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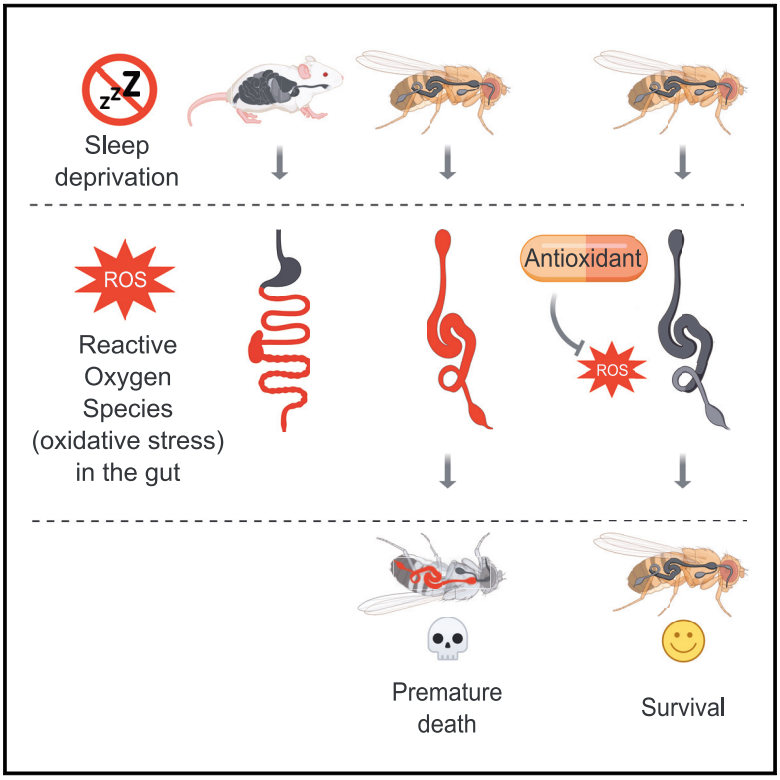
Sleep Loss Can Cause Death through Accumulation of Reactive Oxygen Species in the Gut

Graphical Abstract



Highlights

- Sleep deprivation leads to ROS accumulation in the fly and mouse gut
- Gut-accumulated ROS trigger oxidative stress in this organ
- Preventing ROS accumulation in the gut allows survival without sleep in flies

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In Brief

Sleep deprivation is associated with lethality through accumulation of reactive oxygen species in the gut.

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Sleep Loss Can Cause Death through Accumulation of Reactive Oxygen Species in the Gut

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SUMMARY

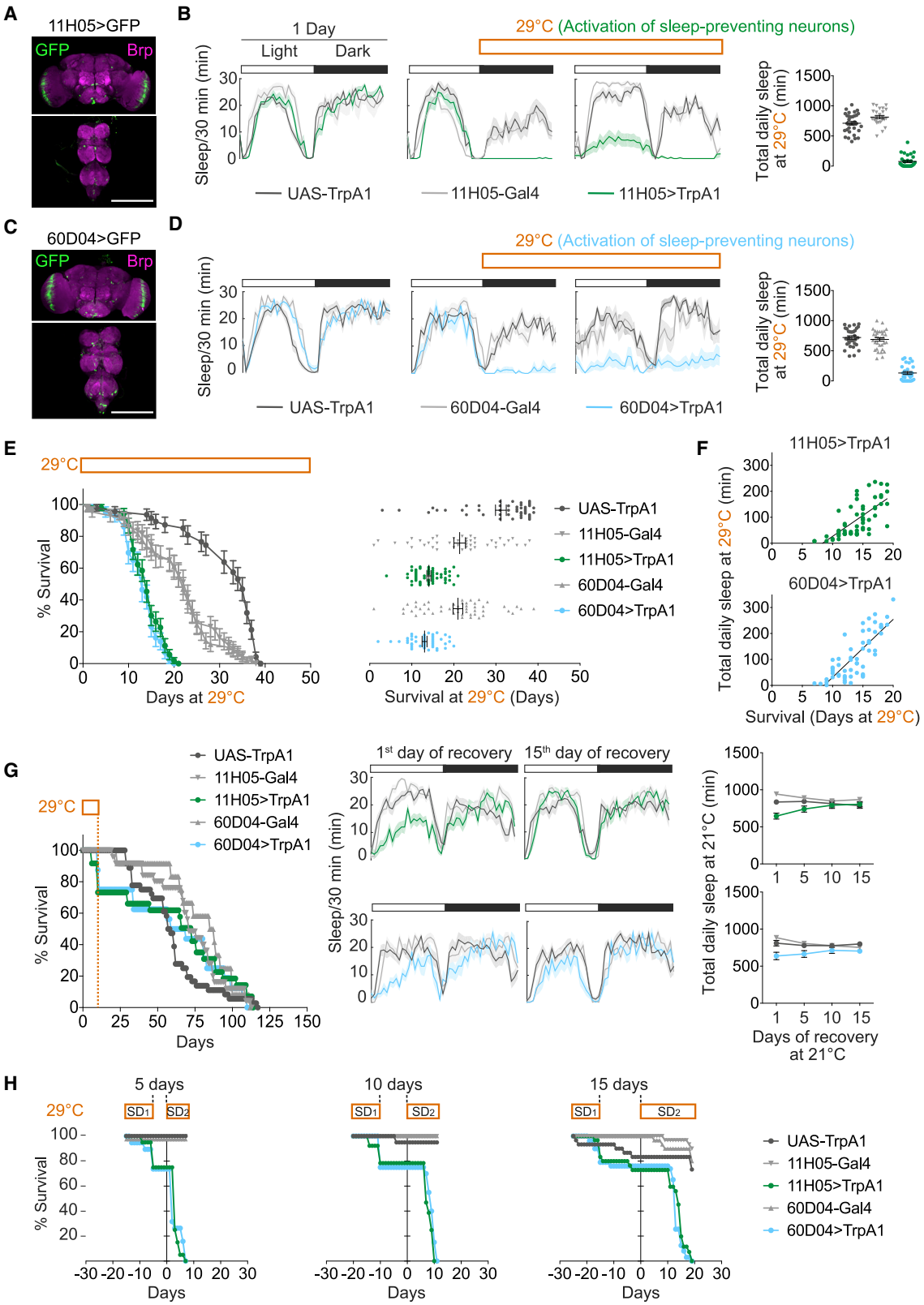
The view that sleep is essential for survival is supported by the ubiquity of this behavior, the apparent existence of sleep-like states in the earliest animals, and the fact that severe sleep loss can be lethal. The cause of this lethality is unknown. Here we show, using flies and mice, that sleep deprivation leads to accumulation of reactive oxygen species (ROS) and consequent oxidative stress, specifically in the gut. ROS are not just correlates of sleep deprivation but drivers of death: their neutralization prevents oxidative stress and allows flies to have a normal lifespan with little to no sleep. The rescue can be achieved with oral antioxidant compounds or with gut-targeted transgenic expression of antioxidant enzymes. We conclude that death upon severe sleep restriction can be caused by oxidative stress, that the gut is central in this process, and that survival without sleep is possible when ROS accumulation is prevented.

INTRODUCTION

Sleep is an all-encompassing state that renders animals immobile and hypo-responsive to stimulation (Campbell and Tobler, 1984). A single answer is unlikely to explain the function of sleep since processes like cognition, immunity, and metabolism are all sleep dependent (Krueger et al., 2016; Zielinski et al., 2016). Countless clinical and experimental studies link insufficient sleep with serious health problems (Chattu et al., 2018; Medic et al., 2017), and sleep restriction can lead to premature death in model organisms, including dogs, rats, cockroaches, and flies (Bentivoglio and Grassi-Zucconi, 1997; Rechtschaffen et al., 1983; Shaw et al., 2002; Stephenson et al., 2007). We wanted to find out specifically what makes sleep required for survival in the most basic sense.

Sleep is generated by neurons, so it has been assumed that death observed with sleep deprivation results from impaired brain function. This idea is supported by the significant cognitive decline noticeable after sleep loss (Donlea, 2019; Killgore, 2010; Krause et al., 2017). Two leading proposals for the brain-related restorative functions of sleep are downscaling of synapses formed during wakefulness (Bushey et al., 2011; de Vivo et al., 2017; Diering et al., 2017; Gilestro et al., 2009; Tononi and Cirelli, 2014) and clearance of harmful substances from interstitial brain areas (Holth et al., 2019; Xie et al., 2013). Because it is difficult to induce these processes during wakefulness or inhibit them during sleep, their contribution to the lethality associated with sleep deprivation is unknown. In addition to impairing cognition, sleep loss leads to dysfunction of the gastrointestinal, immune, metabolic, and circulatory systems (Ali et al., 2013; Irwin, 2019; Khanijow et al., 2015; Koren and Taveras, 2018; McAlpine et al., 2019; Mullington et al., 2010; Tobaldini et al., 2019). It is unclear whether these are secondary consequences of altered nervous system function or direct and independent effects of sleep deprivation. It is also unclear whether any of these impairments contribute to premature death of sleep-deprived animals. Given the diverse system failures, it is important to know whether a single intervention could prevent lethality in sleep-deprived animals or whether the longevity benefits of rescuing one problem would be masked by a host of other consequences incompatible with life. Prevention of death by a single means would argue that the gradual collapse of nearly all major bodily functions derives from a common origin.

One proposed function of sleep is prevention of oxidative stress in the brain (Reimund, 1994). Multiple studies have reported altered antioxidant response in the brain during sleep loss (Alzoubi et al., 2012; D'Almeida et al., 1998; Hill et al., 2018; Kanazawa et al., 2016; Ramanathan et al., 2002; Silva et al., 2004; Singh and Kumar, 2008; Süer et al., 2011; Villafuerte et al., 2015), and it was recently found that sleep loss alters the redox state of several sleep-regulating neurons in the fly brain, influencing their activity (Kempf et al., 2019). Because the brain does not appear to be significantly damaged by sleep deprivation (Cirelli, 2006; Cirelli et al., 1999; de Souza et al., 2012; Eiland et al., 2002; Hipólide et al., 2002), some labs have searched for signs of oxidative stress elsewhere. In rodents, the endogenous antioxidant defense is weakened in the liver (Everson et al., 2005; Pandey and Kar, 2018), and visceral organs show evidence of



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oxidation after long-term sleep restriction (Everson et al., 2014; Pandey and Kar, 2018; Villafuerte et al., 2015). As there are no known localized origins of oxidative molecules during sleep deprivation, it is not known whether this oxidation is a cause or consequence of other damage and whether it is the reason why sleep-deprived animals die.

In our search for factors that directly link sleep loss and death, we took an agnostic approach in terms of anatomy, examining multiple tissues in parallel. Our first model was the fly since flies and mammals share core attributes of sleep (Hendricks et al., 2000; Nitz et al., 2002; Shaw et al., 2000) and since flies require sleep for a normal lifespan (Bushey et al., 2010; Cirelli et al., 2005a; Koh et al., 2008; Pitman et al., 2006; Rogulja and Young, 2012; Shaw et al., 2002; Stavropoulos and Young, 2011). After determining how long it takes for sleep deprivation to cause death, we examined various markers of cell damage in the days leading up to that point. We identified reactive oxygen species (ROS)—unstable, short-lived, and highly reactive molecules—as drivers of cellular damage and lethality during sleep deprivation. Endogenously generated ROS have important signaling roles (Holmström and Finkel, 2014; Li et al., 2018; Lim et al., 2014; Mittler, 2017; Oswald et al., 2018; Owusu-Ansah and Banerjee, 2009; Schieber and Chandel, 2014), but when their levels exceed the cellular antioxidant capacity, a chain of reactions is triggered that results in widespread oxidation (D'Autréaux and Toledano, 2007; Schieber and Chandel, 2014). The free radical forms of ROS are particularly reactive because their valence electrons are unpaired (Dhawan, 2014). To gain stability, they interact with macromolecules (DNA, proteins, lipids), stripping them of electrons and consequently destabilizing them (Evans et al., 2004; Gutteridge and Halliwell, 1990; Halliwell, 2006; Stadtman and Levine, 2003).

Three independent methods of sleep deprivation in the fly led to accumulation of ROS in the gut, triggering oxidative stress in this organ. When deprivation was stopped, ROS and oxidative stress markers gradually cleared. Sleep deprivation in the mouse produced a similar outcome, with ROS accumulating specifically in the small and large intestines and triggering oxidative stress. We show in flies that accumulated ROS are causally linked to decreased survival, as clearing them from the gut (with oral antioxidant compounds or gut-targeted expression of antioxidant enzymes) can allow a normal lifespan with little to no sleep.

RESULTS

Severe Sleep Loss Can Cause Premature Death

To study how severe sleep loss impacts survival, we looked for methods that can continually suppress most sleep. It is difficult to keep animals awake for a long time because a powerful homeostatic mechanism drives recovery of lost sleep; this compensatory mechanism is not well understood but is evident in rebound sleep seen after a night of sleep deprivation (i.e., animals sleep instead of being active) (Allada et al., 2017; Hendricks et al., 2000; Huber et al., 2004; Shaw et al., 2000). As can be intuited (Bringmann, 2019), to reveal the full consequence of sleep loss, it is likely necessary to bypass the mechanisms that drive rebound sleep. Crucially, lack of such drive should not eliminate the physiological need for sleep, the same way that a lack of appetite would not eliminate the need for nutrients.

A common sleep deprivation method in flies relies on thermogenetic stimulation of various neurons whose activity suppresses sleep (Dubowy et al., 2016; Kayser et al., 2014, 2015; Liu et al., 2012, 2016; Nall et al., 2016; Seidner et al., 2015; Sitaraman et al., 2015; Vienne et al., 2016). We tested most Gal4 drivers known to target such neurons, using them to express the heat-activated cation channel TrpA1 (Hamada et al., 2008). At 21°C, TrpA1 is closed, so this temperature was used to raise animals and monitor baseline sleep. Increasing the ambient temperature to 29°C causes TrpA1 to open and neurons to be stimulated as a consequence. Sleep was reduced with all Gal4s, but in most cases, animals could not be kept awake for more than several days (data not shown). Fortunately, some Gal4s have been shown to target neurons whose activation causes severe sleep loss without triggering a compensatory increase in sleep drive; when deprivation is stopped, animals resume normal sleep-wake cycles without seeking extra sleep (Seidner et al., 2015). Consistent with this characterization, such Gal4s were effective at suppressing sleep through life (Figure 1). We used two Gal4s in parallel (Figures 1A–1D) to increase our confidence that whatever phenomena we observe are generalizable. The conclusions we reached with this approach were later supported by other methods of deprivation, including those that trigger sleep rebound.

When TrpA1 was expressed in neurons labeled by 11H05- or 60D04-Gal4 (11H05>TrpA1, 60D04>TrpA1), flies lost ~90% of sleep (Figures 1A–1D). In each case, sleep-deprived animals

Figure 1. Sleep Deprivation Shortens Lifespan

(A) Expression of 11H05-Gal4 in the nervous system, reported by mCD8::GFP. Top: brain. Bottom: ventral nerve cord. Brp, a presynaptic protein, marks the neuropil.

(B) Sleep across 3 days. Day 1, 21°C: sleep amount is the same between the parental controls (11H05-Gal4, UAS-TrpA1) and flies expressing TrpA1 (11H05>TrpA1). Male flies sleep in the middle of the day and at night. When the temperature is raised to 29°C on day 2 (orange), 11H05>TrpA1 flies become nearly sleepless. Mean and SEM (shading). Right: daily sleep in control and experimental flies. Mean and SEM.

(C and D) The same as (A) and (B) but for 60D04-Gal4. Scale bars, 200 μm.

