49], and in flies, starvation suppresses sleep behavior [50] and prolonged sleep is associated with increased starvation resistance [51]. We tested whether neuronal inc-RNAi flies have altered starvation resistance, which reflects altered metabolic energy stores. We found that the mortality rate of inc-
RNAi flies after starvation was intermediate between normally sleeping control flies containing either the elav driver or the UAS-inc-RNAi construct alone (Fig 1D, \( p = 0.0592 \) compared to elav control, \( p = 0.0493 \) compared to inc-RNAi control), suggesting that short sleep does not affect metabolic energy storage in neuronal inc-RNAi animals.

Acute sleep deprivation has also been associated with immune dysfunction in humans, rats, and mice [52–55]. Work in flies has shown that acute sleep deprivation can also augment the immune response [56]. To assay for defects or enhancement in immunity because of chronic short sleep, we injected neuronal inc-RNAi flies with different bacterial pathogens, including Streptococcus pneumoniae, a gram-positive pathogen that has been well characterized in Drosophila (Fig 1E), Providencia rettgeri, a gram-negative natural pathogen found in wild-caught Drosophila (Fig 1F), Listeria monocytogenes, and Staphylococcus aureus (S1B and S1C Fig). In each case, neuronal inc-RNAi flies died at the same rate as one or both of their genetic controls. To further test whether chronically reduced sleep causes deficits in immune function, we examined the response of short-sleeping fumin mutants that lack a functional DAT [44]. We confirmed earlier findings that fumin mutants exhibit short sleep (an approximately 95% reduction in sleep relative to controls) (S1D Fig). We found that fumin mutants responded
variably to these pathogens (S1E–S1H Fig). The lack of a consistent immunity defect across different pathogens in both neuronal inc-RNAi flies and fumin mutants suggests that chronic short sleep does not have a dramatic or common impact on immune function in Drosophila.

**Short sleep via reduction of inc causes sensitivity to oxidative stress**

We next set out to test whether sleep is required to defend against oxidative stress (Fig 2A) [30]. We compared the survival of neuronal inc-RNAi flies relative to controls when subjected to two different treatments that induce oxidative stress by increasing ROS levels (Fig 2B). We first injected neuronal inc-RNAi flies with a lethal dose of paraquat, an herbicide that catalyzes the production of superoxide anions [57]. We found that neuronal inc-RNAi flies died at a significantly faster rate after paraquat injection than controls (Fig 2B, left panel, *p* < 0.0001 relative to either control). To determine whether neuronal inc-RNAi flies have a specific sensitivity to superoxide anions or if they are also sensitive to other forms of oxidative stress, neuronal inc-RNAi flies and controls were fed hydrogen peroxide (H$_2$O$_2$), an oxidant that produces highly reactive hydroxyl radicals and has been shown to alter locomotor activity when fed to flies [58]. Similar to paraquat injection, neuronal inc-RNAi flies were sensitive to H$_2$O$_2$ feeding compared to controls (Fig 2B, right panel, *p* < 0.0001 relative to either control). These results indicate that short-sleeping neuronal inc-RNAi flies are susceptible to oxidative stress.

To verify that oxidative stress sensitivity is caused by the reduction in inc expression, rather than an off-target effect of RNAi, we next tested inc null mutants for paraquat sensitivity. We confirmed that inc null mutants exhibit a 50% reduction in sleep (S2A Fig, *p* < 0.0001 for both inc$^1$ and inc$^2$ mutants, relative to controls), as previously reported [45]. Consistent with neuronal inc-RNAi flies, inc null mutants died faster than controls when injected with paraquat (Fig 2C, *p* < 0.0001 for both inc$^1$ and inc$^2$ mutants, relative to controls). Furthermore, because Inc is a putative adaptor for the Cul3 ubiquitin ligase, we predicted that reduction of neuronal Cul3 activity would also cause paraquat sensitivity. As previously reported [45], neuronal Cul3-RNAi flies exhibit a 60% reduction in sleep (S2B Fig, *p* < 0.0001 relative to either control); here we found that neuronal Cul3-RNAi flies were also sensitive to paraquat injection (Fig 2D, *p* < 0.0001 relative to either control). Thus, chronic short-sleeping inc null mutants and Cul3-RNAi flies are sensitive to oxidative stress induced by elevated ROS levels, similar to neuronal inc-RNAi flies.

*Sensitivity to oxidative stress is common to a diverse group of short-sleeping mutants*

To determine whether sensitivity to oxidative stress is caused specifically by the reduction in inc or Cul3 activity or whether it is more broadly associated with loss of sleep, we next tested for sensitivity to oxidative stress in three different short-sleeping mutants, each carrying mutations in different genes with varied functions: sleepless$^{Δ40}$ (sleepless), DATfumin (fumin), and nACHRα4$^{rye}$ (redeye) (Fig 3A). We first confirmed, as previously reported [42–44], that each mutant spends significantly less time sleeping than its isogenic control (Fig 3B–3D, left panels, *p* < 0.0001 for each; S1C, S3A and S3B Fig). We next tested these short-sleeping mutants for sensitivity to oxidative stress. Relative to controls, we found that each mutant was sensitive to both paraquat injection (Fig 3B–3D, middle panels, *p* < 0.0001 for each) and H$_2$O$_2$ feeding (Fig 3B–3D, right panels, *p* < 0.0001 for each). Thus, our finding that this molecularly diverse set of short-sleeping mutants has a common susceptibility to oxidative challenge raises the possibility that sleep itself is required for proper response to oxidative stress.
Reducing *inc* or *Cul3* expression results in sensitivity to oxidative stress. We investigated whether reduction of *inc* or *Cul3*, either of which causes short sleep, affects the oxidative stress response (A). Neuronal *inc-RNAi* flies died faster than controls after paraquat injection (B, left panel, $p < 0.0001$ compared to either control, $n = 60–80$ flies/genotype) and $H_2O_2$ feeding (B, right panel, $p < 0.0001$ compared to either control, $n = 27–30$ flies/genotype). Similar sensitivity to paraquat was observed in *inc* and *inc* null mutants (C, $p < 0.0001$ for both mutants compared to control, $n = 49–63$ flies/genotype) and neuronal *Cul3-RNAi* flies (D, $p < 0.0001$ compared to either control, $n = 59–60$ flies/genotype). $p$-values were obtained by log-rank analysis. Data from representative experiments are shown. Each experiment was performed at least three times. Raw data from representative experiments are available in S1 Data; raw data from all trials are available upon request. *Cul3*, Cullin-3; *dcr*, UAS-Dicer; *inc*, insomnia; RNAi, RNA interference. 

https://doi.org/10.1371/journal.pbio.2005206.g002
Fig 3. A diverse group of short-sleeping mutants is sensitive to oxidative stress. We asked (A) whether other sleep mutants unrelated to inc or Cul3 share the same sensitivity to oxidative stress. (B–D, left panels) We found that sleepless mutants slept 65% less than controls (B, \( p < 0.0001 \), \( n = 6–10 \) flies/genotype), fumin mutants slept 95% less than controls (C, \( p < 0.0001 \), \( n = 15–16 \) flies/genotype), and redeye mutants slept 50% less than controls (D, \( p < 0.0001 \), \( n = 8–10 \) flies/genotype).
Increasing sleep confers resistance to oxidative stress

Because short-sleeping mutants exhibit sensitivity to oxidative stress, we next tested whether extending sleep duration promotes resistance to oxidative stress. We increased sleep by either genetic manipulation or pharmacological treatment and measured the effect on survival after oxidative challenge. For the genetic approach, we used transgenic flies in which sleep-inducing neurons were activated by the expression of a neuron-activating bacterial sodium channel [21]. For the pharmacological approach, we treated wild-type animals with the sleep-inducing drug Gaboxadol [19,59].