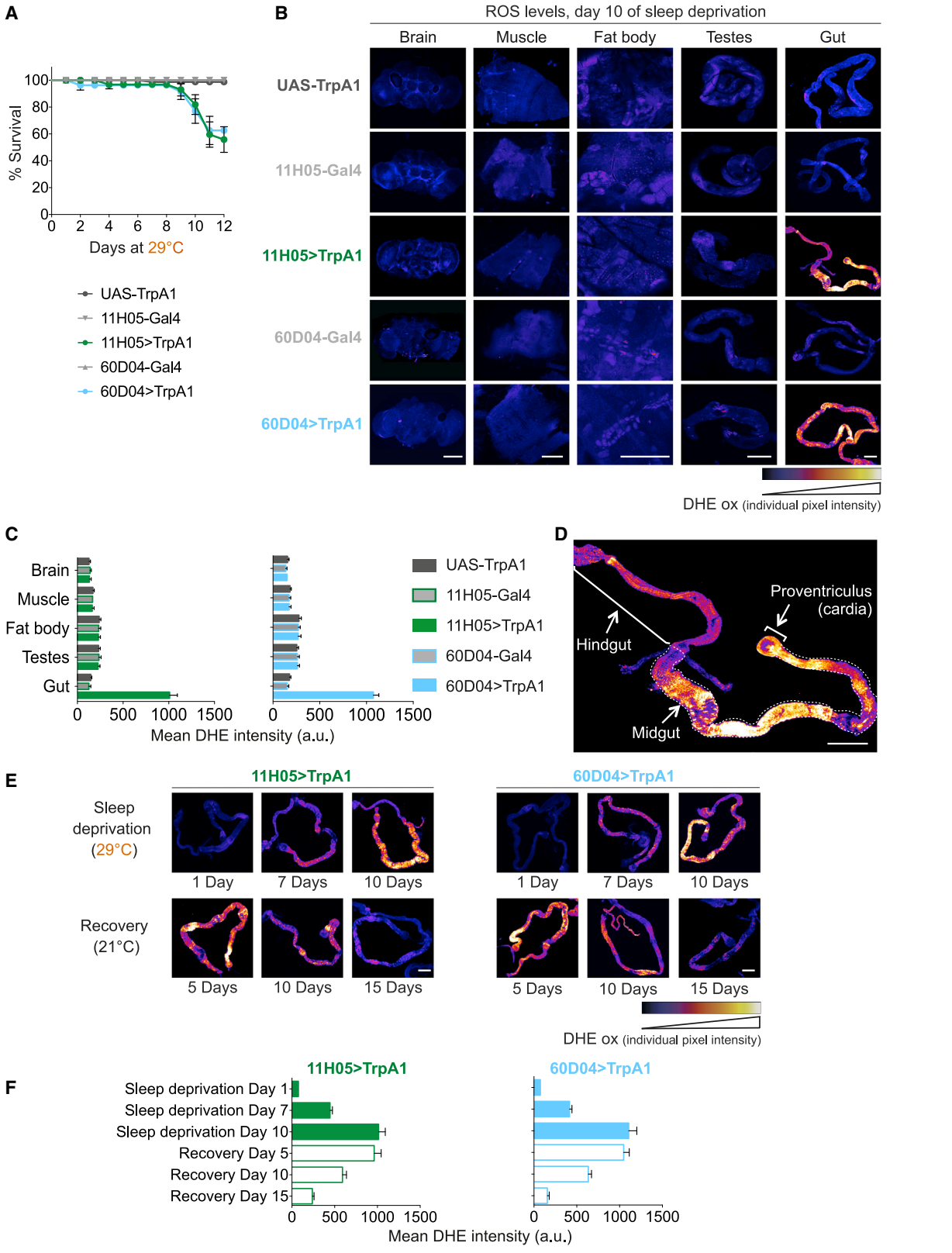
(E) Survival of sleep-deprived (green, blue) and non-deprived flies at 29°C. Left: population. Right: individual survival. Mean and SEM.

(F) Correlation between sleep and lifespan in individuals.

(G) Left: survival of flies allowed to sleep after being deprived for 10 days. Error bars are omitted for clarity. Center and right: sleep during recovery. Mean and SEM.

(H) Flies were sleep-deprived for 10 days (SD1) and allowed to sleep for 5, 10, or 15 days before being deprived again (SD2). Day 0, onset of SD2. Error bars are omitted for clarity.

All survival experiments were performed at least twice. For sample sizes and statistical analyses, see Table S1. See also Figure S2.



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died earlier than their non-deprived parental controls (Figure 1E). High mortality became apparent around day 10 of deprivation (Figures 1E and 1F). Greater sleep loss triggered earlier death in individual flies (Figure 1F). If deprivation was stopped on day 10, the surviving animals established normal sleep-wake cycles and lived as long as the controls (Figure 1G). The lifespan of flies extends at lower temperatures (21°C versus 29°C); regardless, there was no difference in survival between the experimental genotypes and the controls (Figure 1G, left), suggesting that at least some major negative consequences of sleep deprivation are reversible.

To find out how long the impact of sleep deprivation lingers in the body, we deprived flies of sleep for 10 days, allowed different groups of animals to sleep *ad libitum* for different time durations, and then restricted their sleep again. Being allowed to sleep for 5 or 10 days was insufficient to erase the impact of the first bout of deprivation (Figure 1H, first two graphs). If the period of unrestricted sleep was extended to 15 days, flies seemed fully recovered (Figure 1H, last graph), as it took 20 days before they all died (comparable with Figure 1E). These results suggest a gradual accumulation of damage during sleep loss that is also gradually repaired during recovery sleep.

Thermogenetic Sleep Deprivation Causes Accumulation of ROS in the Gut

Because mortality increased around day 10 of sleep deprivation (Figures 1E, 1F, and 2A), we examined various markers of cell damage in the preceding days, throughout the body. Most tissues appeared indistinguishable between sleep-deprived and non-deprived animals, except for one major difference: the guts of deprived animals had increased levels of ROS (Figures 2B and 2C). High ROS levels were mostly observed in the midgut, which spans much of the gut length but has clear boundaries (Figure 2D; Miguel-Aliaga et al., 2018). ROS are inherently unstable because of high reactivity and are consequently difficult to detect. This problem can be circumvented with probes that form stable fluorescent products when oxidized by ROS (Chen et al., 2011; Hardy et al., 2018). Dihydroethidium (DHE) is considered the most sensitive and specific among such probes and is used to detect superoxide radicals in living (non-fixed) tissues. DHE emits blue fluorescence unless oxidized, in which case it intercalates into DNA and fluoresces red (Kalyanaraman et al., 2017). Superoxide is a precursor of other ROS (Babior, 1997; Fridovich, 1997), so DHE fluorescence should probably be taken as a general reporter of reactive species and free radicals. Staining was performed on whole tissues because their small size allows DHE penetration (Vaccaro et al., 2017). A different fluorescent probe, thought to be predominantly oxidized by hydrogen peroxide (Cathcart et al., 1983), also reported an increase in ROS levels specifically in the gut (Figure S1A).

ROS accumulation was gradual, peaking on day 10 of sleep deprivation (Figures 2E, top row, and 2F and data not shown). When deprivation was stopped, ROS levels gradually decreased (Figures 2E, bottom row, and 2F), coming close to baseline after 15 days (Figure 2F), which is also how long it took for survival curves to reset after sleep deprivation was stopped (Figure 1H). ROS levels remained slightly elevated relative to pre-deprivation levels even after 20 days of recovery (data not shown).

The two Gal4 lines used for sleep deprivation have almost no expression in the gut, except for several cells that can be detected with a bright fluorescent nuclear marker (Barolo et al., 2004; Figure S1B). Still, we ensured that our observations did not stem from TrpA1-dependent activation of gut cells. Expression and activation of TrpA1 in different cell populations in the gut (enterocytes, using myo1A-gal4 [Buchon et al., 2013; Jiang et al., 2009], or enteroblasts and intestinal stem cells, using esg-Gal4 [Micchelli and Perrimon, 2006]) did not affect sleep, longevity, or ROS levels (Figures S1C–S1H). We conclude that sleep deprivation is the most likely cause of ROS increase in the gut. To strengthen this conclusion and ensure that ROS accumulation is not limited to methods that do not trigger sleep rebound, we used additional approaches to prevent sleep.

Other Sleep Deprivation Methods Also Lead to ROS Accumulation in the Gut

Several modes of mechanical agitation have been used for acute, short-term sleep deprivation in *Drosophila* (Hendricks et al., 2000; Huber et al., 2004; Kayser et al., 2014; Li et al., 2009; Seugnet et al., 2008; Shaw et al., 2000, 2002). Exposing flies to vibrations did cause sleep reduction, but with high variability across individuals (Figures 3A and 3D) and across days (Figure 3B). The variability was presumably due to habituation but also the fact that the compensatory sleep drive increases in response to mechanical deprivation (Hendricks et al., 2000; Huber et al., 2004; Shaw et al., 2000). Consistent with this interpretation, rebound sleep was observed immediately after the agitation was stopped (Figure 3C). The extreme variability of sleep loss, and the fact that wings and legs were often damaged by shaking, made it difficult to correlate the observed shortening of lifespan (data not shown) with sleep loss. Despite this limitation, we could ask whether sleep loss induced by mechanical deprivation leads to ROS accumulation. Most flies exhibited significant sleep loss on the first day, but their sleep increased subsequently (e.g., Figure 3A, inconsistent sleep loss). A minority of flies consistently lost most of their sleep for up to a week (e.g., Figure 3A, consistent sleep loss). Because they experienced

Figure 2. ROS Accumulate in the Fly Gut upon Prolonged Thermogenetic Sleep Deprivation

(A) Survival of sleep-deprived flies (green, blue) and non-deprived parental controls, initial 12 days. Performed 3 times. Mean and SEM.

(B) Oxidized DHE (DHE ox) reports high levels of reactive oxygen species (ROS) in the gut of sleep-deprived flies (green, blue) on day 10.

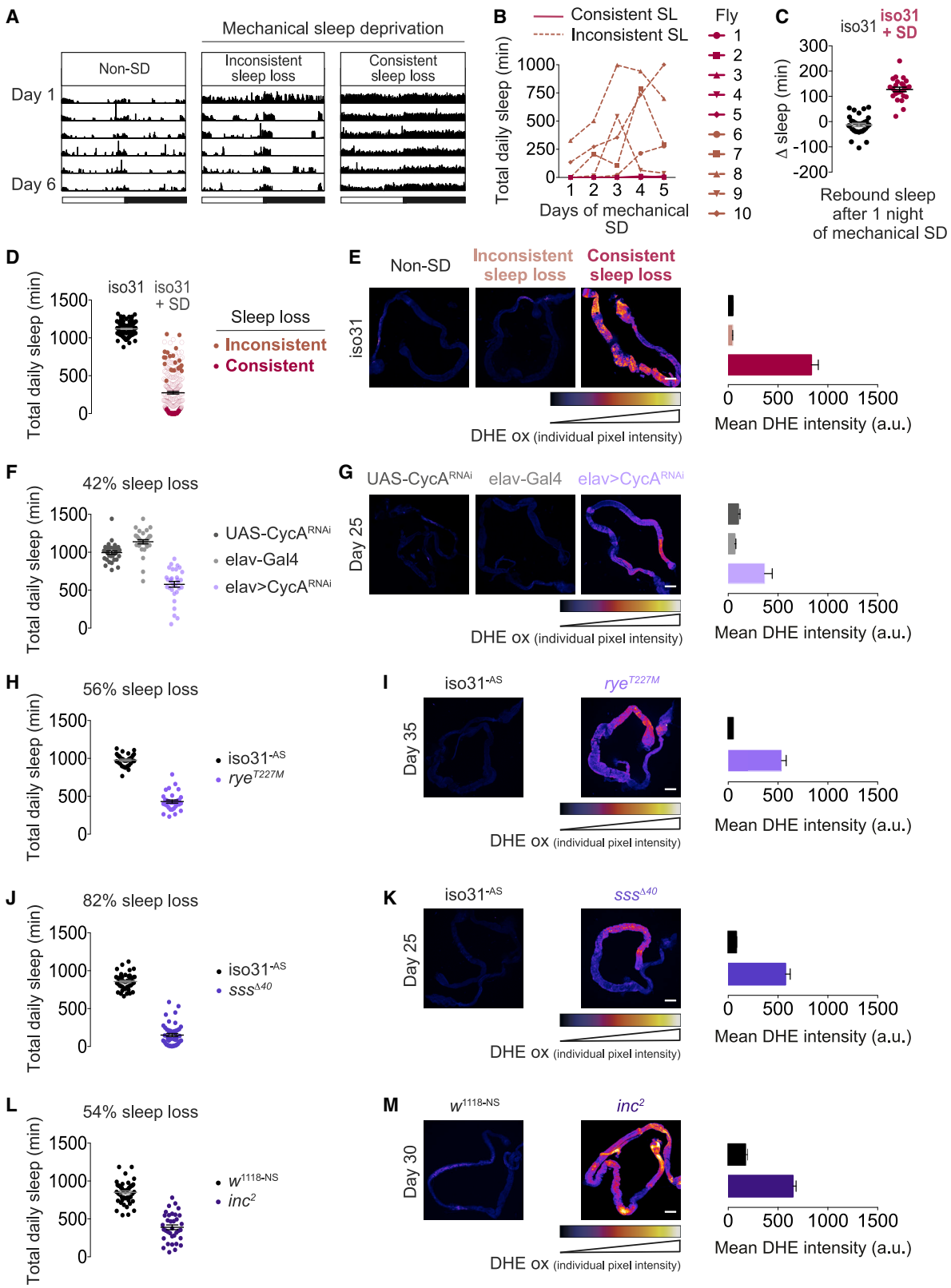
(C) Quantification. Mean and SEM.

(D) Closeup of the gut on day 10 of sleep deprivation. The same image as in (B) (11H05>TrpA1).

(E) Top: ROS in the gut on days 1, 7, and 10 of sleep deprivation (29°C). Bottom: ROS in the gut 5, 10, or 15 days after deprivation was stopped (21°C). Representative images. Scale bars, 200 μm.

(F) Quantification. Mean and SEM.

For sample sizes and statistical analyses, see Table S1. See also Figures S1 and S2.



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identical mechanical agitation but different sleep amounts, animals could be directly compared to reveal the effect of sleep loss on ROS levels. In agreement with the thermogenetic approach, ROS accumulated in the gut and only in animals that experienced sustained sleep loss (Figure 3E). This supports the idea that sleep deprivation itself causes ROS accumulation. The fact that ROS levels increase with deprivation methods that either do (mechanical; Figure 3C) or do not (thermogenetic; Figure 1G, center and right) activate the sleep homeostat supports the notion that the physiological need for sleep is separable from sleep drive (i.e., preventing sleepiness would not obviate the need for sleep).

For the third deprivation method, we used loss-of-function mutations or RNAi against known sleep regulators: Cyclin A (CycA) (Afonso et al., 2015; Rogulja and Young, 2012), Insomniac (Inc) (Li et al., 2017; Pfeiffenberger and Allada, 2012; Stavropoulos and Young, 2011), Redeye (Rye), (Shi et al., 2014), and Sleepless (SSS) (Koh et al., 2008; Shi et al., 2014; Wu et al., 2010, 2014). As in the original studies, sleep was reduced with neuronal RNAi against Cyclin A (elav>CycA^{RNAi}) (Figures 3F and S2A) or mutations in *redeye* (*rye*^{T227M}) (Figures 3H and S2B), *sleepless* (*sss*$^{\Delta 40}$) (Figures 3J and S2C), and *insomniac* (*inc*^{2}) (Figures 3L and S2D). In agreement with the other two deprivation methods, ROS levels were elevated in the gut of these chronically deprived flies (Figures 3G, 3I, 3K, and 3M), reinforcing the idea that insufficient sleep leads to ROS accumulation.

Lifespan was reduced by all of these genetic manipulations (Figures S2A–S2D), as previously described (Koh et al., 2008; Rogulja and Young, 2012; Stavropoulos and Young, 2011). In contrast to *inc* mutants, nervous system-specific depletion of Inc was reported not to shorten lifespan (Hill et al., 2018; Stavropoulos and Young, 2011). These flies (elav>inc^{RNAi}) were indeed not short lived (Figure S2G), but their sleep phenotype (Figure S2E) was milder than that of *inc*^{2} mutants (Figures 3L and S2D) and was not sustained (Figure S2F). Consistent with the no-mortality phenotype, ROS levels were not elevated in elav>inc^{RNAi} flies (Figure S2H), suggesting that they have not accumulated sufficient sleep debt. Sleep loss may not be the only cause of premature death in sleep mutants because the affected proteins function broadly. For example, Inc and SSS function at larval neuromuscular synapses (Li et al., 2017; Wu et al., 2010), and adult *sss* mutants exhibit motor neuron defects and lack coordination (Iyengar and Wu, 2014; Koh et al., 2008; Wu et al., 2010). Nonetheless, insufficient sleep and high ROS levels in the gut are phenotypes shared by these mutants. The general rule seems to be that ROS accumulate when sleep falls below a certain threshold, which correlates with increased mortality. The one verified example of severely sleep-deprived flies with normal longevity supports the proposed ROS-mortality link. Dopamine transporter *fumin* (*fmn*) mutations cause sleep loss without affecting lifespan (Kume et al., 2005; Figures S2I). The guts of *fmn* mutants exhibited normal ROS levels, even after a month of severe sleep loss (Figure S2J). We have not yet uncovered the mechanisms that prevent ROS accumulation in this case; regardless, we conclude that a normal lifespan with little sleep is possible if ROS levels in the gut are kept low.