It was previously shown that total sleep time is increased by constitutively activating neurons in the dorsal Fan-shaped Body (dFB), a sleep-promoting region in the fly brain [21]. We verified this phenotype using a previously established dFB driver (23E10-GAL4) [60] to drive expression of the neuron-activating bacterial sodium channel construct UAS-NaChBac [61] and observed a 40% increase in sleep duration in dFB>NaChBac flies (Fig 4A, left panel, p < 0.0001 relative to either control; S3C Fig). We then subjected dFB>NaChBac flies to oxidative stress by either paraquat injection or H₂O₂ feeding. In both cases, dFB-activated flies died at a slower rate than controls (Fig 4A, middle and right panels, p < 0.001 for each). Thus, genetically activating the dFB to increase sleep promotes resistance to oxidative stress.

To further test whether extended sleep duration can increase survival of acute oxidative stress, we used an independent pharmacological method of sleep induction. Wild-type animals were fed the GABA_A receptor agonist Gaboxadol, which induces sleep in Drosophila [19,59]. We observed a 25% increase in total sleep time in Gaboxadol-treated animals (Fig 4B, left panel, p < 0.001; S3D Fig) and a corresponding increase in resistance to paraquat injection relative to vehicle-fed controls (Fig 4B, right panel, p < 0.0001). Together, these results demonstrate that two different methods of increasing sleep both promote resistance to oxidative stress, consistent with the idea that oxidative stress resistance is a physiological function of sleep (Fig 4C).

Neuronal knockdown of inc causes altered expression of stress response genes

If sleep clears ROS from neurons, one would expect short-sleeping flies to exhibit higher baseline levels of ROS in the brain. Quantitation of ROS in live brains is extremely difficult, possibly due to tight feedback control of ROS levels via the induction of antioxidant gene expression. As an indirect measure of ROS, we measured the expression of genes known to be activated by high levels of ROS by performing quantitative reverse transcription polymerase chain reaction (qRT-PCR) on the heads of neuronal inc-RNAi flies and controls (Fig 5A). These genes include the antioxidant genes superoxide dismutase 1 (SOD1), catalase, the glutathione-S-transferases GSTS1 and GSTO1, and; the mitochondrial stress response genes hsp60,
**Sleep and oxidative stress in Drosophila**

ClpX, and Pink1; and the endoplasmic reticulum stress response gene BiP, which was previously shown to be induced by sleep deprivation [40,62–64]. We found that neuronal *inc*-RNAi flies exhibited increased expression of all of these genes except catalase and BiP (Fig 5B–5I). While neuronal *inc*-RNAi flies had modestly elevated BiP expression in the head (Fig 5I), the difference was not significant. Thus, the increased baseline expression of antioxidant genes and mitochondrial stress genes in neuronal *inc*-RNAi flies is consistent with short sleep causing increased ROS levels in the brain.

---

**Fig 4. Inducing sleep increases resistance to oxidative stress.** (A) *dFB>*NaChBac flies slept 40% more than controls (left panel, \( p < 0.0001 \) compared to either control, \( n = 20 \) flies/genotype) and died slower than controls after either paraquat injection (middle panel, \( p < 0.0001 \) compared to either control, \( n = 79–80 \) flies/genotype) or \( \text{H}_2\text{O}_2 \) feeding (right panel, \( p < 0.001 \) compared to either control, \( n = 31–32 \) flies/genotype). (B) Flies fed the GABA\(_\alpha\) agonist Gaboxadol slept 25% more than controls (left panel, \( p < 0.001, n = 8 \) flies/condition) and died slower than controls after paraquat injection (right panel, \( p < 0.0001, n = 118–119 \) flies/condition). These data support the conclusion (C) that inducing sleep by either genetic or pharmacological means confers oxidative stress resistance. For scatterplots (A–B, left panels), each data point represents average sleep in minutes/day measured across 4–5 days in an individual animal; data are shown as mean ± SEM, \( p \)-values were obtained by ordinary one-way ANOVA followed by a post hoc Tukey test when significance was detected (A–B, left panels) or by log-rank analysis (A–B, middle and right panels). Data from representative experiments are shown. Each experiment was performed at least three times. Raw data from representative experiments are available in S1 Data; raw data from all trials are available upon request. *dFB*, dorsal Fan-shaped Body; GABA\(_\alpha\), \( \gamma\)-aminobutyric acid-A.

https://doi.org/10.1371/journal.pbio.2005206.g004
Overexpression of antioxidant genes in neurons reduces sleep

If one function of sleep is to clear ROS from the brain, then it is plausible that ROS itself may be a factor that triggers sleep, perhaps when it reaches a certain critical threshold. To

Fig 5. Neuronal inc-RNAi heads have increased expression of stress response genes. We investigated whether short sleep affects the expression of three main groups of stress response genes: antioxidant genes, mitochondrial stress genes, and one ER stress gene (A). Neuronal inc-RNAi flies exhibited increased baseline head expression of antioxidant genes SOD1 (B, \(p < 0.001\) compared either control, \(n = 6\) biological replicates per genotype), GSTS1 (C, \(p < 0.05\) compared to either control, \(n = 6\) biological replicates per genotype), and GSTO1 (D, \(p < 0.05\) compared to either control, \(n = 6\) biological replicates per genotype), but normal expression of catalase (E, \(p > 0.05\) compared to either control, \(n = 6\) biological replicates per genotype). Neuronal inc-RNAi flies also exhibited increased basal head expression of mitochondrial stress genes hsp60 (F, \(p < 0.05\) compared to either control, \(n = 6\) biological replicates per genotype), Pink1 (G, \(p < 0.001\) compared to either control, \(n = 6\) biological replicates per genotype), and ClpX (H, \(p < 0.05\) compared to either control, \(n = 5–6\) biological replicates per genotype). The ER chaperone gene BiP was elevated compared to one, but not both, controls (G, \(p < 0.05\) compared to elav driver control, \(n > 0.05\) compared to inc-RNAi control, \(n = 6\) biological replicates per genotype). Expression was normalized to actin. Data are shown as mean ± SEM. Each data point represents an independent biological replicate with 15–20 individual fly heads per biological replicate. \(p\)-values were obtained by ordinary one-way ANOVA followed by a post hoc Tukey test when significance was detected. Raw data from representative experiments are available in S1 Data; raw data from all trials are available upon request. ER, endoplasmic reticulum; GST, glutathione-S-transferase; hsp60, heatshock protein 60; Pink1, PTEN-induced putative kinase 1; inc, insomniac; RNAi, RNA interference; SOD1, superoxide dismutase 1.

![Fig 5. Neuronal inc-RNAi heads have increased expression of stress response genes.](https://doi.org/10.1371/journal.pbio.2005206.g005)
determine whether neuronal ROS levels play a role in the regulation of sleep, we reduced ROS levels in the brains of otherwise wild-type flies by driving neuronal overexpression of the antioxidant genes catalase, SOD1, or SOD2 using the elav-Gal4 driver (Fig 6A). SOD1 or SOD2 overexpression resulted in a significant reduction in the total amount of sleep, with an average decrease in total sleep of 10% and 16%, respectively (Fig 6B, p < 0.05 compared to either control; S3F and S3G Fig). catalase overexpression resulted in a similar trend but did not reach significance compared to the driver control (Fig 6B, S3E Fig). Our observation that reducing neuronal ROS levels reduces sleep amount suggests that ROS levels reflect sleep need and play a role in the regulation of sleep (Fig 6C).

**Discussion**

Although sleep appears to be evolutionarily conserved across all animal species [1–3], the physiological function of sleep remains unclear. Our data show that chronic sleep restriction sensitizes flies to two types of oxidative stress: paraquat injection and H$_2$O$_2$ feeding (Figs 2 and 3). Conversely, increasing sleep through either genetic or pharmacological methods promotes
resistance to oxidative stress (Fig 4). Thus, our data suggest that one important function of sleep is defense against oxidative stress.

The molecular mechanisms underlying the susceptibility of short-sleeping mutants to acute oxidative stress and whether this susceptibility is due to the effects of oxidative stress on the brain or other, nonneuronal tissues of the body remains unclear. It is possible that increased baseline ROS levels in neurons or other tissues sensitize short sleepers to acute oxidative stress. Other investigators have found that accumulation of cellular ROS was associated with susceptibility to acute oxidative challenge [65,66]. Chronic sleep loss may lead to accumulated mitochondrial damage that, in the presence of an acute oxidative stress, triggers cell death pathways. Another possibility is that short sleepers are less able to detect or respond to acute oxidative challenge in specific tissues. Testing these hypotheses will be an important focus for future investigation.

Our data also suggest that short-sleeping animals accumulate higher baseline ROS levels in the brain. While ROS levels in the brain are difficult to measure directly, we observed increased expression of antioxidant and mitochondrial stress response genes in the heads of short-sleeping neuronal inc-RNAi flies, consistent with increased ROS levels in the brain. Other studies have similarly observed that sleep-deprived animals display increased expression of genes induced by high ROS levels. Induction of the antioxidant regulator cap’n’collar (cnc) was observed in fly heads when flies were exposed to recurrent sleep fragmentation [67], and its mammalian homolog Nuclear factor (erythroid-derived 2)-like 2 (nrf2) was reported to be induced in the cerebral cortex of mice after 6 hours of sleep deprivation [68]. Sleep deprivation has also been associated with activation of the unfolded protein response in the ER in fly heads and mouse brains [40,62–64]. Because both the ER- and mitochondrial unfolded protein responses can be induced by high levels of ROS, we hypothesize that both genetic and environmental sleep loss increase baseline ROS levels that, depending on the specific method of sleep deprivation, genetic background, and tissue tested, are reflected in the activation of different response pathways.

Finally, we found that increasing antioxidant gene expression in the brain causes short sleep, suggesting that decreasing neuronal ROS levels will promote the wake state. Emerging evidence demonstrates that ROS can act as crucial signaling molecules in a number of biological processes [69,70], and it has been demonstrated that injecting an oxidant into the rat brain induces sleep [71]. One study showed modest effects of lifelong, low-dose paraquat feeding on sleep in flies [72]. Thus, ROS levels, either directly or indirectly through the activation of oxidative stress responses, appear to induce sleep.

Taken together, our results support a model for a bidirectional relationship between sleep and oxidative stress, in which one function of sleep is to act as an antioxidant for both the body and the brain, increasing the organism’s resistance to acute oxidative challenge and reducing ROS levels in the brain; moreover, neuronal ROS play a role in the regulation of sleep and wake states (Fig 6C). Thus, with chronic sleep restriction, the animal accumulates higher ROS levels in the brain and is sensitive to acute oxidative stress.

Identifying the physiological functions and key regulators of sleep is critical to understanding the negative effects on health associated with chronic sleep restriction. In the United States, average sleep time is steadily decreasing [73], and one third of adults sleep less than the recommended 7 hours per night [38]. Sleep restriction is correlated with a variety of diseases [12,13], many of which are also associated with oxidative stress [74–78]. Sleep disturbances have been implicated as a predictor for Alzheimer, Parkinson, and Huntington’s diseases [79–82], and in all of these diseases, oxidative damage has been reported in the brains of patients postmortem [83–85]. Because oxidative stress can induce protein misfolding and aggregation through protein damage, neuronal accumulation of ROS is a plausible contributing factor in the pathogenesis of neurodegenerative diseases. Thus, understanding the role of sleep in defense against
oxidative stress and the role of ROS in regulating sleep could provide much-needed insight into the pathology and treatment of neurodegenerative diseases.

**Materials and methods**

Results from all experiments are summarized in S1 Table in the Supporting information, and raw data are available upon request.

**Fly strains and rearing conditions**

The following flies were used to manipulate inc and Cul3 as described previously [45]: UAS-inc-RNAi (VDRC stock #18225), elav<sup>C155</sup>-Gal4, UAS-Dicer (dcr) (Bloomington stock #24651), inc<sup>1</sup> deletion mutant, and inc<sup>2</sup> transposon insertion mutant (CG32810<sup>f00285</sup>), all in the same genetic background (w<sup>1118</sup> iso31 or Bloomington stock #5905), along with the isogenic iso31 strain used for outcrossing. UAS-Cul3-RNAi (NIG stock #11861R-2) was in the NIG w<sup>1118</sup> background and compared to its isogenic control. For neuronal Cul3 knockdown experiments, the UAS-Dicer line (Bloomington stock #24651) was crossed into the elav<sup>C155</sup>-Gal4 line. Parental controls used for experiments were obtained by crossing expression driver (e.g., elav-Gal4) and RNAi construct (e.g., UAS-inc-RNAi) lines to the outcrossed wild-type line (e.g., iso31) for heterozygous controls, accounting for differences in complex phenotypes affected by genetic background. In case the absence of the white gene, which encodes an ABC transporter, has an effect on survival after paraquat or H<sub>2</sub>O<sub>2</sub> exposure, red-eyed controls were used with the red- and orange-eyed inc<sup>1</sup> and inc<sup>2</sup> mutants; these w<sup>+</sup> controls were generated by outcrossing w<sup>+</sup> from an Oregon-R background for eight generations with the iso31 stock (Bloomington stock #5905).

redeye, sleepless<sup>A40</sup> (imprecise excision mutants), and their corresponding background-matched controls were obtained from Amita Sehgal (University of Pennsylvania). sleepless<sup>A40</sup> was used instead of sleepless<sup>P1</sup> because sleepless<sup>P1</sup> flies were sensitive to CO<sub>2</sub>, which made paraquat injection experiments difficult to interpret. Male sleepless<sup>A40</sup> flies also exhibited some wounding sensitivity, whereas females did not, so female sleepless<sup>A40</sup> flies were used in the paraquat injection experiments (S4 Fig). Male sleepless<sup>A40</sup> were used in H<sub>2</sub>O<sub>2</sub> feeding experiments. fumin mutants and their background-matched controls were obtained from Rob Jackson (Tufts University).

UAS-NaChBac [61] was obtained from Paul Shaw (Washington University, St. Louis, MO) and 23E10-Gal4 [60] was obtained from Jeffrey Donlea (University of Oxford); both were outcrossed for eight generations with the iso31 stock. As described above, parental controls used for experiments were obtained by crossing expression driver (23E10-Gal4) and transgene construct (UAS-NaChBac) lines to the outcrossed wild-type line (iso31) for heterozygous controls.

The following stocks were obtained from the Bloomington Stock Center (BDSC, Bloomington, IN) and outcrossed 6–8 generations into the iso31 background: UAS-SOD1 (#24754), UAS-SOD2 (#24492), and UAS-cat (#24621).