Cellular Evidence of Oxidative Stress upon Sleep Deprivation

ROS can destroy cellular macromolecules through oxidation (Evans et al., 2004; Gutteridge and Halliwell, 1990; Stadtman and Levine, 2003). As predicted from their high ROS levels, guts from sleep-deprived flies showed extensive evidence of oxidative stress (Figures 4A and 4B). Oxidation is harmful to DNA (Cadet and Wagner, 2013; Evans et al., 2004). In flies (Madigan et al., 2002; Rogakou et al., 1999) and mammals (Rogakou et al., 1998), DNA double-strand breaks can be detected with antibodies that recognize histone H2A phosphorylation (γH2Av, γH2Ax in mammals) (Lake et al., 2013), a modification triggered by the DNA damage response (Scully and Xie, 2013). This approach revealed widespread DNA damage in the gut (Figures 4A and 4B). Oxidation also triggers adaptive pathways (Espinosa-Diez et al., 2015; Ma, 2013; Navarro-Yepes et al., 2014), and there was an increase in stress granules (RNA-protein aggregates that halt translation and protect mRNA) (Buchan and Parker, 2009; Chen and Liu, 2017) and lysosomes (organelles that degrade damaged cell components) (Filomeni et al., 2015; Figures 4A and 4B). Sustained oxidation can ultimately cause cells to die (Redza-Dutordoir and Averill-Bates, 2016); markers of apoptosis and necrosis were observed throughout the gut (Figures 4A and 4B). Although these signs of cellular damage fit the notion of oxidative stress where ROS are the inducers of damage, there are other interpretations; in some contexts, ROS can be downstream of cell-death-triggering signals (Kanda et al., 2011; Pérez et al., 2017a; Zhang et al., 2009). Arguing that cellular damage and cell death are a consequence rather than the cause of ROS increase, all oxidative stress markers appeared only after ROS began accumulating (Figures 4C and 4D). When sleep deprivation was stopped and ROS levels gradually decreased, markers of oxidative stress decreased as well (Figures S3A and S3B). Because the gut has high regenerative capacity (Amcheslavsky et al., 2009; Buchon et al., 2009b; Jiang et al., 2009; van der Flier and Clevers, 2009), the damaged cells were likely replaced.

Figure 3. Other Methods of Sleep Suppression Also Lead to ROS Accumulation in the Gut

(A) Mechanical sleep deprivation varies in efficiency across individual flies. Each panel, 6 days of locomotor activity for an individual. Vertical ticks, 5-min locomotor activity bins.

(B) Efficiency of mechanical deprivation varies across days in individuals (SL, sleep loss).

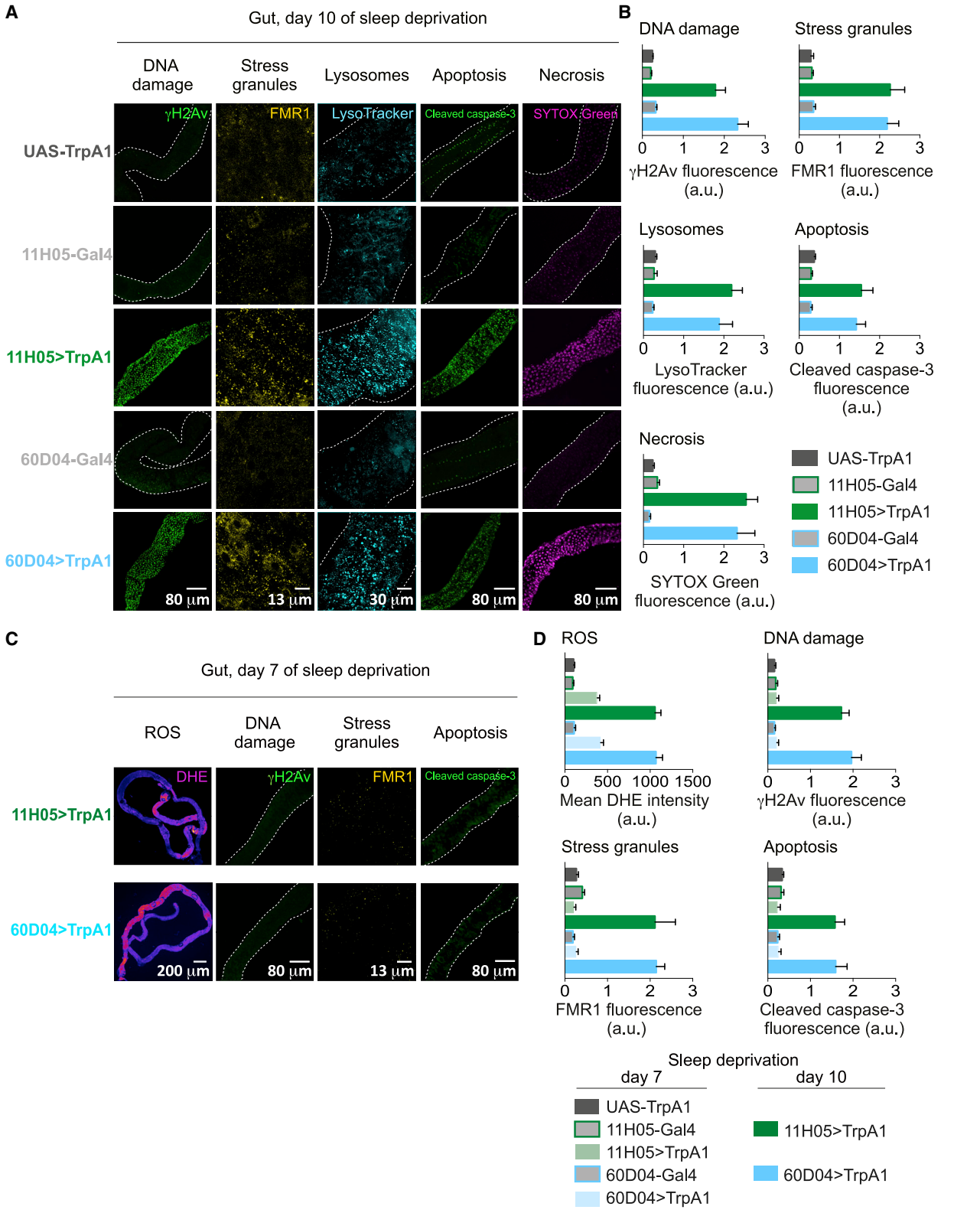
(C) Sleep rebound after 12 h of mechanical deprivation (SD) in isogenized (iso31) flies.

(D) Total amount of daily sleep in shaken (iso31 + SD) and non-shaken (iso31) flies for 6 days.

(E) Only in flies experiencing consistent (sustained) sleep loss, ROS accumulate in the gut. Day 7.

(F–M) Reduced sleep and high ROS levels in the gut of elav>CycA^{RNAi} flies (F and G) and *rye*^{T227M} (H and I), *sss*$^{\Delta 40}$ (J and K), and *inc*^{2} (L and M) mutants. Representative images. Scale bars, 200 μm. Mean and SEM.

For sample sizes and statistical analyses, see Table S1. See also Figure S2.



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The widespread cell death raised the possibility that the gut becomes structurally damaged and leaky, which we tested with a commonly used method based on addition of unabsorbable blue dye to food. If the intestinal barrier is abnormally permeable, such as in old flies, the entire body turns blue due to an open circulatory system (Rera et al., 2011). With this method, we could not detect a difference between deprived and non-deprived animals (Figure S3C), which suggests that gut permeability was not majorly altered. We noticed that the guts of sleep-deprived animals seemed to contain more food (Figure S3C). This was due not to impaired excretion (Figure S3D, right) but to increased food intake (Figure S3D, left), consistent with other sleep studies in humans and model organisms (Baud et al., 2013; Everson et al., 1989; Greer et al., 2013; Koban et al., 2008; Markwald et al., 2013; Mavanji et al., 2013; Newman et al., 2009; Rechtschaffen and Bergmann, 1995). We later present examples of sleep-deprived animals that do not eat more than controls but still have high ROS levels, which indicates that overeating is not the cause of ROS accumulation.

Sleep Deprivation Leads to ROS Accumulation and Oxidative Stress in the Mammalian Gut

We tested the generalizability of our findings in a mammalian model. With gentle but continuous mechanical stimulation, C57BL/6J mice were deprived of sleep for up to 5 days (Figure 5). In each repetition of the experiment, 5 mice were housed in a deprivation setup from Pinnacle Technology (Hines et al., 2013; Naidoo et al., 2014) where a slowly rotating bar caused them to move more frequently. As controls, 5 mice were housed in an identical setup but without the bar rotating. Sleep deprivation was confirmed with electroencephalogram (EEG)/electromyogram (EMG) (Figure 5A), although the recordings were taken in singly housed mice and might be under-representing the extent of sleep loss in group-housed mice. Group housing caused animals to disturb each other (our observation): if one mouse was awake, it likely disrupted the sleep of other mice.

Internal organs from sleep-deprived and non-deprived mice were processed in parallel; they were harvested, sliced, and probed for ROS using DHE. Compared with the non-deprived controls, sleep-deprived animals had elevated ROS levels specifically in the small and large intestines (Figures 5B and 5C). The increase in the small intestine was obvious after 2 days of deprivation, whereas the increase in the large intestine (colon) was less obvious because of the seemingly higher basal ROS levels (Figures 5B and 5C). There were no evident changes in ROS in the other examined tissues, even after 5 days (Figures 5B and 5C). Sleep-depriving a different mouse strain (CBA/CaJ) produced a similar outcome, with ROS levels high in the small and large intestines but not in the brain or liver (Figures S4A–S4C). When deprivation was stopped, mice engaged in rebound sleep (Figure S4D), supporting our earlier conclusion that ROS accumulation occurs regardless of the presence or absence of compensatory sleep pressure.

Markers of DNA damage, stress granules, and cell death were not observed after 2 days but could be clearly detected in the small and large intestines after 5 days of sleep deprivation (Figure 5D), again indicating that ROS are a cause rather than a consequence of cellular damage. The weight of sleep-deprived mice dropped by ~10% during the first 24 h of deprivation and then remained stable for the rest of the experiment (Figure S4E, left). Food intake was comparable with the controls (Figure S4E, right), suggesting that the increase in ROS levels is not caused by excessive eating. Despite the evidence mentioned earlier, food intake is not always increased during sleep restriction (Barf et al., 2012; Caron and Stephenson, 2010). As would be consistent with another study (Koban et al., 2008), the severity and length of sleep deprivation that we imposed on mice may not have been sufficient to appreciably change their appetite. Yet, these conditions were sufficient to induce ROS accumulation in the gut. We conclude that the impact of sleep loss on the gut is evolutionarily conserved and is separable from food consumption.

ROS Neutralization Extends the Survival of Sleep-Deprived Animals

Our results reveal a correlation between sleep deprivation, ROS accumulation, and premature death. To test causality, we asked whether clearing ROS could extend survival without increasing sleep itself. Through food supplementation, we tested 53 individual compounds known to have antioxidant properties, identifying 11 that allowed a normal or near-normal lifespan (Figures 6A and 6B). These included compounds that directly neutralize ROS through electron donation (e.g., lipoic acid, quercetin, and 2,2,6,6-tetramethylpiperidine 1-oxyl [TEMPO]) as well as compounds that boost the expression of endogenous antioxidant enzymes (e.g., N-acetyl-L-cysteine [NAC] and sodium phenylbutyrate [PBA]). Figure 6B shows examples of survival curves for three rescuing compounds: melatonin, lipoic acid, and NAD. The compounds that were able to rescue survival were effective at clearing ROS (Figure 6C and data not shown), unlike the non-rescuing compounds we examined (Figures S5A and S5B). Rescue of survival was achieved without increasing sleep itself; all animals remained sleep deprived when supplemented with antioxidants (if anything, average sleep was reduced further) (Figure 6D). This was true even for melatonin, primarily known for its effects on the mammalian circadian clock (Pfeffer et al., 2018). Melatonin does not seem to play a major role in controlling circadian rhythmicity or sleep in flies (Hardeland and Poeggeler, 2003). A lesser known fact is that melatonin is a potent antioxidant (Tan et al., 2015), produced at high levels in the vertebrate gut (Bubenik, 2002). In flies, the rate-limiting enzyme in melatonin biosynthesis, arylalkylamine N-acetyltransferase, is highly

Figure 4. Sleep Deprivation Causes Oxidative Stress in the Gut

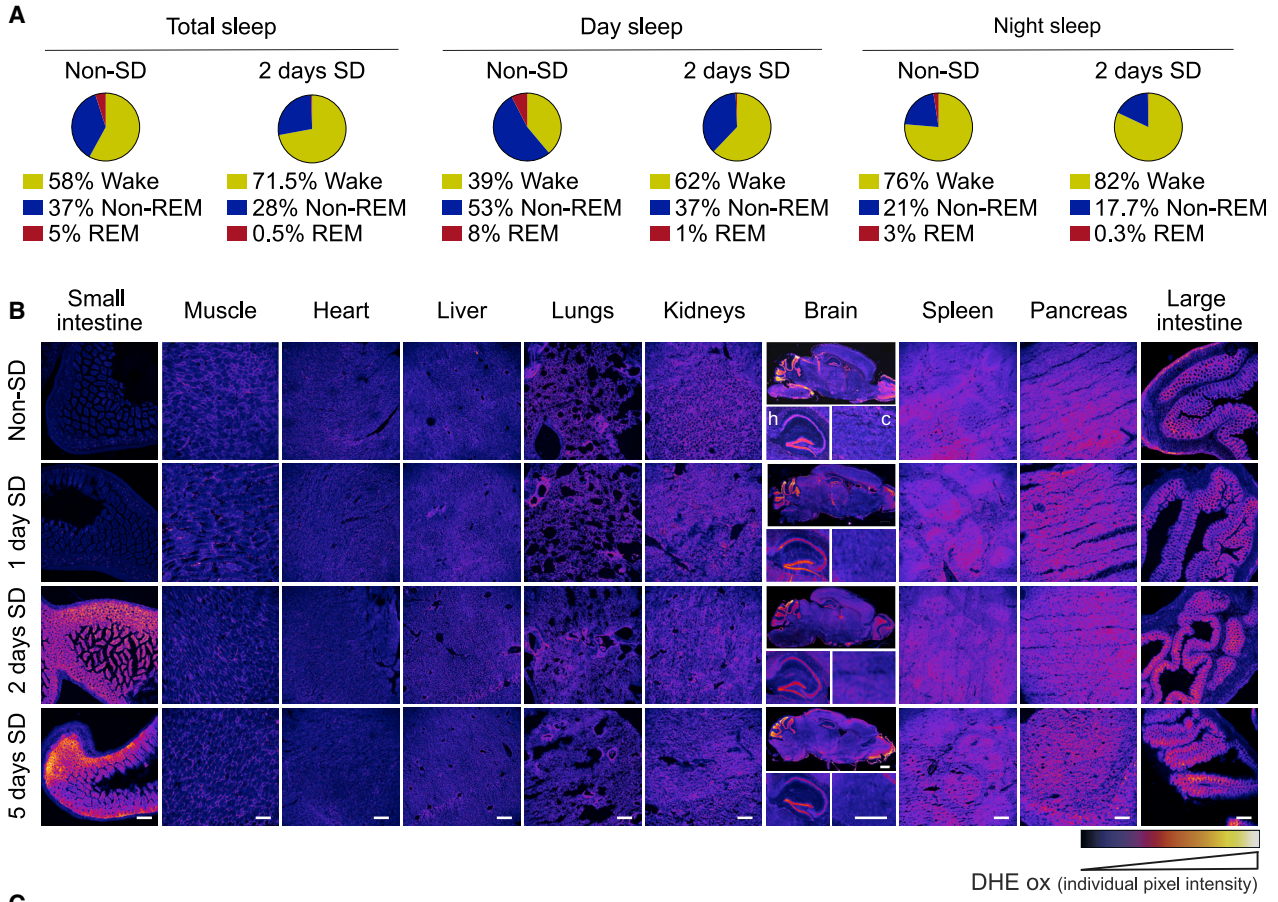
(A) Guts from sleep-deprived flies (green, blue) show high levels of oxidative stress markers. Some guts are outlined in white to help visualization.

(B) Quantification of oxidative stress markers in the guts of sleep-deprived (green, blue) and non-deprived flies.

(C) Oxidative stress markers are elevated only after 10 days of sleep deprivation, whereas ROS levels are already elevated on day 7.

(D) Quantification of ROS and oxidative stress markers in the guts of sleep-deprived (green, blue) and non-deprived flies. Mean and SEM.

For sample sizes and statistical analyses, see Table S1. See also Figure S3.



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expressed in the gut (Brodbeck et al., 1998). A recent study showed that circulating melatonin levels decrease with sleep deprivation in mice (Gao et al., 2019). In agreement with our findings, the authors found that melatonin supplementation attenuates mucosal injury and dysbiosis in the gut (Gao et al., 2019). Based on our data, this effect of melatonin could be attributed to its antioxidant properties.

Together, these results draw a direct connection between sleep deprivation-induced oxidative stress and mortality. If antioxidants can mean the difference between life and death for sleep-deprived animals, one might predict life extension under non-deprived conditions. Indeed, some groups have observed beneficial effects of antioxidants on the healthspan and lifespan of flies (Kang et al., 2002). However, supplementation with melatonin, lipoic acid, NAD, or PBA, at doses that had rescuing effects during sleep deprivation, did not affect the survival of non-deprived animals (Figure S5C). This argues for a context-dependent value of antioxidants.

Gut-Targeted Expression of Antioxidant Enzymes Prevents Oxidative Damage and Extends the Survival of Sleep-Deprived Animals

Compounds given through food easily reach gut cells, but we could not exclude the possibility that they also reach other organs and have some protective effects there. To directly test the role of the gut, we expressed antioxidant enzymes in this tissue. This required two separate transgenic expression systems: one in neurons for thermogenetic sleep deprivation and the other in the gut for enzyme expression. The LexA/LexAop system (Lai and Lee, 2006) is similar to the Gal4/UAS system (Brand and Perrimon, 1993). There are no available LexA drivers specific to the gut, whereas such Gal4 drivers exist, so we searched for LexAs to use for sleep deprivation.