All flies were raised at room temperature on standard molasses food (5.85% cornmeal, 2.675% yeast, 0.575% agar, 3% v/v blackstrap molasses, 0.14% methylparaben, 0.5% v/v propionic acid) and kept on cornmeal food (4% cornmeal, 2.15% yeast, 9% dextrose, 0.75% agar, 0.095% methylparaben) post-eclosion in a temperature- (25˚C) and humidity- (55%) controlled incubator with a 12-hour light–dark cycle. Four- to ten-day-old males were used for all experiments, unless otherwise noted.

**Sleep analysis and starvation assay**

Individual flies were loaded into plastic tubes containing cornmeal food and allowed to acclimate for 1 day. Sleep was monitored for 4–5 days using Drosophila Activity Monitors (either
DAM2s or DAM5s, an older model of DAM5M with a single beam per tube) (Trikinetics, Waltham, MA). Activity was recorded as beam-breaks in 1-minute bins and analyzed using PySolo software [86] or Microsoft Excel, with sleep defined as a 5-minute period of inactivity. Graphing and statistical analysis were performed using GraphPad Prism (survival assays and scatterplots) and PySolo (24-hour sleep profiles). When comparing two groups: an unpaired t test was performed when standard deviations were similar, and an unpaired t test with Welch’s correction was performed when standard deviations were not similar (F test \( p < 0.5 \)). When comparing three groups, a one-way ANOVA was performed and followed by a post hoc Tukey test to compare means when significance was detected.

For starvation assays, flies were transferred to tubes containing 1% agar and loaded into *Drosophila* Activity Monitors. Time of death was determined by complete loss of movement.

**Lifespan**

Flies were collected on the day of eclosion and allowed to mate overnight. Total flies per genotype ranged from 74 to 225. Numbers were roughly equivalent for each group within different trials. Males were separated into groups of 20 per vial. Flies were transferred to new vials every 2–7 days and scored for death at time of transfer. Lifespan experiments were performed in at least two independent trials.

**Bacterial and paraquat injections**

Injections were carried out with a pulled glass capillary needle. A custom-made microinjector (Tritech Research, Los Angeles, CA) was used to inject 50 nL of liquid into the abdomen of each fly. Volume was calibrated by measuring the diameter of the expelled drop under oil. Death was assayed visually at least daily, with a typical \( n = 60 \) for both bacterial infections and paraquat injections. For each experiment, a smaller set of flies was injected with vehicle alone to ensure that wounding caused minimal death.

The following bacterial strains were used for injections: *S. pneumoniae* (strain SP1, a streptomycin-resistant variant of D39) obtained from Elizabeth Joyce (University of California, San Francisco, CA) was grown standing in Brain Heart Infusion media (BHI, Teknova, Hollister, CA) at 37°C with 5% CO\(_2\), frozen into aliquots with 10% glycerol, pelleted and resuspended upon thawing, and injected at an OD\(_{600}\) of 0.015–0.05; *P. rettgeri* (strain Dmel, a natural pathogen isolated from wild-caught *D. melanogaster* [87]) obtained from Brian Lazzaro (Cornell University) was grown shaking in LB at 37°C and injected at an OD\(_{600}\) of 0.003–0.005; *L. monocytogenes* (strain 10403S) obtained from Julie Theriot (Stanford University) was grown standing in BHI at 37°C and injected at an OD\(_{600}\) of 0.075–0.2; and *S. aureus* strain 12600 (ATCC) was grown shaking in BHI at 37°C and injected at an OD\(_{600}\) of 0.0001–0.001. Postinjection, flies were kept in a 29°C incubator for the remainder of the experiment to allow for optimal infection, with the exception of *P. rettgeri* injection, in which case optimal infection was achieved at 25°C. All OD\(_{600}\) measurements were made using a Genesys 10S Vis Spectrophotometer (ThermoScientific, Waltham, MA), blanked against the corresponding sterile media for the given culture. Cultures were then diluted in sterile media to the desired OD.

For paraquat injections, paraquat (methyl viologen hydrate, Fisher Scientific, Hampton, NH) was dissolved in water to a concentration of 3–5 mM. Paraquat solution was either stored at 4°C for up to 1 month or frozen in aliquots and thawed as needed. For every experimental genotype treated with paraquat injection, we conducted mock injections with ddH\(_2\)O to control for wounding sensitivity (S4 Fig).
H$_2$O$_2$ feeding assays

These assays were performed in two ways. In one method, flies were transferred to vials containing a folded Kimwipe soaked with 1.5 mL of a 5% sucrose, 1%–4% H$_2$O$_2$ solution. Thirty percent H$_2$O$_2$ (Sigma-Aldrich, St. Louis, MO) was diluted in ddH$_2$O to a concentration of 1%–4% depending on the death rate for the given genotype, titrated to complete death within several days. Flies were flipped onto a freshly soaked Kimwipe every 2 days and death was assayed visually and recorded daily. This method allows very rapid setup (typical experiment used 40 flies/genotype) but provides relatively low-resolution survival kinetics. In the second method, flies were transferred to 5 mm tubes containing a piece of a soaked Kimwipe and loaded into Drosophila Activity Monitors, in which case death was determined by a complete loss of movement. Control flies were kept on 5% sucrose alone to ensure that death did not occur by starvation or desiccation. This method provides high-resolution survival kinetics but requires more time-intensive setup (typical experiment used 30 flies/genotype). We found that all our results for short-sleeping mutants were consistent between the two methods.

Survival curves

Survival curves for starvation assays, lifespan experiments, bacterial infections, paraquat injections, and H$_2$O$_2$ feeding assays are all plotted as Kaplan-Meier graphs. Log-rank analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). All experiments were performed with a minimum of three independent trials and yielded statistically similar results, except where noted. Graphs and p-values in figures are from representative trials.

qRT-PCR

Age-matched, 6–8-day-old flies were anesthetized on ice and decapitated between ZT2 and ZT5. Fifteen to twenty heads per sample were homogenized in TRIzol (Invitrogen), and a phenol-chloroform extraction was performed to isolate nucleic acids. Samples were treated with DNase (Invitrogen, Carlsbad, CA) to isolate RNA and then diluted to a concentration of about 60 ng/μL. RevertAid First Strand cDNA synthesis kit (ThermoFisher, Waltham, MA) was used to convert RNA to cDNA. Quantitative RT-PCR was performed using a Bio-Rad CFX Connect Real-Time qPCR machine, with Express Sybr GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) and the following primer sets:

$SODI$:
For: GGAGTCGGTGATGTTGACCT
Rev: GGAGTCGGTGATGTTGACCT

$GSTS1$:
For: CACCAGACATTTTCGATGGCT
Rev: ACGACTGCAATTTTTAGACGGA

$GSTO1$:
For: ACGACTGCAATTTTTTAGACGGA
Rev: CCGATCGCCGGGAGTTCATGTA

catalase:
For: TTCTGGTTATCCCGGTGGAC
Rev: GGTAATGGCACCAGGAGAAA

$hsp60$:
For: TGATGCTGATCTCGTCAAGC
Rev: TACTCGGAGGGTGTGTCCTC

ClpX:
For: AAAATGCTCGAAGGCAAG

PLOS Biology | https://doi.org/10.1371/journal.pbio.2005206 July 12, 2018 15 / 22
Rev: TTGAGACGACGTGCAGATAG
Pink1:
For: TCGGTGTCAATGTAGTGC
Rev: CCACCTCGGAAGATTCCACTGC
BiP:
For: GCTATTGCCTACGGTCTGGA
Rev: CATCACACGCTGATCGAAGT
actin:
For: TTGTCTGGGCAAGAGGATCAG
Rev: ACCACTCGCATCTTGACATTC

Analysis was performed using the Standard Curve method. Total cDNA concentration was normalized to actin expression. Data are represented as mean ± SEM. Five to six biological replicates (containing 15–20 heads each) per experiment.