After screening through ~150 sparsely expressed LexAs from the FlyLight collection (Jenett et al., 2012; Pfeiffer et al., 2010), we identified two (50A07- and 68A07-LexA) that allow long-term and consistent thermogenetic sleep deprivation (Figures 7A, 7B, S6A, and S6B). The two lines show little expression in the gut (Figure S6C). Similar to what we observed using Gal4s, sleep deprivation with LexAs lead to premature death (Figure S6D). 50A07-LexA caused nearly complete sleep loss and precipitated death faster than 68A07-LexA, which caused ~85% sleep loss (Figures 7B, S6B, and S6D). 50A07-LexA-dependent deprivation was not accompanied by increased food intake (Figure S6E), which was contrary to the Gal4-dependent sleep deprivation but similar to the mouse experiments, reinforcing the idea that excessive food consumption is not at the root of sleep deprivation-triggered lethality. We combined 50A07-LexA with two independent Gal4 drivers expressed throughout the midgut in the most prevalent cell type, nutrient-absorbing enterocytes (the widespread pattern of oxidative stress in the gut pointed to enterocytes as the most likely cell type involved). The Gal4s used were mex1-Gal4 (Phillips and Thomas, 2006; Figure 7C, left) and myo1A-Gal4 (Buchon et al., 2013; Jiang et al., 2009; Figure S7A). mex1-Gal4 shows no expression in the central nervous system (Figure 7C, left); myo1A-Gal4 has been reported not to be expressed in the brain (Takeishi et al., 2013), but with a more sensitive detection method (a brighter fluorescent reporter coupled with immunolabeling), we detected some expression there (Figure S7A; also reported by Dai et al., 2019). Because of this, and because of a previous report suggesting oxidative stress in neurons of sleep-deprived flies (Hill et al., 2018), we also attempted to rescue survival using elav-Gal4, a driver that is expressed in most neurons and largely excluded from the gut (Figure 7C, right; Chen et al., 2016). This allowed us to compare survival between sleep-deprived animals overexpressing antioxidant enzymes throughout the gut (mex1-Gal4 and myo1A-Gal4) versus the nervous system (elav-Gal4).

Gut-targeted overexpression of superoxide dismutase 1 or 2 (SOD1 and SOD2, respectively) or catalase (CAT) using mex1-Gal4 (Figure 7D, top left) or myo1A-Gal4 (Figure S7B) rescued the survival of animals sleep-deprived with 50A07-LexA. The presence of myo1A-Gal4 (and, to a lesser extent, mex1-Gal4) somewhat reduced the long-term efficiency of sleep deprivation, independent of transgene expression (i.e., sleep levels gradually increased over the lifetime in any animal that had myo1A-Gal4 [Figure S7C, top left] or mex1-Gal4 [Figure S7C, top right]) for reasons we do not understand. Regardless, during the initial 11 days (the time it took for the sleep-deprived animals with no expression of transgenic antioxidant enzymes to die), average sleep loss was only weakly affected by myo1A-Gal4 (Figure S7C, bottom left) and not affected by mex1-Gal4 (Figure S7C, bottom right). Moreover, loss of sleep throughout the lifespan was comparable between sleep-deprived flies overexpressing antioxidant enzymes with mex1-Gal4 and the sleep-deprived controls (Figure 7D, bottom left).

In contrast to gut-targeted overexpression, nervous system-targeted overexpression of antioxidant enzymes did not have a major rescuing effect, although lifespan was extended by several days (Figure 7D, top right). This is consistent with the absence of noticeable ROS accumulation in the nervous system during sleep deprivation (Figures 2B and 2C). The minor rescue obtained with elav-Gal4 did not result from an increase in sleep (Figure 7D, bottom right) and could mean that there is some contribution of the nervous system to lethality, but it could also result from the sparse elav-Gal4 expression in the gut (Chen et al., 2016).

Figure 5. ROS Accumulate in the Mouse Gut upon Sleep Deprivation

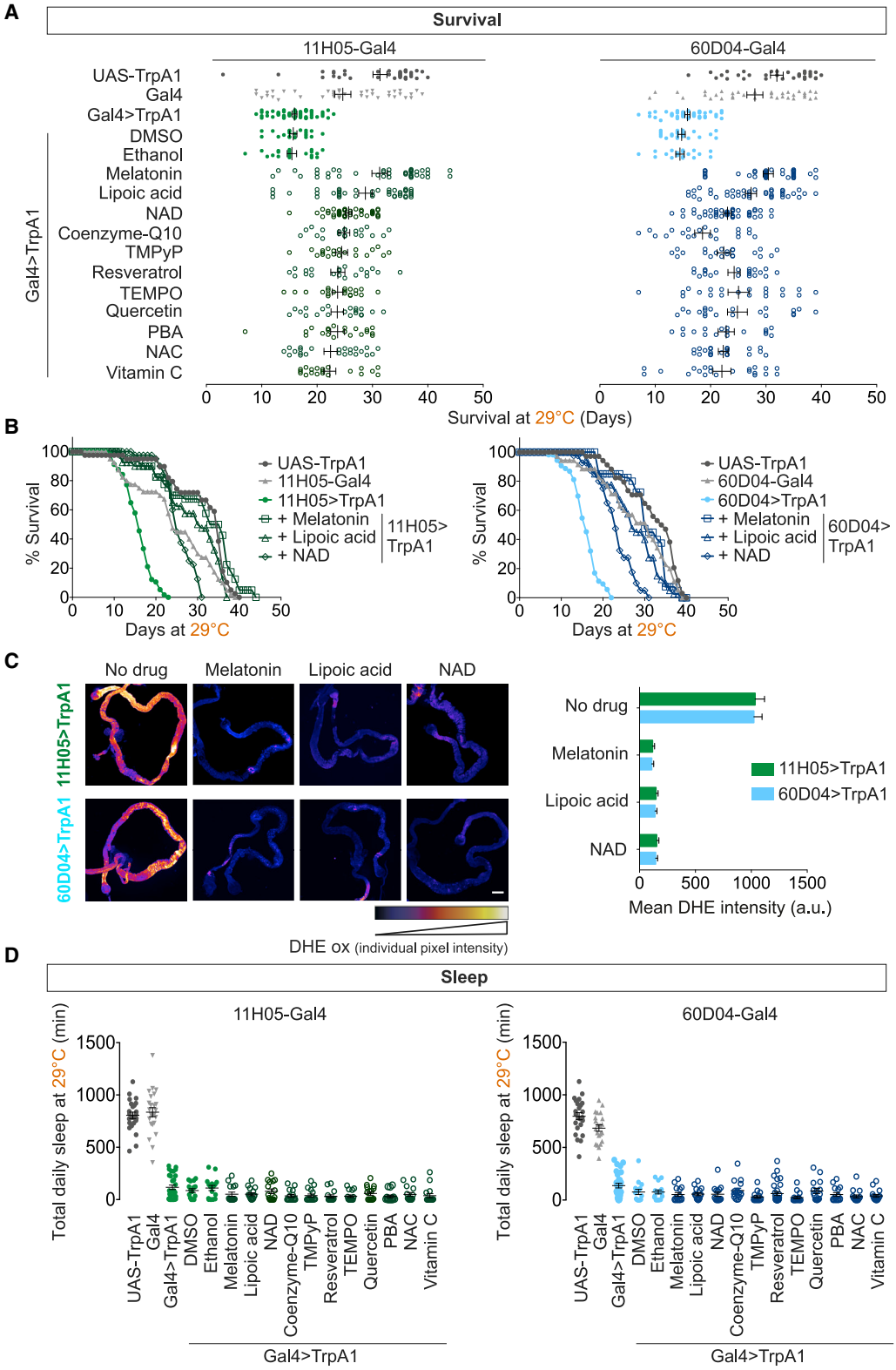
(A) Rapid eye movement (REM) sleep, non-REM sleep, and wakefulness in sleep-deprived (SD) and non-deprived (non-SD) C57BL/6J mice, reported by EEG/EMG.

(B) High ROS levels are seen in the small and large intestines after 2 days of sleep deprivation. Brain: h, hippocampus; c, cortex.

(C) Quantification of ROS levels in tissues from sleep-deprived and non-deprived mice.

(D) Markers of oxidative stress are elevated in the small and large intestine only after 5 days of sleep deprivation. Representative images. Scale bars: brain, 1 mm; other tissues, 200 μm. Mean and SEM.

For sample sizes and statistical analyses, see Table S1. See also Figure S4.



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With mex1- or myo1A-Gal4-driven overexpression of antioxidant enzymes, ROS levels in the gut were brought down to baseline (SOD1 or SOD2 overexpression) or even seemingly below (CAT overexpression) (Figures 7E, left, and S7D and S7F, left, and S7G). DNA damage and apoptosis were no longer detectable (Figures 7E, center and right, and S7E and S7F, center and right, and S7H), directly demonstrating that ROS are the cause of cellular damage in the gut of sleep-deprived flies. The efficacy of the mex1- or myo1A-Gal4-dependent rescue suggests that enterocytes are the major source of ROS in the gut upon sleep deprivation. With elav-Gal4-driven overexpression of antioxidant enzymes, ROS levels in the gut remained high (Figure S6F). When flies were sleep-deprived with the other LexA line (68A07-LexA), and SOD1, SOD2, or CAT were overexpressed in the gut using myo1A-Gal4, lifespan was also extended (Figures S6G and S6H). The results of these rescue experiments reinforce our conclusion that the gut is the main source of lethal ROS during severe sleep loss (Figure 7F).

DISCUSSION

Our study uncovered a cause of death upon experimental sleep deprivation. One caveat of this and other sleep deprivation studies in animal models is the accompanying overall increase in physical activity that could contribute to ROS production through higher energy expenditure. We do not think that this is a major factor behind the observed phenomena because sleep is not merely a period of immobility. Metabolic changes associated with normal daily sleep-wake cycles are separable from changes in locomotion (Bonnet et al., 1991; Stahl et al., 2017), and increased locomotion by itself does not seem to contribute significantly to the increased metabolic rate and energy expenditure during experimental sleep deprivation (Barf et al., 2012; Bergmann et al., 1989; Caron and Stephenson, 2010). Additionally, intense physical activity increases the levels of ROS in muscles (Powers and Jackson, 2008), but we did not detect changes in this tissue, suggesting that our observations do not stem simply from physical exhaustion. The second caveat is that we were able to study survival and demonstrate rescue only with thermogenetic deprivation because only this approach allowed consistent and sustained sleep suppression without any obvious side effects. Regardless, ROS accumulation was observed with different methods of suppressing sleep and at different temperatures. Two of these methods (thermogenetics and mutations in sleep genes) suppress sleep without triggering (or weakly triggering) an increase in homeostatic sleep pressure, but mechanical agitation robustly engages the homeostat. From this, we conclude that the physiological need for sleep can be separated from the compensatory sleep drive, consistent with the fact that other drives, such as hunger (Luquet et al., 2005), are separable from the physiological need they represent.

How does sleep deprivation lead to ROS accumulation? Why does this take place in the gut? It might be that ROS clearance is a daily sleep function, but it is also possible that sleep deprivation generates unique adverse conditions that lead to ROS accumulation. ROS levels may result from increased production, decreased elimination, or both. Prolonged wakefulness could affect the gut directly, but ROS accumulation could also be a consequence of signaling from the nervous system or other tissues.

The vast majority of ROS (~90%) are generated during mitochondrial oxygen-dependent ATP synthesis (Balaban et al., 2005), making this organelle an obvious candidate for the relevant ROS source. Metabolism increases during sleep disruption (Barf et al., 2012; Bonnet et al., 1991; Caron and Stephenson, 2010; Everson et al., 1989; Everson and Wehr, 1993; Stahl et al., 2017; Valenti et al., 2017), and metabolic regulation may have been an original sleep function. The concentration of atmospheric oxygen changed significantly several times in Earth's history, which is thought to have led to eukaryotic and multicellular life (El Albani et al., 2010; Sessions et al., 2009). Oxygen use increased the efficiency of ATP production (Stamati et al., 2011) but came with the price of having to neutralize reactive and potentially toxic oxygen derivatives (Fischer et al., 2016; Taverne et al., 2018). Entering a resting state characterized by reduced metabolism could temper ROS production by decreasing the rate of redox reactions. The gut appeared early in evolution (Stainier, 2005) and could be uniquely sensitive to metabolic perturbations. It is not clear, however, whether the gut has any special energetic demands (Aiello and Wheeler, 1995) that explain the increase in ROS seen there. Moreover, increasing mitochondrial activity in the gut can actually reduce ROS levels and extend lifespan (Rera et al., 2011). Another potential source of ROS is the endoplasmic reticulum (ER), where ROS are routinely made as byproducts of oxidative protein folding (Shimizu and Hendershot, 2009). ER stress (the accumulation of misfolded or unfolded proteins) can lead to oxidative stress, as increased demand for protein folding leads to greater ROS production; ER stress also induces mitochondria to produce more ROS (Cao and Kaufman, 2014; Santos et al., 2009; Zeeshan et al., 2016). Previous studies found that sleep-deprived brains show evidence of unfolded protein response (Brown and Naidoo, 2010; Brown et al., 2017; Hakim et al., 2015; Naidoo et al., 2005, 2007; Shaw et al., 2000), which might in turn trigger a response in the gut (Berendzen et al., 2016; Schinzel and Dillin, 2015; Taylor and Dillin, 2013; Zhang et al., 2018). Alternatively, sleep deprivation could directly induce ER stress in the gut. In both scenarios, ER stress in the gut may lead to oxidative stress in this tissue.

Figure 6. Clearance of ROS Prevents Death of Thermogenetically Sleep-Deprived Flies

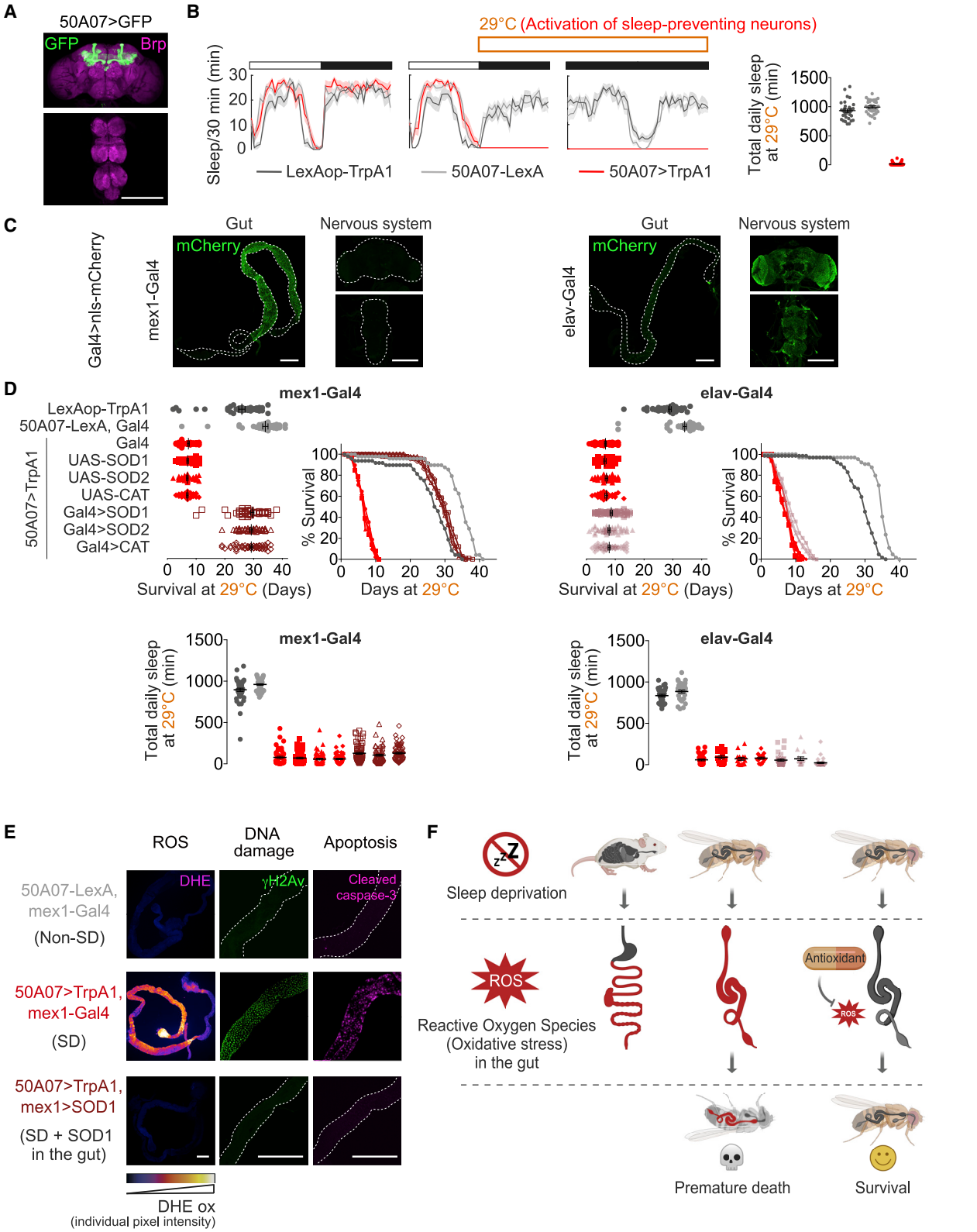
(A) Individual survival. Sleep-deprived flies (11H05>TrpA1, 60D04>TrpA1) die earlier than the parental controls (11H05-Gal4, 60D04-Gal4, UAS-TrpA1). Solvents (ethanol, DMSO) do not affect survival. Supplementation with antioxidants (melatonin–vitamin C) extends the survival of sleep-deprived flies. Mean and SEM.

(B) Example survival curves for three rescuing compounds. Error bars are omitted for clarity.

(C) ROS are cleared from the gut by the compounds that rescue survival. Day 10. Representative images. Scale bar, 200 μm. Mean and SEM.

(D) Sleep is not promoted by the rescuing antioxidants. Mean and SEM.

Survival experiments were performed 2–3 times. For sample sizes and statistical analyses, see Table S1. See also Figure S5.



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Gut homeostasis depends on physiological levels of locally -produced ROS (Campbell and Colgan, 2019; Pérez et al., 2017b) that regulate stem cell proliferation (Buchon et al., 2009a; Hochmuth et al., 2011; Jones et al., 2013; Patel et al., 2019; Wang et al., 2014; Xu et al., 2017) and function in intestinal immunity (Buchon et al., 2009a; Ha et al., 2005, 2009; Jones et al., 2013; Jones and Neish, 2017; Kim and Lee, 2014). A significant fraction of intestinal ROS are made through the action of the conserved enzymes Nox and Duox (Aviello and Knaus, 2018; Ha et al., 2005; Jones et al., 2013). Nox is hyperactivated by dysbiosis (Iatsenko et al., 2018), alteration of the gut microbial profile (Levy et al., 2017). This might be relevant because sleep deprivation reportedly affects gut microbiota (Benedict et al., 2016; El Aidy et al., 2019; Gao et al., 2019; Ma et al., 2019; Poroyko et al., 2016), though conflicting evidence exists (Zhang et al., 2017). Intestinal Nox is also activated by stressors other than dysbiosis (Patel et al., 2019), and sleep deprivation could be one such stressor. The gut has the ability to receive distress signals from other tissues (Berendzen et al., 2016; Liu and Jin, 2017; Ulgherait et al., 2014; Zhang et al., 2018) and initiate protective responses. For example, sterile external wounds can cause a ROS increase in the fly gut, which promotes enterocyte turnover and improves survival (Takeishi et al., 2013). ROS levels could similarly start increasing as a protective response in the gut during periods of insufficient sleep. Under natural conditions, animals likely fall asleep before ROS reach dangerously high levels. We consider this scenario possible because antioxidant compounds sometimes accelerated death of sleep-deprived animals (if given too early or at doses that were too high, data not shown).

ROS could accumulate not only through increased production but decreased clearance. Inadequate neutralization coupled with constant production in the gut (Campbell and Colgan, 2019; Jones and Neish, 2017; Pérez et al., 2017b) could explain gradual accumulation of these molecules. Nrf2 (CnC in flies) is a conserved master antioxidant regulator whose basal activity is higher in the fly gut than in other tissues (Sykiotis and Bohmann, 2008). A transcription factor, Nrf2 becomes stabilized and translocates into the nucleus only under oxidative conditions to induce expression of antioxidant and cytoprotective genes (Ma, 2013; Sykiotis and Bohmann, 2008; Tonelli et al., 2018). A defect in this mechanism could result in gut-biased ROS accumulation. At least several antioxidant compounds that rescued the survival of sleep-deprived flies can act on Nrf2 (Rochette et al., 2013; Vriend and Reiter, 2015), supporting the idea of potential Nrf2 dysfunction during sleep loss.

Why could we and others (Cirelli, 2006; Cirelli et al., 1999; D'Almeida et al., 1997; Eiland et al., 2002; Gopalakrishnan et al., 2004; Hipólide et al., 2002) not detect any significant oxidative damage in sleep-deprived brains? The nervous system has high energetic demands, and neuronal ATP production is almost certain to rise during prolonged wakefulness, which is expected to elevate ROS levels. The lack of damage, as proposed previously (Cirelli, 2006), might be at least partially explained by efficient activation of antioxidant and cytoprotective mechanisms in sleep-deprived brains (Brown et al., 2017; Cirelli et al., 2004, 2005b, 2006; Cirelli and Tononi, 2000; Hill et al., 2018; Jones et al., 2008; Naidoo et al., 2005; Nikonova et al., 2010; Shaw et al., 2000; Terao et al., 2003, 2006). Oxidation regulates the activity of a small group of sleep-regulating neurons in flies (Kempf et al., 2019), but it is likely critical to keep oxidation generally low in neurons because they cannot be replaced, in contrast to gut cells which undergo constant turnover (accelerated by tissue damage) (Amcheslavsky et al., 2009; Buchon et al., 2009; Jiang et al., 2009; Micchelli and Perrimon, 2006; Miguel-Aliaga et al., 2018; Ohlstein and Spradling, 2006; van der Flier and Clevers, 2009). Because the gut is plastic in terms of proliferation, recovery from oxidative damage when deprivation is stopped may rely mostly on turning cells over rather than repairing them. It will be informative to test whether sleep promotes active repair of cellular damage in the gut, similar to the way it facilitates DNA damage repair in neurons (Bellesi et al., 2016; Zada et al., 2019).

Regardless of how or why they accumulate during insufficient sleep, excessive intestinal ROS are lethal, consistent with previous reports (Ha et al., 2009; Iatsenko et al., 2018; Lee et al., 2013). The widespread cellular damage we observed was not accompanied by the breakdown of the intestinal barrier seen in older animals (Rera et al., 2011, 2012), suggesting that sleep deprivation is not simply accelerated aging. To strengthen this conclusion, other hallmarks of aging, such as intestinal dysplasia (Biteau et al., 2010; Jasper, 2020), should be examined, especially given that ROS regulate intestinal stem cell proliferation (Biteau et al., 2008; Buchon et al., 2009a; Choi et al., 2008; Hochmuth et al., 2011; Jones et al., 2013; Patel et al., 2019; Wang et al., 2014; Xu et al., 2017). Oxidative damage might interfere with the absorptive function of the gut, and the increased feeding seen during sleep loss (Baud et al., 2013; Everson et al., 1989; Koban et al., 2008; Mavanji et al., 2013; Newman et al., 2009; Rechtschaffen and Bergmann, 1995) could be an attempt to compensate for nutrient deficiency. In agreement with this

Figure 7. Overexpression of Antioxidant Enzymes in the Gut Prevents Death of Thermogenetically Sleep-Deprived Flies

(A) Expression of 50A07-LexA in the nervous system, reported by mCD8::GFP

(B) Sleep across 3 days. Raising the temperature to 29°C (orange) causes sleep loss in 50A07>TrpA1 flies (these experiments were done in darkness because the efficiency of deprivation was greater than in light-dark cycles). Mean and SEM. Right: daily sleep in controls and experimental flies across the lifespan. Mean and SEM.

(C) Expression pattern of mex1-Gal4 (left) and elav-Gal4 (right) in the gut and the central nervous system, visualized with nls-mCherry. Scale bars, 200 μm.

(D) Top: survival of sleep-deprived flies overexpressing antioxidant enzymes (SOD1, SOD2, or CAT) in the gut (mex1-Gal4, left) or the nervous system (elav-Gal4, right). Dot plot, survival for individuals flies. Mean and SEM. Curves, population survival. Error bars are omitted for clarity. Bottom: daily sleep of sleep-deprived flies overexpressing antioxidant enzymes (SOD1, SOD2, or CAT) in the gut (left) or the nervous system (right).

(E) Overexpression of SOD1 in the gut of SD flies (50A07>TrpA1, mex1>SOD1) prevents ROS accumulation and oxidative stress. Representative images. Scale bars, 200 μm. Guts are outlined for visualization. Survival experiments were performed 3 times.

(F) Graphic summary. The model was created with BioRender.

For sample sizes and statistical analyses, see Table S1. See also Figures S6 and S7.

idea, a high-calorie diet can delay death of sleep-deprived rats (Everson and Wehr, 1993).

Accumulated intestinal ROS might have systemic effects, for example by altering the gut microbial profile through direct bactericidal action or through modulation of gut immunity (Campbell and Colgan, 2019; Ha et al., 2005; Yardeni et al., 2019). Since microbiota regulate health and lifespan (Clark et al., 2015; Clark and Walker, 2018; Heintz and Mair, 2014; Smith et al., 2017; Thaiss et al., 2016), this might be a factor in early mortality. Intestinal ROS could also trigger a systemic inflammatory response independently of microbiota (Wu et al., 2012). Because ROS are important signaling molecules at physiological levels (Holmström and Finkel, 2014; Jones, 2006; Li et al., 2018; Lim et al., 2014; Mittler, 2017; Oswald et al., 2018; Owusu-Ansah and Banerjee, 2009; Schieber and Chandel, 2014), lethality could also result from disruption of the normal ROS-dependent processes.

Severe conditions were presented here, but mild deprivation also leads to ROS accumulation in the gut, albeit slower (data not shown). This means that our results are likely relevant for chronic insufficient sleep in humans, especially considering that, among the many pathologies associated with sleep restriction (Chattu et al., 2018), gastrointestinal diseases feature prominently (Ali et al., 2013; Khanijow et al., 2015; Parekh et al., 2018) and that several gastrointestinal disorders are characterized by abnormal ROS levels (Bhattacharyya et al., 2014; Campbell and Colgan, 2019; Pérez et al., 2017b). The risk of colon cancer is elevated by insufficient or untimely sleep (Schernhammer et al., 2003; Thompson et al., 2011); intestinal ROS might be tumorigenic through several mechanisms (Bhattacharyya et al., 2014), such as damaging DNA (Evans et al., 2004; Markowitz and Bertagnolli, 2009) or causing inflammation (Aviello and Knaus, 2017; Lasry et al., 2016). Inflammation is frequently observed with insufficient sleep (Irwin, 2019; Mullington et al., 2010) and is increasingly recognized as a driver of disease (Chung et al., 2009; Elinav et al., 2013; Gao and Hong, 2008; Libby, 2007; Rea et al., 2018). Inflammation could also trigger ROS production by immune cells (Mittal et al., 2014), leading to oxidation of other organs. With a longer deprivation protocol than we describe in mice, oxidative DNA damage was indeed seen, in addition to the small intestine, in the lung and the liver of rats (Everson et al., 2014). We recognize that what kills extremely sleep-deprived animals might not reflect what sleep does daily. Regardless, our findings demystify the observation that extreme sleep loss can cause death and show that it is possible to decrease the cost of forced wakefulness.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
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- EXPERIMENTAL MODELS AND SUBJECT DETAILS
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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cell.2020.04.049.

A video abstract is available at https://doi.org/10.1016/j.cell.2020.04.049#mmc2.

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AUTHOR CONTRIBUTIONS

A.V. and D.R. conceived and initiated the study. A.V., Y.K.D., K.N., E.A.P., C.L., and D.R. designed the experiments and analyzed the data. A.V., Y.K.D., K.N., E.A.P., and C.L. performed the experiments. A.V., Y.K.D., and D.R. wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS

A patent to A.V. and D.R. is pending ("A method for treating damage induced by sleep deprivation").

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fly Stocks		
11H05-Gal4	BDSC (Bloomington Drosophila Stock Center)	RRID: BDSC_45016
60D04-Gal4	BDSC	RRID: BDSC_45356
UAS-mCD8::GFP	Gift from Michael Crickmore	N/A
UAS-TrpA1	BDSC	RRID: BDSC_26263
iso31	Ryder et al., 2004	N/A
elav-Gal4; UAS-Dcr2	Gift from Nicholas Stavropoulos	N/A
UAS-CycA^{RNAi}	NIG (National Institute of Genetics)	5940R-1; FBgn0000404
iso31^{-AS}	Gift from Amita Sehgal	N/A
rye^{T227M}	Gift from Amita Sehgal	N/A
sss$^{\Delta 40}$	Gift from Amita Sehgal	N/A
w$^{1118\text{-}NS}$	Gift from Nicholas Stavropoulos	N/A
inc^{2}	Gift from Nicholas Stavropoulos	N/A
50A07-LexA	BDSC	RRID: BDSC_53538
68A07-LexA	BDSC	RRID: BDSC_61586
LexAop-mCD8::GFP	BDSC	RRID: BDSC_32203
LexAop-TrpA1	Burke et al., 2012 (via Michael Crickmore)	N/A
mex1-Gal4 (Chromosome X insertion)	Gift from Claire Thomas	N/A
elav-Gal4	BDSC	RRID: BDSC_458
myo1A-Gal4	Gift from Norbert Perrimon	N/A
UAS-nls-mCherry	BDSC	RRID: BDSC_38424
UAS-SOD1	BDSC	RRID: BDSC_33605
UAS-SOD2	BDSC	RRID: BDSC_24494
UAS-CAT	BDSC	RRID: BDSC_24621
UAS-RedStinger	Gift from Michael Crickmore	N/A
esg-Gal4	BDSC	RRID: BDSC_26816
UAS-inc^{RNAi}	Gift from Nicholas Stavropoulos	N/A
w$^{1118\text{-}RJ}$	Gift from Rob Jackson	N/A
fmn^{rec19}	Gift from Rob Jackson	N/A
LexAop-RedStinger	Gift from Michael Crickmore	N/A
Antibodies		
chicken anti-GFP	Aves	Cat# GFP-1020; RRID: AB_10000240
mouse anti-Bruchpilot	DHSB (Developmental Studies Hybridoma Bank)	Cat# nc82; RRID: AB_2314866
mouse anti-γH2Av	DHSB	Cat# UNC93-5.2.1; RRID: AB_2618077
mouse anti-FMR1	DHSB	Cat# anti-dFMR1, 5A11; RRID: AB_528252
rabbit anti-cleaved Caspase 3	Cell Signaling Technology	Cat# 9661; RRID: AB_2341188
mouse anti-γH2Ax	Cell Signaling Technology	Cat# 2577; RRID: AB_2118010
mouse anti-TIA1	Santa-Cruz	Cat# sc-166247; RRID: AB_2201545
rabbit anti-dsRed	Takara Bio	Cat# 632496; RRID: AB_10013483
Alexa Fluor 488 donkey anti-mouse	Molecular Probes	Cat# A-21202; RRID: AB_141607
Alexa Fluor 488 donkey anti-rabbit	Molecular Probes	Cat# A-21206; RRID: AB_2535792

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alexa Fluor 555 donkey anti-rabbit	Molecular Probes	Cat# A-31572; RRID: AB_162543
Alexa Fluor 568 donkey anti-mouse	Thermo Fisher Scientific	Cat# A10037; RRID: AB_2534013
Alexa Fluor 647 donkey anti-mouse	Molecular Probes	Cat# A-31571; RRID: AB_162542
Alexa Fluor 488 donkey donkey anti-chicken	Jackson ImmunoResearch Labs	Cat# 703-545-155; RRID: AB_2340375
Antioxidant Compounds		
Melatonin	Sigma-Aldrich	Cat# M5250; CAS: 73-31-4
Lipoic acid	Sigma-Aldrich	Cat# T5625; CAS: 1077-28-7
Nicotinamide adenine dinucleotide (NAD)	Sigma-Aldrich	Cat# N0632; CAS: 20111-18-6
Coenzyme-Q10	Sigma-Aldrich	Cat# C9538; CAS: 303-98-0
5,10,15,20-Tetrakis(1-methyl-4-pyridinio) porphyrin tetra(*p*-toluenesulfonate) (TMPyP)	Sigma-Aldrich	Cat# 323497; CAS: 36951-72-1
Resveratrol	VWR	CAS: 501-36-0
2,2,6,6-Tetramethylpiperidine 1-oxyl (TEMPO)	Sigma-Aldrich	Cat# 176141; CAS: 2226-96-2
Quercetin	Sigma-Aldrich	Cat# Q4951; CAS: 117-39-5
Sodium phenylbutyrate (PBA)	Sigma-Aldrich	Cat# SML0309; CAS: 1716-12-7
N-Acetyl-L-cysteine (NAC)	Sigma-Aldrich	Cat# A7250; CAS: 616-91-1
Vitamin C	Sigma-Aldrich	Cat# A5960; CAS: 50-81-7
Ebselen	Sigma-Aldrich	Cat# E3520; CAS: 60940-34-3
Glutathione	Sigma-Aldrich	Cat# PHR1359; CAS: 70-18-8
Methylene blue	Sigma-Aldrich	Cat# 28514; CAS: 122965-43-9
Other		
Agar	Moorhead	Cat# 41084
BSA	Gemini	Cat# 700-101P; CAS: 9048-46-8
C&B metabond quick adhesive cement system	Parkell	Cat# S380
Cornmeal (Degermed yellow)	Bunge	Cat# CCM 250
DAPI Fluoromount-G	SouthernBiotech	Cat# 0100-20
DHE	Sigma-Aldrich	Cat# 37291; CAS: 104821-25-2
DMSO	VWR	Cat# 97063-136; CAS: 67-68-5
Ethanol (pure)	Koptec	Cat# V1001; CAS: 64-17-5
Hydrophobic Barrier Pen	Vector Laboratories	Cat# H-4000; RRID: AB_2336517
KPL 10X Dulbeco's PBS	SeraCare	Cat# 5460-0030
Loctite 404 Instant Adhesive	Fisher Scientific	Cat# NC0619400
LysoTracker Red	Invitrogen	Cat# L12492
Microscope coverslips	Electron Microscopy Sciences	Cat# 72230-01
Microscope coverslips	VWR	Cat# 48393-251
Microscope glass slides Super Frost Plus	VWR	Cat# 48311-703
Microscope glass slides	Electron Microscopy Sciences	Cat# 63421-10
Normal goat serum	Jackson ImmunoResearch	Cat# 005-000-121; RRID: AB_2336990
O.C.T (Tissue-Tek)	Electron Microscopy Sciences	Cat# 62550-01
PFA (16%)	Electron Microscopy Sciences	Cat# 15710; CAS: 30525-89-4
Prolong Gold antifade reagent	Invitrogen	Cat# 1942345
Proprionic acid	Fisher	Cat# A258-500; CAS: 79-09-4
Sugar (premium pure cane granulated)	Domino	
Schneider's medium	GIBCO	Cat# 21720-001
SYTOX Green	Invitrogen	Cat# S7020
Tegosept	Genesee	Cat# 20-258