Gaboxadol and antioxidant feeding

Gaboxadol hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in water and added to melted cornmeal food to a final concentration of 0.1–0.2 mg/mL. Flies were flipped onto Gaboxadol-containing food for 3 days prior to paraquat injection and remained on Gaboxadol-containing food postinjection. Control food was made by adding the appropriate amount of vehicle alone (H\textsubscript{2}O) to melted cornmeal food.

Supporting information

S1 Fig. Neuronal inc-RNAi flies and fumin mutants are short sleeping and do not display a global immunity defect. (A) Twenty-four-hour sleep plot for neuronal inc-RNAi flies and controls. Neuronal inc-RNAi flies died at the same or a slightly slower rate than genetic controls after injection with Listeria monocytogenes (B, \(p = 0.09\) compared to elav control, \(p = 0.04\) compared to inc-RNAi control, \(n = 62–63\) flies/genotype) and died at the same rate as controls after injection with Staphylococcus aureus (C, \(p > 0.05\) compared to either control, \(n = 19–21\) flies/genotype). (D) Twenty-four-hour sleep plot for fumin mutants and controls. fumin mutants died slower than controls after injection with Streptococcus pneumoniae (E, \(p < 0.01\), \(n = 96–98\) flies/genotype), died faster than controls after injection with Providencia rettgeri (F, \(p < 0.0001\), \(n = 89–91\) flies/genotype), died slower than controls after injection with L. monocytogenes (G, \(p < 0.01\), \(n = 77–79\) flies/genotype), and died at the same rate as controls after injection with S. aureus (H, \(p > 0.05\), \(n = 94–100\) flies/genotype). \(p\)-values were obtained by log-rank analysis. Data from representative experiments are shown. Each experiment was performed at least three times. Raw data from representative experiments are available in S1 Data; raw data from all trials are available upon request. \(inc\), insomniac; RNAi, RNA interference.

(TIF)

S2 Fig. Reduction of inc or Cul3 causes short sleep. \(inc^1\) and \(inc^2\) null mutants slept about 50% less than controls (A, \(p < 0.0001\) for both mutants, \(n = 20–22\) flies/genotype). elav;; dcr> Cul3-RNAi flies slept about 60% less than controls (B, \(p < 0.0001\) compared to either control, \(n = 40–42\) flies/genotype). Each data point in scatterplots (left) represents average sleep in minutes/day measured across 4–5 days in an individual animal. Data are shown as mean ± SEM. \(p\)-values were obtained by ordinary one-way ANOVA followed by a post hoc Tukey test. Twenty-four-hour sleep plots (right) show sleep profiles for mutants and controls averaged over a 4–5-day period. Data from representative experiments are shown. Each experiment was performed at least three times. Raw data from representative experiments are available in S2 Data.
available in S1 Data; raw data from all trials are available upon request. Cul3, Cullin-3; dcr, UAS-Dicer; inc, insomniac; RNAi, RNA interference.

S3 Fig. Twenty-four-hour sleep plots for short-sleeping mutants. Shown here are the 24-hour sleep plots, averaged over 4–5 days, for the indicated short-sleeping flies, with their relevant controls. (A) sleepless mutants and controls; relates to Fig 3B. (B) redeye mutants and controls; relates to Fig 3D. (C) dFB>NaChBac flies and controls; relates to Fig 4A. (D) Gaboxadol-fed flies compared with vehicle only; relates to Fig 4B. (E–G) Neuronal overexpression of Catalase, SOD1, and SOD2, compared with controls; relates to Fig 6. Raw data from representative experiments are available in S1 Data; raw data from all trials are available upon request. dFB, dorsal Fan-shaped Body; SOD, superoxide dismutase.

S4 Fig. Paraquat injection controls. Shown here are representative H2O-injected wounding controls for each of the genotypes subjected to paraquat injection: (A) neuronal inc-RNAi (relates to Fig 2B); (B) inc null mutants (relates to Fig 2C); (C) neuronal Cul3-RNAi (relates to Fig 2D); (D) sleepless mutants (relates to Fig 3B); (E) fumin mutants (relates to Fig 3C); (F) redeye mutants (relates to Fig 3D); (G) dFB>NaChBac flies (relates to Fig 4A); and (H) iso31 controls (relates to Fig 4B). In all cases, flies injected with paraquat died significantly faster (p < 0.5 by log-rank analysis) than H2O-injected controls. Raw data from representative experiments are available in S1 Data; raw data from all trials are available upon request. Cul3, Cullin-3; dFB, dorsal Fan-shaped Body; inc, insomniac; RNAi, RNA interference.

S1 Table. Data summary. Summary of results from experimental trials. Raw data from representative experiments are available in S1 Data; raw data from all trials are available upon request.

Acknowledgments
We thank Shirasu-Hiza and Canman lab members for support, productive discussions, and feedback on this manuscript; Charlotte Wayne for technical support; and Amita Sehgal, Rob Jackson, Paul Shaw, and Jeff Donlea for fly lines. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study.

Author Contributions
Conceptualization: Vanessa M. Hill, Julie C. Canman, Mimi Shirasu-Hiza.
Formal analysis: Vanessa M. Hill, Gunter B. Sissoko, Ifeoma S. Irobunda, Stephen Leong, Mimi Shirasu-Hiza.
Funding acquisition: Mimi Shirasu-Hiza.
Investigation: Vanessa M. Hill, Reed M. O’Connor, Gunter B. Sissoko, Ifeoma S. Irobunda, Stephen Leong, Julie C. Canman, Mimi Shirasu-Hiza.
Methodology: Nicholas Stavropoulos.
Project administration: Mimi Shirasu-Hiza.

Supervision: Vanessa M. Hill, Julie C. Canman, Mimi Shirasu-Hiza.

Validation: Vanessa M. Hill, Reed M. O’Connor, Gunter B. Sissoko, Ifeoma S. Irobunda, Stephen Leong.

Visualization: Vanessa M. Hill.

Writing – original draft: Vanessa M. Hill, Julie C. Canman, Mimi Shirasu-Hiza.

Writing – review & editing: Vanessa M. Hill, Reed M. O’Connor, Julie C. Canman, Nicholas Stavropoulos, Mimi Shirasu-Hiza.

References

1. Campbell SS, Tobler I. Animal sleep: A review of sleep duration across phylogeny. Neurosci Biobehav Rev. 1984; 8: 269–300. https://doi.org/10.1016/0149-7634(84)90054-X PMID: 6504414

2. Cirelli C, Tononi G. Is sleep essential? PLoS Biol. 2008; 6: 1605–1611. https://doi.org/10.1371/journal.pbio.0060216 PMID: 18752355

3. Joiner WJ. Unraveling the Evolutionary Determinants of Sleep. Curr Biol. Elsevier Ltd; 2016; 26: R1073 —R1087. https://doi.org/10.1016/j.cub.2016.08.068 PMID: 27780049

4. Huber R, Hill SL, Holladay C, Biesiadcki M, Tononi G, Cirelli C. Sleep homeostasis in Drosophila melanogaster. Sleep. 2004; 27: 628–639. https://doi.org/10.1093/sleep/27.4.628 PMID: 15282997

5. Sauer S, Herrmann E, Kaiser W. Sleep deprivation in honey bees. J Sleep Res. 2004; 13: 145–152. https://doi.org/10.1111/j.1365-2869.2004.00393.x PMID: 15175094