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Triton X-100	Amresco	Cat# M143; CAS: 9002-93-1
Vectaschield with DAPI	Vector Laboratories	Cat# H-1200; RRID: AB_2336790
Yeast torula	MP Biomedicals	Cat# 903085

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dragana Rogulja (dragana_rogulja@hms.harvard.edu).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Data are available from the corresponding author on request, and sleep analysis codes are available on github at https://github.com/CrickmoreRoguljaLabs/SleepAnalysis.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Drosophila melanogaster

All *Drosophila* stocks used in this study are listed in the key resources table. Mutant flies were compared to wild-type controls of the same genetic background. Flies were maintained on cornmeal-agar medium in 12 hours light:12 hours dark cycles (12:12 LD), unless indicated otherwise. The cornmeal-agar medium was composed of agar (7.3%, Spectrum), yeast (12.7%, MP Biomedicals), sugar (56%, Domino), and cornmeal (24%, Bunge). To make 4 L of cornmeal-agar medium, water temperature was brought to ~50°C. Agar, yeast, sugar, and cornmeal were added slowly, with constant stirring to prevent clumping. Temperature was increased until bubbles formed (around 97°C), after which the heat was turned off. Once temperature dropped to 75°C, propionic acid (100 ml) and Tegosept + ethanol (95.2 g in 367.2 ml) were added and water was adjusted to compensate for evaporation. The mix was left for 15 minutes before pouring into vials, to allow homogenization. All experiments were performed using adult males and carried out in incubators purchased from Tritech Research Inc. (standard DigiTherm model) and Percival Scientific Inc. (DR36VL model).

Mice

2-month-old male C57BL/6J (JAX 000664) and CBA/CaJ (JAX 000654) mice were housed according to standard protocols approved by the Harvard University Standing Committee on Animal Care in accordance with federal guidelines. On arrival to our animal facility, mice were housed in pathogen-free cages (max 5 per cage) under 12:12 LD cycles, at 23°C and 50% humidity. They were given standard Pico Lab Rodent Diet 20 # 5053, and water.

METHOD DETAILS

Drosophila melanogaster

Locomotor activity and sleep monitoring

Locomotor activity and sleep were tracked using commercially available *Drosophila* Activity Monitors (DAMs, TriKinetics). Individual males were placed into 65 mm-long glass tubes (TriKinetics) containing ~20 mm of cornmeal-agar food. Locomotor activity data were collected from the DAM System (TriKinetics) using DAM5M monitors, unless indicated otherwise. Each DAM5M monitor measures locomotor activity of 32 flies. 4 independent infrared beams are positioned across each tube. As a fly walks back and forth, it interrupts the infrared beams, leaving a record of when and where it moved. Total movement counts across the 4 beams were recorded and summed every minute. In experiments using mechanical sleep deprivation and RNAi against Cyclin A ($CycA^{RNAi}$), DAM2 activity monitors were used. These monitors also measure activity of up to 32 individual flies, but there is only 1 infrared beam to monitor movement, potentially under-reporting total movements. Data from DAM5M or DAM2 monitors were then analyzed using a custom MATLAB software (available on github at https://github.com/CrickmoreRoguljaLabs/SleepAnalysis). A sleep episode was defined as inactivity lasting at least five minutes, based on the original characterization of fly sleep (Andretic and Shaw, 2005; Nitz et al., 2002; Shaw et al., 2000).

For each genotype/condition, average daily sleep was calculated for the entire lifespan, unless indicated otherwise. Locomotor activity data were collected at either 25°C (*redeye* (rye^{T227M}), *sleepless* ($sss^{\Delta 40}$), *insomniac* (inc^{2}) and $fumin^{rec19}$ (*fmn*) mutants, *insomniac* RNAi (inc^{RNAi}) and mechanical sleep deprivation); 27°C (RNAi against Cyclin A ($CycA^{RNAi}$)), or 29°C (Thermogenetic deprivation).

Glass tubes were replaced every 2-3 days to avoid food desiccation. For antioxidant feeding assays, the antioxidant compounds were added to food at concentrations listed below.

Sleep deprivation

Thermogenetic sleep deprivation was performed using the Gal4/UAS and LexA/LexAop systems. Flies were raised in 12:12 LD cycles at 21°C until day 4-5 post-eclosion, after which males were loaded into TriKinetics glass tubes and placed in DAM5M monitors. After one day of baseline sleep recording at 21°C, temperature was raised to constant 29°C, which triggered sleep deprivation. Deprivation was done in 12:12 LD cycles, except for the experiments involving the LexA/LexAop system – these were done in constant darkness because deprivation using the LexA lines was more consistent in the absence of light.

Mechanical sleep deprivation was performed at 25°C using a multi-tubes vortexer (VWR) modified by TriKinetics to house DAM2 activity monitors. Flies were raised at 25°C (12:12 LD) until day 4-5 post-eclosion, when males were loaded into tubes. After a day of baseline sleep recording, the multi-tubes vortexer delivered 2 s-long vibrations at random intervals centered around 60 s (±30 s). The intensity of the vortexer was set to 7.

Sleep rebound was measured after one night (12 hours, ZT12-ZT24) of mechanical sleep deprivation. Rebound sleep (Δ sleep) was calculated for each fly by subtracting the amount of sleep on the morning before deprivation (ZT0-ZT4) from the amount of sleep on the morning (ZT0-ZT4) following the deprivation.

Lifespan assay

Survival was measured on cornmeal-agar medium. In all survival experiments, males were transferred to fresh vials or tubes every 2-3 days, without using CO_2. Each survival experiment was performed at least twice. For thermogenetic sleep-deprivation experiments, crosses were kept at 21°C (12:12 LD). Newly eclosed flies were collected every two days and kept at 21°C (12:12 LD) until day 4-5 post-eclosion. Males were then transferred to 29°C under either 12:12 LD cycles (for deprivation experiments using Gal4/UAS) or to constant darkness (for deprivation experiments using LexA/LexAop). For *CyclinA* RNAi (*CycA*^{RNAi}) experiments, crosses were kept at 25°C (12:12 LD). Newly eclosed males (day 0-1 post-eclosion) were transferred to 27°C (12:12 LD), as in the original publication. *redeye* (*rye*^{T227M}), *sleepless* (*sss*$^{\Delta 40}$), *insomniac* (*inc*^{2}) and *fumin*^{rec19} (*fmn*) mutants, as well as crosses of *insomniac* RNAi (*inc*^{RNAi}) were maintained at 25°C (LD 12:12). Newly eclosed males (0-1 days post-eclosion) were collected and kept at 25°C (LD 12:12) until death. For genetic rescue of LexA-dependent thermogenetic sleep deprivation and for *redeye* (*rye*^{T227M}) and *sleepless* (*sss*$^{\Delta 40}$) mutants, single flies were loaded into TriKinetics glass tubes and placed in TriKinetics DAM2 or DAM5M monitors to assess survival (only locomotor data from DAM5M monitors were used for calculating sleep amount, due to better resolution. Death was determined on the day when locomotor activity stopped permanently, as evident by locomotor activity recordings. At least 15 males per genotype per experiment were tested. In all the other survival experiments, males were placed into standard fly vials. At least 3 vials per genotype per experiment were used, with 10-20 animals per vial. We initially tested if survival depended on the number of animals per vial (10 versus 15 versus 20) and on whether the flies were grouped in vials or placed individually in TriKinetics glass tubes, and did not observe any difference (data not shown). Survival with antioxidant feeding was measured as described above, but with single flies in TriKinetics glass tubes. At least 10 males per genotype per condition per experiment were tested. Flies were counted as dead if they failed to move (e.g., leg movements) in response to taps on the side of the vials or tubes.

Antioxidant Feeding

Individual antioxidant compounds were diluted in the appropriate solvent (H_2O, DMSO or EtOH) and added to the cornmeal-agar food. Animals were put on this food after 6 complete days at 29°C (Day 12 post-eclosion, Figures 6 and S5), and transferred to fresh tubes every 2-3 days (without using CO_2) to avoid compound degradation and food desiccation. Antioxidants were used at the following final concentrations: Coenzyme-Q10, 1 mM (in EtOH); Ebselen, 1 mM (in DMSO); Glutathione, 5 mM (in DMSO); Lipoic acid, 2 mM (in EtOH); Melatonin, 100 μg/ml (in EtOH); Methylene blue, 5 mM (in H_2O); NAC, 20 mM (in H_2O); NAD, 10 mM (in DMSO); PBA 20 mM (in H_2O); Quercetin 5 mM (in DMSO); Resveratrol, 10 mM (in DMSO); TEMPO, 10 mM (in DMSO); TMPyP, 10 mM (in H_2O); Vitamin C, 40 mM (in H_2O).

Food intake measurement in thermogenetically sleep-deprived flies

After 5 days (Gal4-dependent sleep deprivation) or 4 days (LexA-dependent sleep deprivation) at 29°C, males were transferred from standard cornmeal-agar medium to otherwise identical media containing 2% (wt/vol) FD&C Blue #1. 24 hours later, feeding on blue food was interrupted by freezing the vials at −20°C. Frozen flies were transferred to Eppendorf tubes (5 males per tube), decapitated (to prevent the eye pigment from interfering with the subsequent measurement), and homogenized with a motorized pestle (VWR) in 50 μL 1X-PBS + 1% Triton X-100. Samples were centrifuged to clear the debris and the absorbance of the supernatant was measured at 660 nm (A660) on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Flies fed regular non-blue food were used as controls, and their A660 values were subtracted from experimental values. Serial dilutions of the dye were used to generate a standard curve. The dye concentration of each sample was determined using the linear fit of the standard curve. For each genotype, a minimum of 15 males were tested.

Smurf assay

FD&C blue #1 dye was added at a concentration of 2.5% (wt/vol) to standard cornmeal-agar medium. Males were maintained on dyed medium from the first day of sleep deprivation until death. Smurf flies are characterized by blue coloration present outside the digestive tract. We did not observe any Smurf phenotypes in sleep-deprived flies : the blue coloration stayed restricted to the gut.

Gut Immunohistochemistry

Adult males were anesthetized on ice and briefly washed with 70% ethanol before tissue dissection in 1X-PBS (Phosphate Buffered Saline). Guts were fixed for 1 hour on a shaker at room temperature in fixative containing 4% paraformaldehyde (PFA) in Phosphate-Buffered Saline (PBS). Samples were then washed 3 times for 20 minutes each in PBS, and incubated overnight in PBS/0.5% Triton X-100 + 2% BSA, at 4°C. Staining with primary antibodies was carried out for ~24 hours at 4°C in PBS/0.5% Triton X-100 + 2% BSA. Samples were then washed 3 times for 20 minutes in PBS/0.5% Triton X-100, and incubated for 2 hours at room temperature with secondary antibodies diluted in PBS/0.5% Triton X-100 + 2% BSA. Three 20-minute final washes were performed in PBS. Guts were mounted between glass slides and coverslips (Electron Microscopy Sciences) using either Vectashield Antifade Mounting medium with DAPI (Vector Laboratories) in the case of anti-FMR1 staining, or Prolong Gold Antifade mounting medium (Invitrogen) for other stainings. All samples were imaged on a Leica SP8 confocal microscope (as described below).

Primary antibodies used were: rabbit anti-cleaved caspase 3 (1:100, Cell Signaling Technology), mouse anti-FMR1 (1:60, DSHB), chicken anti-GFP (1:1000, Aves), mouse anti-γH2Av (1:40, DSHB), rabbit anti-dsRed that recognizes mCherry (1:100, Takara Bio). Secondary antibodies used were: Alexa Fluor 488 donkey anti-mouse, Alexa Fluor 488 donkey anti-rabbit, Alexa Fluor 555 donkey anti-rabbit, Alexa Fluor 568 donkey anti-mouse, Alexa Fluor 568 donkey anti-rabbit (Invitrogen Molecular Probes, 1:1000); Alexa Fluor 488 donkey anti-chicken (Jackson ImmunoResearch Labs, 1:100).

Brain Immunohistochemistry

Adult males were anesthetized on ice and briefly washed with 70% ethanol before dissection in PBS. Dissected brains were fixed for 25 minutes on a shaker at room temperature, in fixative containing 4% PFA in PBS. Samples were then washed 3 times for 20 minutes each in PBS/0.2% Triton X-100 and incubated overnight in PBS/0.2% Triton X-100 + 2% BSA at 4°C. Staining with primary antibodies was carried out for ~48 hours at 4°C in PBS/0.2% Triton X-100 + 2% BSA (~72 hours when Bruchpilot staining was included). Samples were then washed 3 times for 20 minutes each in PBS/0.2% Triton X-100, and incubated for ~48 hours with secondary antibodies diluted in PBS/0.2% Triton X-100 + 2% BSA. Finally, three 20-minute washes were performed using PBS/0.2% Triton X-100 followed by a 20-minute wash in PBS. Brains were mounted between glass slides and coverslips (Electron Microscopy Sciences) in Prolong Gold Antifade mounting medium (Invitrogen), and imaged on a Leica SP8 confocal microscope (as described below).

Primary antibodies used were: chicken anti-GFP (1:1000, Aves), mouse anti-Bruchpilot (1:7, DSHB), rabbit anti-dsRed that recognizes mCherry (1:100, Takara Bio). Secondary antibodies used were: Alexa Fluor 488 donkey anti-chicken (Jackson ImmunoResearch Labs, 1:100); Alexa Fluor 488 donkey anti-mouse, Alexa Fluor 568 donkey anti-rabbit, Alexa Fluor 568 donkey anti-mouse, Alexa Fluor 647 donkey anti-mouse, (Invitrogen Molecular Probes, 1:1000).

Expression Patterns of Gal4 and LexA Lines

Expression patterns of 11H05-Gal4, 60D04-Gal4, 50A07-LexA, and 68A07-LexA in the nervous system were visualized with a membrane-targeted GFP, mCD8::GFP (Lee and Luo, 1999), stained with anti-GFP antibodies. Antibodies against Bruchpilot (Brp), a presynaptic cytoskeletal protein (Wagh et al., 2006), were used to visualize the nervous system. The expression of these lines in the gut was visualized with the bright nuclear fluorescent reporter RedStinger (Barolo et al., 2004) that did not require antibody staining.

Expression patterns of mex1-Gal4, myo1A-Gal4 and elav-Gal4 in the gut and the nervous system were visualized with nls-mCherry (Caussinus et al., 2008) (stained with anti-dsRed antibodies that recognize mCherry). We used nls-mCherry here because expression of RedStinger or nls-GFP in the whole nervous system seemed to be toxic to the flies, and we wanted the same marker in the gut and the brain for direct comparison.

ROS imaging

In situ ROS detection was performed using either dihydroethidium (DHE) (Sigma-Aldrich) or 2′,7′-dichlorofluorescein (H2DCF) (Sigma-Aldrich) following previously described protocols (Owusu-Ansah et al., 2008; Rera et al., 2011). For DHE staining, flies were anesthetized on ice and dissected in Schneider's insect medium (GIBCO). Whole tissues were then incubated on a shaker at room temperature with 60 μM DHE for 7 minutes in the dark. Before mounting, samples were washed three times for 5 minutes in Schneider's medium and once in PBS for 5 minutes, using a shaker at room temperature. For H2DCF, flies were anesthetized using CO_2 and dissected in PBS. Tissues were then incubated on a shaker at room temperature with 10 μM H2DCF for 15 minutes in the dark. Before mounting, samples were washed three times for 5 minutes each in PBS using a shaker at room temperature.