6. Stephenson R, Chu KM, Lee J. Prolonged deprivation of sleep-like rest raises metabolic rate in the Pacific beetle cockroach, Diploptera punctata (Eschscholtz). J Exp Biol. 2007; 210: 2540–2547. https://doi.org/10.1242/jeb.005322 PMID: 17601958

7. Vyazovskiy V V, Tobler I. Theta activity in the waking EEG is a marker of sleep propensity in the rat. Brains Res. 2005; 1050: 64–71. https://doi.org/10.1016/j.brainres.2005.05.022 PMID: 15973563

8. Ganguly-Fitzgerald I, Donlea J, Shaw PJ. Waking experience affects sleep need in Drosophila. Science (80-). 2006; 313: 1775–1781. https://doi.org/10.1126/science.1130405 PMID: 16990546

9. Tartar JL, Ward CP, McKenna JT, Thakkar M, Arrigoni E, McCarley RW, et al. Hippocampal synaptic plasticity and spatial learning are impaired in a rat model of sleep fragmentation. Eur J Neurosci. 2006; 23: 2739–2748. https://doi.org/10.1111/j.1460-9568.2006.04868.x PMID: 16817877

10. Rechtschaffen A, Bergmann BM. Sleep deprivation in the rat: an update of the 1989 paper. Sleep. 2002; 25: 18–24. PMID: 11833856

11. Shaw PJ, Tononi G, Greenspan RJ, Robinson DF. Stress response genes protect against lethal effects of sleep deprivation in Drosophila. Nature. 2002; 417: 287–291. https://doi.org/10.1038/417287a PMID: 12015603

12. Nedeltcheva A V, Scheer FAJL. Metabolic effects of sleep disruption, links to obesity and diabetes. Curr Opin Endocrinol Diabetes Obes. 2014; 21: 293–298. https://doi.org/10.1097/MED.0000000000000082 PMID: 24937041

13. Cappuccio FP, Cooper D, Delia L, Strazzullo P, Miller MA. Sleep duration predicts cardiovascular outcomes: A systematic review and meta-analysis of prospective studies. European Heart Journal. 2011. pp. 1484–1492. https://doi.org/10.1093/eurheartj/ehr007 PMID: 21300732

14. Faraut B, Boudjelta KZ, Vanhamme L, Kerkhofs M. Immune, inflammatory and cardiovascular consequences of sleep restriction and recovery. Sleep Med Rev. Elsevier Ltd; 2012; 16: 137–149. https://doi.org/10.1016/j.smrv.2011.05.001 PMID: 21835655

15. Ferrie JE, Kivimaki M, Akbaraly TN, Singh-Manoux A, Miller MA, Gimoeno D, et al. Associations Between Change in Sleep Duration and Inflammation: Findings on C-reactive Protein and Interleukin 6 in the Whitehall II Study. Am J Epidemiol. 2013; 178: 956–961. https://doi.org/10.1093/aje/kw7072 PMID: 2381012

16. Baglioni C, Battagliese G, Faige B, Spiegelhalder K, Nissen C, Voderholzer U, et al. Insomnia as a predictor of depression: A meta-analytic evaluation of longitudinal epidemiological studies. Journal of Affective Disorders. 2011. pp. 10–19. https://doi.org/10.1016/j.jad.2011.01.011 PMID: 21300408

17. Hublin C, Partinen M, Koskenvuo M, Kaprio J. Sleep and mortality: A population-based 22-year follow-up study. Sleep. 2007; 30: 1245–1253. https://doi.org/10.1093/sleep/30.10.1245 PMID: 17969458
18. Gallicchio L, Kalesan B. Sleep duration and mortality: A systematic review and meta-analysis. J Sleep Res. 2009; 18: 148–158. https://doi.org/10.1111/j.1365-2869.2008.00732.x PMID: 19645960

19. Dissel S, Angadi V, Kirszenblat L, Suzuki Y, Donlea J, Klose M, et al. Sleep restores behavioral plasticity to drosophila mutants. Curr Biol. Elsevier Ltd; 2015; 25: 1270–1281. https://doi.org/10.1016/j.cub.2015.03.027 PMID: 25913403

20. Bushey D, Huber R, Tononi G, Cirelli C. Drosophila Hyperkinetic Mutants Have Reduced Sleep and Impaired Memory. J Neurosci. 2007; 27: 5384–5393. https://doi.org/10.1523/JNEUROSCI.0108-07.2007 PMID: 17507560

21. Donlea JM, Thimgan MS, Suzuki Y, Gottschalk L, Shaw PJ. Inducing Sleep by Remote Control Facilitates Memory Consolidation in Drosophil a. Science (80-). 2011; 332: 1571–1576. https://doi.org/10.1126/science.1202249 PMID: 21700877

22. Gais S. Sleep after learning aids memory recall. Learn Mem. 2006; 13: 259–262. https://doi.org/10.1101/lm.132106 PMID: 16741280

23. Borbely AA. A two process model of sleep regulation. Human Neurobiology. 1982. pp. 195–204. https://doi.org/10.1111/jsr.12371 PMID: 7185792

24. Allada R, Cirelli C, Sehgal A. Molecular mechanisms of sleep homeostasis in flies and mammals. Cold Spring Harb Perspect Biol. 2017; 9: 1–20. https://doi.org/10.1101/cshperspect.a027730 PMID: 28432135

25. Joiner WJ, Crocker A, White BH, Sehgal A. Sleep in Drosophil a is regulated by adult mushroom bodies. Nature. 2006; 441: 757–760. https://doi.org/10.1038/nature04811 PMID: 16760980

26. Wu M, Robinson JE, Joiner WJ. SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission. Curr Biol. Elsevier Ltd; 2014; 24: 621–629. https://doi.org/10.1016/j.cub.2014.02.026 PMID: 24613312

27. Liu S, Liu Q, Tabuchi M, Wu MN. Sleep drive is encoded by neural plastic changes in a dedicated circuit. Cell. Elsevier Inc.; 2016; 165: 1347–1360. https://doi.org/10.1016/j.cell.2016.04.013 PMID: 27212237

28. Donlea JM, Alam MN, Szymusiak R. Neuronal substrates of sleep homeostasis; lessons from flies, rats and mice. Curr Opin Neurobiol. Elsevier Ltd; 2017; 44: 228–235. https://doi.org/10.1016/j.conb.2017.05.003 PMID: 28628804

29. Reimund E. The free radical flux theory of sleep. Med Hypotheses. 1994; 43: 231–233. https://doi.org/10.1016/0306-9877(94)90071-X PMID: 7838006

30. D’Almeida V, Lobo LL, Hipólido DC, de Oliveira a C, Nobrega JN, Tufik S. Sleep deprivation induces brain region-specific decreases in glutathione levels. Neuroreport. 1998; 9: 2853–2856. https://doi.org/10.1097/00001756-199808240-00031 PMID: 9700133

31. Eiland MM, Ramanathan L, Gulyani S, Siegel JM, Eiland MM, Ramanathan L, et al. Increases in amino-cupric-silver staining of the supraoptic nucleus after sleep deprivation. Brain Res. 2002; 945: 1–8. https://doi.org/10.1016/S0006-8993(02)02448-4 PMID: 12113945

32. Ramanathan L, Gulyani S, Nienhuis R, Siegel JM. Sleep deprivation decreases superoxide dismutase activity in rat hippocampus and brainstem. Neuroreport. 2002; 13: 1387–1390. https://doi.org/10.1097/00001756-200208070-00007 PMID: 12167758