Samples from each independent experiment were mounted between glass slides and coverslips (Electron Microscopy Sciences) using Vectashield Antifade Mounting medium with DAPI (Vector Laboratories) and immediately imaged with identical settings on a Leica SP8 confocal microscope (as described below).

LysoTracker staining

Lysosomal foci were detected using LysoTracker (Invitrogen) following a protocol from Ulgherait et al. (2014). Flies were anesthetized on ice and dissected in cold PBS. Tissues were then incubated for 1.5 minutes, protected from light, in 1 μM LysoTracker Red at room temperature on a shaker. Before mounting, five 30-s washes in PBS were performed at room temperature on a shaker. Samples were mounted between glass slides and coverslips (Electron Microscopy Sciences) using Vectashield Antifade Mounting medium with DAPI (Vector Laboratories), and imaged immediately with the same intensity settings on a Leica SP8 confocal microscope (as described below).

SYTOX Green necrosis assay

Necrosis was detected with SYTOX Green nucleic acid stain (Invitrogen) following a protocol from Lee et al. (2016). Flies were anesthetized on ice and dissected in cold PBS. Guts were incubated in 1 μM final concentration of SYTOX Green and 4% PFA for 40 minutes at room temperature on a shaker. Tissues were then washed three times for 20 minutes with PBS at room temperature on a shaker, and mounted between glass slides and coverslips (Electron Microscopy Sciences) using Vectashield Antifade Mounting medium with DAPI (Vector Laboratories). Samples were imaged on a Leica SP8 confocal microscope (as described below).

Fluorescent microscopy

Images were acquired with a Leica SP8 confocal microscope. A minimum of 7 samples per genotype was scored for each experiment and at least 2 independent experiments were performed. Laser, filter and gain settings remained constant within each independent experiment. Channels were scanned sequentially using these objectives: 10x for DHE (ROS) imaging and the expression pattern of mex1-Gal4, elav-Gal4 and myo1A-Gal4 in the whole fly gut (nls-mCherry; Figures 7C and S7A); 20x oil for all other expression patterns, γH2Av, cleaved caspase-3 and SYTOX Green imaging; 40x oil for FMR1 and LysoTracker imaging. Confocal Z stacks were analyzed using Fiji, maximum projections are shown.

Image quantification

Image quantification was performed using Fiji. For quantification of total ROS levels, pixel intensities of Z stacks (sum slices) were used. The mean of the summed DHE or H2DCF intensities averaged from each tissue was used for statistical analysis. For quantification of immunostainings, pixel intensities of Z stacks (sum slices) were used. The mean of the summed pixel intensities averaged from each tissue and normalized to the area of selection was used for statistical analysis.

Mice

Sleep deprivation

For continuous sleep deprivation, mice were placed into a restriction chamber (Pinnacle Technology 9000-K-S) in which a rotating bar, placed at short distance above the cage floor, is kept under constant but gentle motion to limit sleep. The bar was programed to move at speed 7 and to alternate between clockwise and counter-clockwise rotations. Age-matched control non-deprived mice were placed into a similar-sized chamber but without the bar rotating. Control and sleep-restricted mice were housed on autoclaved corncob bedding (8B Bed-o'Cobs 1/8'' 1.25 cu ft, Scott Pharma Inc% # 4B) where they had *ad libitum* access to food (Pico Lab Rodent Diet 20 # 5053) and water. For each condition (control and sleep deprived), 5 males were placed in each cage 48 hours before the beginning of the sleep deprivation protocol to allow for acclimation. For each independent experiment, one mouse was taken out from each cage after 1, 2, and 5 days of sleep deprivation for dissections. At least 2 independent experiments were performed for each mouse strain and for each tissue analyzed. Mouse weights were recorded over the deprivation period. Each day, the same amount of food was provided to the sleep-restricted and non-restricted groups.

Sleep monitoring and scoring

To monitor sleep, a single male was placed in a restriction chamber (Pinnacle Technology 9000-K-S) equipped with a 3-channel tethered EEG/EMG system (Pinnacle Technologies 8200-K1-iSL). Head-mounted preamplifiers (Pinnacle Technologies 8201) were implanted surgically. For electroencephalography/electromyography (EEG/EMG), mice were anesthetized with isoflurane and mounted onto a stereotactic apparatus (Kopf) for surgery. The head was shaved, a 1.5 cm rostral-caudal incision was made starting from approximately 3.5 mm anterior of bregma, and the EEG/EMG headmount was adhered onto the surface of the dry skull with Loctite 404 Instant Adhesive (Fisher Scientific #NC0619400). Screws (Pinnacle Technologies 8209, 8212) were drilled into the skull at the corresponding holes in the headmount for hippocampal and cortical EEG recordings. To insert the EMG wires, small pockets in the nuchal muscles were made. C&B Metabond Adhesive Luting Cement (Parkell) was applied to the headmount to protect and insulate EEG/EMG leads. The skin around the headmount was sutured and mice were allowed to recover for at least a week. Implanted mice were tethered to the 3-channel EEG/EMG system in the restriction chamber and allowed to acclimate for 2 days before recording. Recordings were acquired in standard conditions for 48 hours with no rotation of the bar, followed by 48 hours with the bar rotating (and then by 24 hours with no rotation of the bar for rebound experiments).

Quantification of Sleep Restriction

Sleep recordings were scored in 10-s epochs over 48 hours of normal sleep and sleep restriction, with the sleep analysis software Sirenia Sleep Pro. Analysis was semi-automated using both threshold scoring and cluster scoring. Non-REM EEG epochs were scored as low frequency (0.5-4 Hz range), high amplitude (+/− 150-250 μV) delta waves with low-amplitude EMG waves. REM EEG epochs contained mixed frequencies with predominantly theta waves (6-9 Hz range), low amplitude (+/− 50-100 μV) and flat EMG waves. Wake epochs were scored as high frequency (8-50 Hz), low amplitude (+/− 50-100 μV) EEG waves with high-amplitude EMG. Automated scored epochs were visually inspected to ensure that each epoch was scored according to our definitions of wake, non-REM, and REM, and epochs that were not scored by the set rules were manually scored. Epochs that contained movement artifacts were removed.

Sleep rebound was measured after 2 consecutive days of sleep deprivation. Rebound sleep was calculated by subtracting the amount of sleep (REM or NREM) on the day before deprivation from the amount of sleep on the day following deprivation.

ROS measurement

Control and sleep-deprived mice were sacrificed at 1, 2, or 5 days after the onset of sleep restriction. Tissues (small intestine, quadriceps muscle, heart, lungs, liver, kidneys, brain, large intestine, spleen, pancreas) were collected and placed in room temperature

PBS before embedding in O.C.T (Tissue-Tek). Embedded tissues were immediately placed on dry ice and sectioned on a Leica cryostat within 6 hours post-dissection. 30 μm sections were mounted on microscope glass slides (VWR) and incubated for 30 minutes at 37°C with 10 μM DHE (Sigma-Aldrich). Samples were washed three times for 5 minutes each with PBS before mounting. Sections from each independent experiment were mounted between glass microscope slides and coverslips (VWR) with DAPI Fluoromount-G (Southern Biotech), and imaged immediately with identical settings on a Leica SP8 confocal, or Olympus VS120 slide scanner (brain sections only).

Immunohistochemistry

Control and sleep-deprived mice were sacrificed 2 or 5 days after the onset of sleep restriction. Mice were perfused with ice-cold PBS followed by 4% PFA. The small and large intestines were dissected, post-fixed for 24 hours in 4% PFA followed by 20% sucrose for cryoprotection, and embedded in O.C.T (Tissue-Tek). Embedded tissues were placed at −80°C until sectioning. 30 μm tissue sections were obtained using a Leica cryostat and mounted on a microscope glass slide (VWR). Sections were permeabilized for 30 minutes in PBS/0.2% Triton X-100 and incubated for 2 hours in PBS/0.2% Triton X-100 + 5% normal goat serum (Jackson ImmunoResearch Labs). Samples were then immunostained for 48 hours with primary antibodies at 4°C in PBS/0.2% Triton X-100 + 5% normal goat serum. Three 20-minute washes were performed with PBS/0.2% Triton X-100 before incubation with secondary antibodies for 2 hours in PBS/0.2% Triton X-100 + 5% normal goat serum at room temperature. Three final 20-minute washes were performed with PBS/0.2% Triton X-100, after which samples were mounted between glass microscope slides and coverslips (VWR) with DAPI Fluoromount-G (Southern Biotech), and imaged on a Leica SP8 confocal microscope (as described below).

Primary antibodies used were: rabbit anti-cleaved caspase-3 (1:100, Cell Signaling Technology), mouse anti-γH2Ax (1:500, Cell Signaling Technology), mouse anti-TIA1 (1:300, Santa Cruz). Secondary antibodies used were: Alexa Fluor 488 donkey anti-mouse, Alexa Fluor 555 donkey anti-rabbit (Invitrogen Molecular Probes, 1:500).

Fluorescent microscopy

Samples were visualized and images acquired with either a Leica SP8 confocal microscope (all tissues except the brain for DHE, and all immunostainings) or an Olympus VS120 slide scanner (brain sections only). A minimum of 12 samples per tissue was scored for each independent experiment and at least 2 independent experiments were performed for each tissue. Laser, filter and gain settings remained constant within each independent experiment, and channels were scanned sequentially using a 10x objective. Confocal Z stacks and whole slide scanner images were analyzed using Fiji.

For quantification of total ROS levels, pixel intensities of Z stacks (sum slices) were used for all tissues except the brain (see below). The mean of the summed DHE intensities from each sample was used for statistical analysis.

For ROS measurement in the brain, whole slide scanning was used and quantification was performed using pixel intensities of the fluorescent signal. The mean DHE intensity from each sample was used for statistical analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Graphpad Prism software 7 (GraphPad Software Inc.), except for bootstrap analysis of median survival (adjusted for multiple comparisons with Bonferroni's method) that was done using a custom MATLAB software program. Data are presented as mean and SEM unless indicated otherwise. Please see Table S1 for detailed statistical analysis of each figure, including sample sizes and tests used.

CellPress

Supplemental Figures

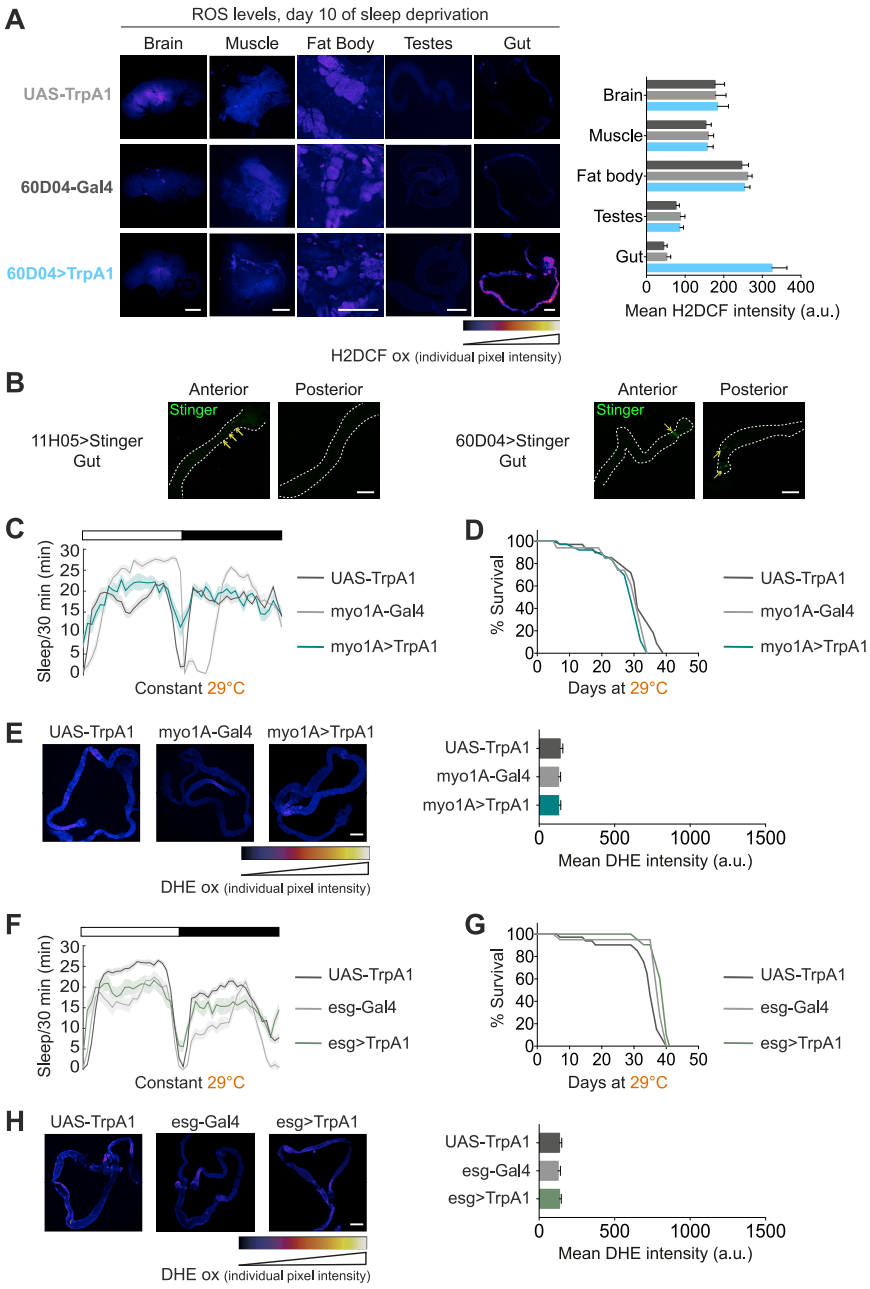

Figure S1. A Second Fluorescent Reporter Also Indicates a ROS Increase in the Gut When Sleep Is Restricted; Expression of TrpA1 in the Gut Does Not Cause ROS Accumulation, Related to Figures 1 and 2

(A) Oxidized H2DCF (H2DCF ox) reports an increase in ROS levels in the gut of sleep-deprived flies (blue) relative to the parental controls on day 10 of sleep deprivation. Mean and SEM. (B) Expression of 11H05-Gal4 and 60D04-Gal4 in the gut, visualized with a nuclear fluorescent reporter RedStinger. The two Gal4s label few cells in the anterior and posterior midgut (arrows). Guts outlined in white to help visualization. (C)-(H) Expression and activation of TrpA1 in different gut cell populations (enterocytes (myo1A-Gal4, see also Figure S7) or intestinal stem cells and enteroblasts (esg-Gal4)) does not affect sleep (C) and (F), mean and SEM (shading)); longevity (D) and (G), error bars omitted for clarity); or ROS levels (E) and (H), mean and SEM). Representative images. All scale bars, 200 μm. All survival experiments were performed twice. Sample sizes and statistics, Table S1.