33. Silva RH, Abílio VC, Takatsu AL, Kameda SR, Grassl C, Chehin AB, et al. Role of hippocampal oxidative stress in memory deficits induced by sleep deprivation in mice. Neuropharmacology. 2004; 46: 895–903. https://doi.org/10.1016/j.neuropharm.2003.11.032 PMID: 15033349

34. D’Almeida V, Hipólido DC, Azzalí LA, Lobo LL,Junqueira VB, Tufik S. Absence of oxidative stress following paradoxical sleep deprivation in rats. Neurosci Lett. 1997; 235: 25–28. Available: http://www.ncbi.nlm.nih.gov/pubmed/9389587 PMID: 9389587

35. Cirelli C, Shaw PJ, Rechtschaffen A, Tononi G. No evidence of brain cell degeneration after long-term sleep deprivation in rats. Brain Res. 1999; 840: 184–193. https://doi.org/10.1016/S0006-8993(99)01768-0 PMID: 10517970

36. Gopalakrishnan A, Ji LL, Cirelli C. Sleep deprivation and cellular responses to oxidative stress. Sleep J Sleep Disord Res. 2004; 27: 27–35. Available: http://ezproxy.lib.uh.edu/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=psyh&AN=2004-17199-006&site=ehost-live%5Cn http://ccirelli@wisc.edu

37. Liu Y, Wheaton AG, Chapman DP, Cunningham TJ, Lu H, Croft JB. Prevalence of Healthy Sleep Duration among Adults—United States, 2014. MMWR Morb Mortal Wkly Rep. 2016; 65: 137–141. https://doi.org/10.15585/mmwr.mm6506a1 PMID: 26890214
39. Hendricks JC, Finn SM, Panckeri KA, Chavkin J, Williams JA, Sehgal A, et al. Rest in Drosophila Is a Sleep-like State. Neuron. 2000; 25: 129–138. https://doi.org/10.1016/S0896-6273(00)80877-6 PMID: 10707978

40. Shaw PJ. Correlates of Sleep and Waking in Drosophila melanogaster. Science (80-). 2000; 287: 1834–1837. https://doi.org/10.1126/science.287.5459.1834

41. Cirelli C, Bushey D, Hill S, Huber R, Kreber R, Ganetzky B, et al. Reduced sleep in Drosophila Shaker mutants. Nature. 2005; 434: 1087–1092. https://doi.org/10.1038/nature03486 PMID: 15858664

42. Koh K, Joiner WJ, Wu MN, Yue Z, Smith CJ, Sehgal A. Identification of SLEEPLESS, a Sleep-Promoting Factor. Science (80-). 2008; 321: 372–376. https://doi.org/10.1126/science.1155942 PMID: 18635795

43. Shi M, Yue Z, Kuryatov A, Lindstrom JM, Sehgal A. Identification of SLEEPLESS, a Sleep-Promoting Factor. Science (80-). 2008; 321: 372–376. https://doi.org/10.1126/science.1155942 PMID: 18635795

44. Kume K. Dopamine Is a Regulator of Arousal in the Fruit Fly. J Neurosci. 2005; 25: 7377–7384. https://doi.org/10.1523/JNEUROSCI.2048-05.2005 PMID: 16093388

45. Stavropoulos N, Young MW. Insomniac and cullin-3 regulate sleep and wakefulness in drosophila. Neuron. Elsevier Inc.; 2011; 72: 964–976. https://doi.org/10.1016/j.neuron.2011.12.003 PMID: 22196332

46. Pfeifferberger C, Allada R. Cul3 and the BTB Adaptor Insomniac Are Key Regulators of Sleep Homoeostasis and a Dopamine Arousal Pathway in Drosophila. PLoS Genet. 2012; 8. https://doi.org/10.1371/journal.pgen.1003003 PMID: 23055946

47. Harbison ST, Negron YLS, Hansen NF, Lobell AS. Selection for long and short sleep duration in Drosophila melanogaster reveals the complex genetic network underlying natural variation in sleep. PLoS Genet. 2017; 13: e1007098. https://doi.org/10.1371/journal.pgen.1007098 eCollection 2017 Dec. PMID: 29240764

48. Taheri S, Lin L, Austin D, Young T, Mignot E. Short sleep duration is associated with reduced leptin, elevated ghrelin, and increased body mass index. PLoS Med. 2004; 1: 210–217. https://doi.org/10.1371/journal.pmed.0010062 PMID: 15602591

49. Wang Y, Carreras A, Lee S, Hakim F, Zhang SX, Nair D, et al. Chronic sleep fragmentation promotes obesity in young adult mice. Obesity. 2014; 22: 758–762. https://doi.org/10.1002/oby.20616 PMID: 24039209

50. Murakami K, Yurgel ME, Stahl BA, Masek P, Mehta A, Heidker R, et al. Translin Is Required for Metabolic Regulation of Sleep. Curr Biol. 2016; 26: 972–980. https://doi.org/10.1016/j.cub.2016.02.013 PMID: 27020744

51. Masek P, Reynolds LA, Bollinger WL, Moody C, Mehta A, Murakami K, et al. Altered regulation of sleep and feeding contributes to starvation resistance in Drosophila melanogaster. J Exp Biol. 2014; 217: 3122–3132. https://doi.org/10.1242/jeb.103309 PMID: 24948636

52. Prather AA, Janicki-Deverts D, Hall MH, Cohen S. Behaviorally Assessed Sleep and Susceptibility to the Common Cold. Sleep. 2015; 38: 1353–1359. https://doi.org/10.5655/sleep.49686 PMID: 26118561

53. Irwin MR, McClintick J, Costlow C, Fortner M, White J, Gillin JC. Partial night sleep deprivation reduces natural killer and cellular immune responses in humans. FASEB J. 1996; 10: 643–653. PMID: 8621064

54. Lungato L, Gazarini ML, Paredes-Gamero EJ, Tufik S, D’Almeida V. Paradoxical sleep deprivation impairs mouse survival after infection with malaria parasites. Malar J. 2015; 14. https://doi.org/10.1186/s12936-015-0690-7 PMID: 25927919

55. Zagera A, Andersen ML, Ruiz FS, Antunes IB, Tufik S. Effects of acute and chronic sleep loss on immune modulation of rats. Am J Physiol Regul Integr Comp Physiol. 2007; 293: R504—9. https://doi.org/10.1152/ajpregu.00105.2007 PMID: 17409265

56. Xu T-H, Williams JA. Increased sleep promotes survival during a bacterial infection in Drosophila. Sleep. 2014; 37: 1077–86, 1086A—1086D. https://doi.org/10.5665/sleep.3764 PMID: 24882902

57. Cochemé HM, Murphy MP. Complex I is the major site of mitochondrial superoxide production by paraquat. J Biol Chem. 2008; 283: 1786–1798. https://doi.org/10.1074/jbc.M708597200 PMID: 18039652

58. Grover D, Ford D, Brown C, Hoe N, Erdem A, Tavarié S, et al. Hydrogen peroxide stimulates activity and alters behavior in Drosophila melanogaster. PLoS ONE. 2009; 4. https://doi.org/10.1371/journal.pone.0007580 PMID: 19862323

59. Berry JA, Cervantes-Sandoval I, Chakraborty M, Davis RL. Sleep Facilitates Memory by Blocking Dopamine Neuron-Mediated Forgetting. Cell. Elsevier Inc.; 2015; 161: 1656–1667. https://doi.org/10.1016/j.cell.2015.06.027 PMID: 26073942

60. Jenett A, Rubin GM, Ngo TTB, Shepherd D, Murphy C, Dionne H, et al. A GAL4-Driven Line Resource for Drosophila Neurobiology. Cell Rep. 2012; 2: 991–1001. https://doi.org/10.1016/j.celrep.2012.09.011 PMID: 23063364
61. Luan H. Functional Dissection of a Neuronal Network Required for Cuticle Tanning and Wing Expansion in Drosophila. J Neurosci. 2006; 26: 573–584. https://doi.org/10.1523/JNEUROSCI.3916-05.2006 PMID: 16407556

62. Naidoo N, Giang W, Galante RJ, Pack AI. Sleep deprivation induces the unfolded protein response in mouse cerebral cortex. J Neurochem. 2005; 92: 1150–1157. https://doi.org/10.1111/j.1471-4159.2004.02952.x PMID: 15715665

63. Naidoo N, Casiano V, Cater J, Zimmerman J, Pack AI. A role for the molecular chaperone protein BiP/GRP78 in Drosophila sleep homeostasis. Sleep. 2007; 30: 557–565. PMID: 17552370

64. Brown MK, Strus E, Naidoo N. Reduced sleep during social isolation leads to cellular stress and induction of the unfolded protein response. Sleep. 2017; 40. https://doi.org/10.1093/sleep/zsx095 PMID: 28541519

65. Tang S, Le PK, Tse S, Wallace DC, Huang T. Heterozygous mutation of Opa1 in Drosophila shortens lifespan mediated through increased reactive oxygen species production. PLoS ONE. 2009; 4. https://doi.org/10.1371/journal.pone.0004492 PMID: 19221591

66. Yang Y, Gehrke S, Haque ME, Imai Y, Kosek J, Yang L, et al. Inactivation of Drosophila DJ-1 leads to impairments of oxidative stress response and phosphatidylinositol 3-kinase/Akt signaling. Proc Natl Acad Sci U S A. 2005; 102: 13670–13675. https://doi.org/10.1073/pnas.0504610102 PMID: 16155123

67. Williams MJ, Perland E, Eriksson MM, Carlsson J, Erlandsen D, Laan L, et al. Recurrent sleep fragmentation induces insulin and neuroprotective mechanisms in middle-aged flies. Front Aging Neurosci. 2016; 8: 1–11.

68. Nikonova EV, Naidoo N, Zhang L, Romer M, Cater JR, Scharf MT, et al. Changes in components of energy regulation in mouse cortex with increases in wakefulness. Sleep. 2010; 33: 889–900. https://doi.org/10.1093/sleep/33.7.889 PMID: 20614849

69. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Curr Biol. Elsevier; 2014; 24: R453—R462. https://doi.org/10.1016/j.cub.2014.03.034 PMID: 24845678

70. Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, et al. ROS signaling: The new wave? Trends Plant Sci. Elsevier Ltd; 2011; 16: 300–309. https://doi.org/10.1016/j.tplants.2011.03.007 PMID: 21482172

71. Ikeda M, Ikeda-Sagara M, Okada T, Clement P, Urade Y, Nagai T, et al. Brain oxidation is an initial process in sleep induction. Neuroscience. 2005; 130: 1029–1040. https://doi.org/10.1016/j.neuroscience.2004.09.057 PMID: 15652998

72. Koh K, Evans JM, Hendricks JC, Sehgal A. A Drosophila model for age-associated changes in sleep: wake cycles. Proc Natl Acad Sci. 2006; 103: 13843–13847. https://doi.org/10.1073/pnas.0605930103 PMID: 16938867

73. Jean-Louis G, Kripke DF, Ancoli-Israel S, Klauber MR, Sepulveda RS. Sleep duration, illumination, and activity patterns in a population sample: effects of gender and ethnicity. Biol Psychiatry. 2000; 47: 921–927. https://doi.org/10.1016/S0006-3223(99)00169-9 PMID: 10807965

74. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circulation Research. 2010. pp. 1058–1070. https://doi.org/10.1161/CIRCRESAHA.110.223545 PMID: 21030723

75. Kayama Y, Raaz U, Jagger A, Adam M, Schellinger IN, Sakamoto M, et al. Diabetic cardiovascular disease induced by oxidative stress. International Journal of Molecular Sciences. 2015. pp. 25234–25263. https://doi.org/10.3390/ijms161025234 PMID: 26512646

76. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. Am J Cardiol. 2003; 91: 7–11. https://doi.org/10.1016/S0002-9149(02)03144-2

77. Youn J-YY, Siu KL, Dob HE, Itani H, Harrison DG, Cai H. Role of vascular oxidative stress in obesity and metabolic syndrome. Diabetes. 2014; 63: 2344–2355. https://doi.org/10.2337/db13-0719 PMID: 24550188

78. Carvajal K, Balderas-Villalobos J, Bello-Sanchez MD, Phillips-Farfán B, Molina-Muñoz T, Aldana-Quintero H, et al. Ca2+ mishandling and cardiac dysfunction in obesity and insulin resistance: Role of oxidative stress. Cell Calcium. 2014. pp. 408–415. https://doi.org/10.1016/j.ceca.2014.08.003 PMID: 25168907

79. Sterniczuk R, Theou O, Rusak B, Rockwood K. Sleep Disturbance is Associated with Incident Dementia and Mortality. Curr Alzheimer Res. 2013; 10: 767–775. https://doi.org/10.2174/1567205013109990134 PMID: 23905991

80. Lim ASP, Kowgier M, Yu L, Buchman AS, Bennett DA. Sleep Fragmentation and the Risk of Incident Alzheimer’s Disease and Cognitive Decline in Older Persons. Sleep. 2013; 36: 1027–1032. https://doi.org/10.5665/sleep.2802 PMID: 23814339

81. Schenck CH, Boeve BF, Mahowald MW. Delayed emergence of a parkinsonian disorder or dementia in 81% of older men initially diagnosed with idiopathic rapid eye movement sleep behavior disorder: A 16-
82. Lazar AS, Panin F, Goodman AOG, Lazic SE, Mason SL, et al. Sleep deficits but no metabolic deficits in premanifest Huntington's disease. Ann Neurol. 2015; 78: 630–648. https://doi.org/10.1002/ana.24495 PMID: 26224419

83. Allan Butterfield D, Castegna A, Lauderback CM, Drake J. Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. Neurobiology of Aging. 2002. pp. 655–664. https://doi.org/10.1016/S0197-4580(01)00340-2 PMID: 12392786

84. Dexter DT, Carter CJ, Wells FR, Javoy-Agid F, Agid Y, Lees A, et al. Basal Lipid Peroxidation in Substantia Nigra Is Increased in Parkinson's Disease. J Neurochem. 1989; 52: 381–389. https://doi.org/10.1111/j.1471-4159.1989.tb09133.x PMID: 2911023

85. Sorolla MA, Reverter-Branchat G, Tamarit J, Ferrer I, Ros J, Cabisco E. Proteomic and oxidative stress analysis in human brain samples of Huntington disease. Free Radic Biol Med. 2008; 45: 667–678. https://doi.org/10.1016/j.freeradbiomed.2008.05.014 PMID: 18588971

86. Gilestro GF, Cirelli C. PySolo: A complete suite for sleep analysis in Drosophila. Bioinformatics. 2009; 25: 1466–1467. https://doi.org/10.1093/bioinformatics/btp237 PMID: 19369499

87. Galac MR, Lazaro BP. Comparative pathology of bacteria in the genus Providencia to a natural host, Drosophila melanogaster. Microbes Infect. 2011; 13: 673–683. https://doi.org/10.1016/j.micinf.2011.02.005 PMID: 21354324