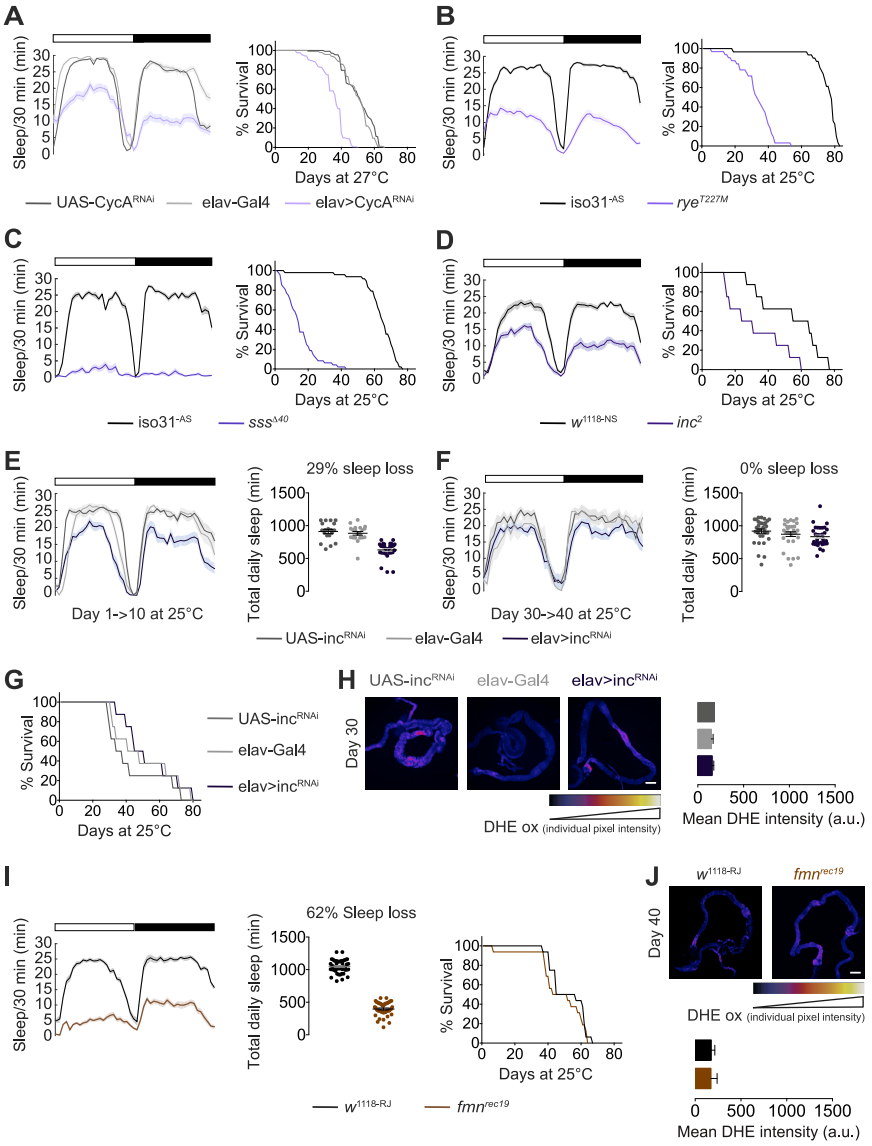

Figure S2. Sleep Reduction and Shortened Lifespan in elav>CycA^{RNAi} Flies and *rye*^{T227M}, *sss*$^{\Delta 40}$, and *inc*^{2} Mutants; Sleep Loss Is Not Sustained in elav>inc^{RNAi} Animals; *fmn* Mutants Are Sleep-Deprived but Show No ROS Accumulation and Have Normal Lifespan, Related to Figures 1, 2, and 3

(A)-(D) Reduced daily sleep and decreased lifespan in elav>CycA^{RNAi} flies (A), *rye*^{T227M} mutants (B), *sss*$^{\Delta 40}$ mutants (C) and *inc*^{2} mutants (D). Sleep plots, mean and SEM (shading); survival plots, error bars omitted for clarity. (E), (F) Sleep loss is not sustained in flies expressing *inc*^{RNAi} in neurons (elav>inc^{RNAi}). Mean and SEM. (G), (H) Survival (error bars omitted for clarity) and ROS levels (mean and SEM) are normal in elav>inc^{RNAi} flies. (I), (J) *fmn* mutants experience severe sleep loss (mean and SEM) but show no ROS increase (mean and SEM) in the gut and do not die prematurely (error bars omitted for clarity). Representative images are shown. Scale bars, 200 μm. All survival experiments were performed 2-3 times. Sample sizes and statistics, Table S1.

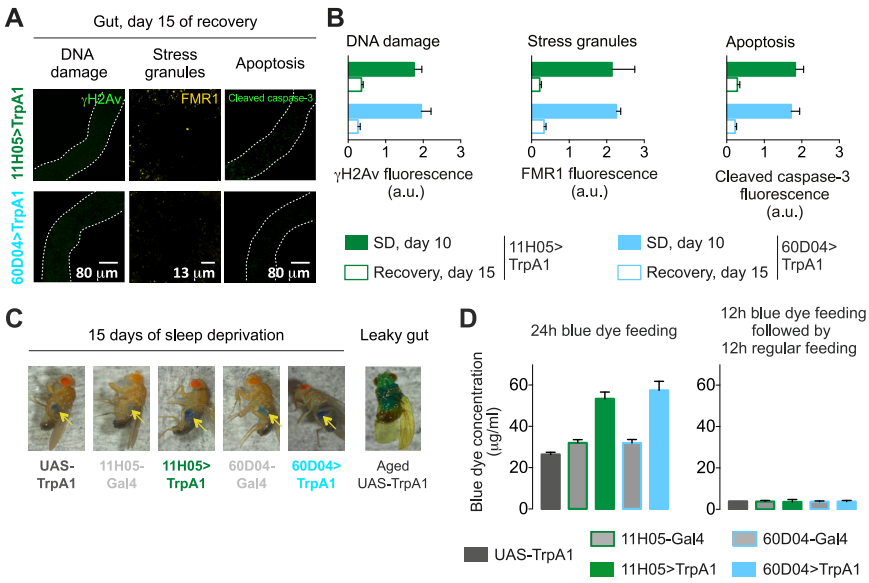

Figure S3. Oxidative Stress in the Gut Decreases When Sleep Deprivation Is Stopped; Gut Permeability Is Not Majorly Altered upon Sleep Deprivation, Related to Figure 4

(A), (B) Levels of oxidative stress markers in the gut decrease after 15 days of recovery from sleep deprivation (SD). (C) The gut does not become leaky upon sleep deprivation (i.e., absence of a "Smurf" phenotype). Aged UAS-TrpA1 fly used as positive control (the gut becomes leaky with age). (D) Left, daily food intake increases in sleep-deprived flies (11H05>TrpA1 and 60D04>TrpA1) compared to the parental controls (11H05-Gal4, 60D04-Gal4, UAS-TrpA1). Right, blue dye almost completely cleared from the gut of both sleep-deprived (11H05>TrpA1 and 60D04>TrpA1) and non-deprived (11H05-Gal4, 60D04-Gal4, UAS-TrpA1) flies after 12 hours of feeding with regular (non-dyed) food. Representative images. Some guts are outlined in white to help visualization. Mean and SEM. Sample sizes and statistics, Table S1.

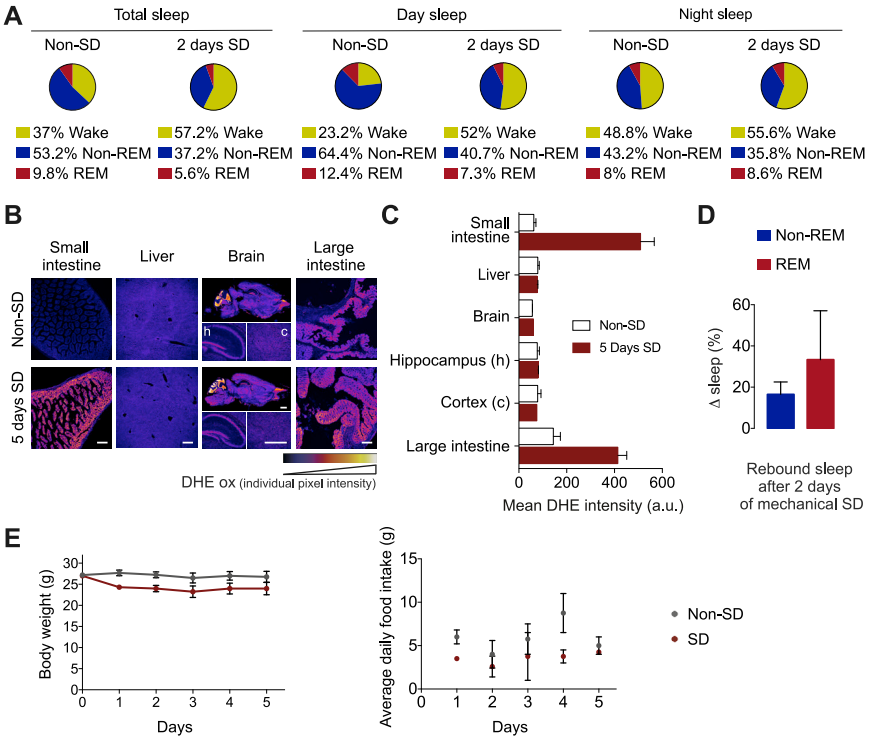

Figure S4. Sleep Deprivation Using a Second Mouse Strain Also Leads to ROS Accumulation in the Small and Large Intestines, Related to Figure 5

(A) REM sleep, Non-REM sleep, and wakefulness in sleep-deprived (SD) and non-deprived (Non-SD) CBA/CaJ mice, as reported by EEG/EMG recordings. (B), (C) DHE ox reports high levels of ROS in the small and large intestines of sleep-deprived CBA/CaJ mice. Brain: h, hippocampus; c, cortex. Representative images. Scale bars, brain, 1 mm; other tissues, 200 μm. (D) When deprivation is stopped (here, after two days), sleep rebound (Non-REM and REM) is observed. (E) Body weight (left) and food intake (right) of sleep deprived and non-deprived mice. Mean and SEM. Sample sizes and statistics, Table S1.

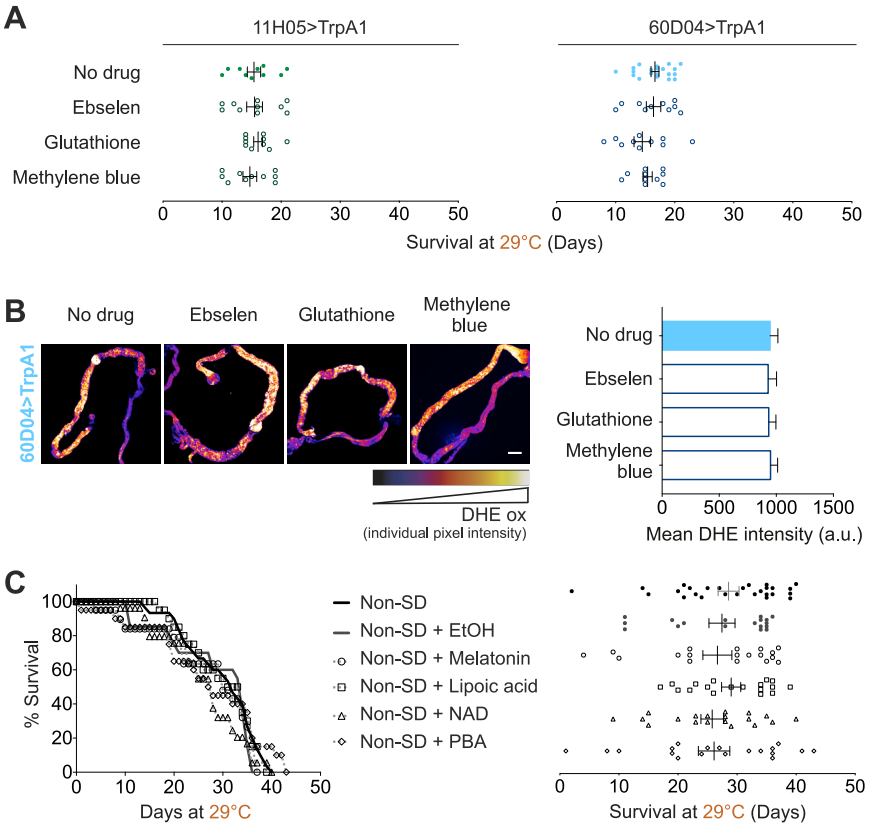

Figure S5. Examples of Antioxidant Compound Treatments that Fail to Extend Survival and to Reduce ROS Levels in the Gut, Related to Figure 6

(A) Example survival data for antioxidant treatments that failed to rescue survival of sleep-deprived flies (all such compounds were tested at several concentrations). Mean and SEM. (B) ROS levels remain high in the guts of sleep-deprived flies treated with the non-rescuing antioxidants. Representative images. Scale bar, 200 μm. Mean and SEM. (C) No lifespan extension with antioxidants in non-deprived (UAS-TrpA1) flies. Left, population survival (error bars omitted for clarity); right, survival data for individual flies (mean and SEM). All survival experiments were performed twice. Sample sizes and statistics, Table S1.

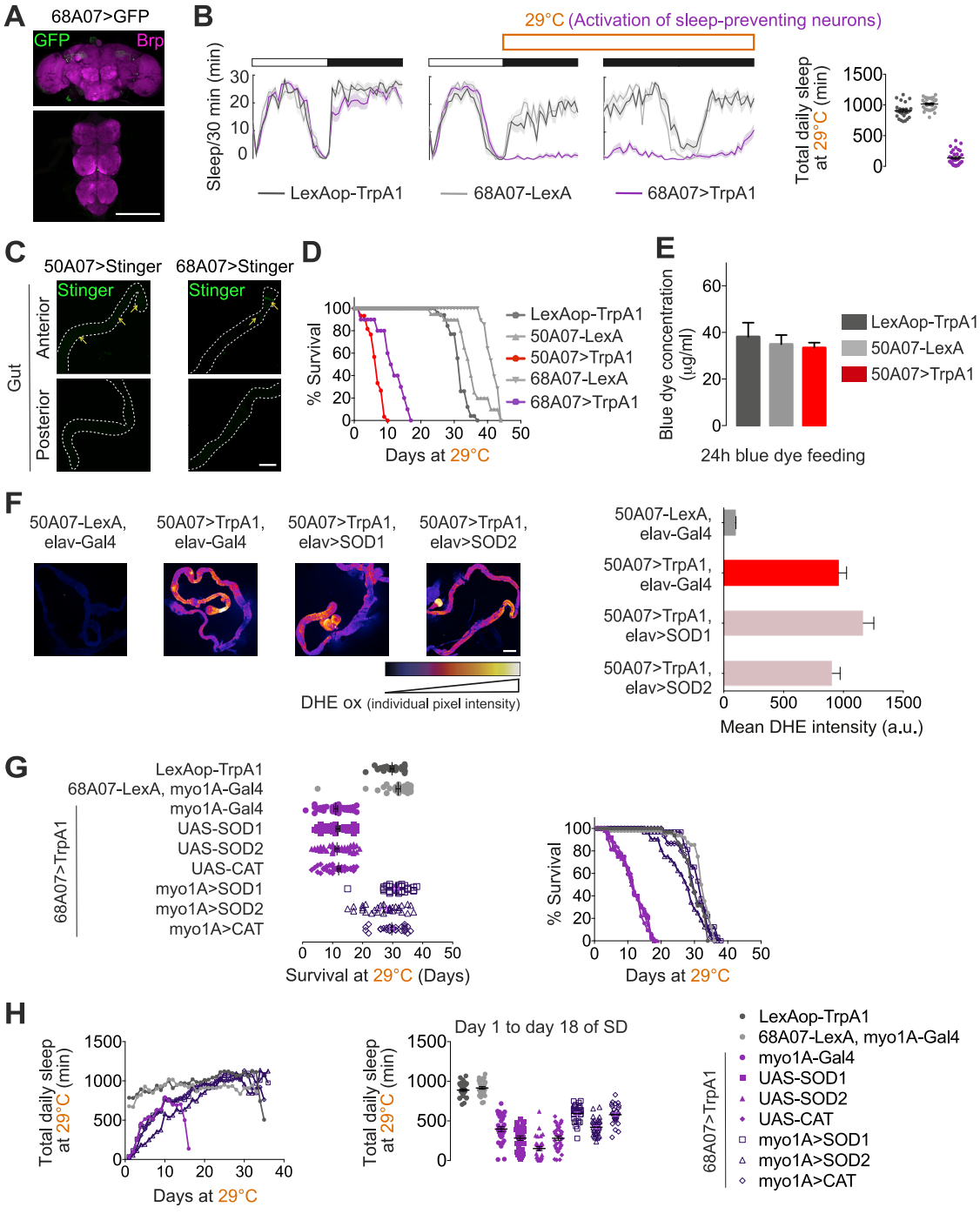

Figure S6. Overexpression of Antioxidant Enzymes in the Gut Promotes Survival of Sleep-Deprived Animals, Related to Figure 7

(A) Expression of 68A07-LexA in the nervous system reported by mCD8::GFP. Brp, neuropil marker. (B) Sleep across three days. Day 1, 21°C: sleep amount is the same between parental controls (68A07-LexA and LexAop-TrpA1) and animals expressing TrpA1 in neurons labeled by 68A07-LexA (68A07>TrpA1). When temperature is raised to 29°C on day 2 (orange), 68A07>TrpA1 flies lost most of their sleep. In experiments involving LexAs, flies were kept in constant darkness after temperature was raised to 29°C because sleep deprivation was more consistent than in light-dark cycles. Mean and SEM (shading). Right, daily sleep in controls and experimental flies across lifespan. Mean and SEM. (C) Expression of 50A07-LexA and 68A07-LexA in the gut, visualized with a nuclear fluorescent reporter RedStinger. The two LexA lines label few cells in the anterior and posterior midgut (arrows). Guts outlined in white to help visualization. (D) Survival of sleep-deprived (red and purple) and non-deprived animals at 29°C in constant darkness. Error bars omitted for clarity. (E) Daily food intake is not different in sleep-deprived flies (50A07>TrpA1) compared to parental controls (LexAop-TrpA1 and 50A07-LexA). Mean and SEM. (F) Overexpressing antioxidant enzymes (SOD1 or SOD2) in neurons throughout the nervous system using elav-Gal4 fails to prevent ROS accumulation in the gut. Mean and SEM. (G) Extended survival of sleep-

(legend continued on next page)

deprived flies (68A07>TrpA1) expressing transgenic antioxidant enzymes (SOD1, SOD2 or CAT) in the gut with myo1A-Gal4. Left, survival for individual flies (mean and SEM); right, population survival (error bars omitted for clarity). (H) Daily sleep across lifespan (left, error bars omitted for clarity) or during the first 18 days of sleep deprivation (the time it took for the sleep-deprived flies to die if they had no transgenic expression of antioxidant enzymes) (right, mean and SEM) of sleep-deprived flies (68A07>TrpA1) expressing transgenic antioxidant enzymes (SOD1, SOD2 or CAT) in the gut. Representative images. Scale bars, 200 μm. All survival experiments were performed 2-3 times. Sample sizes and statistics, Table S1.

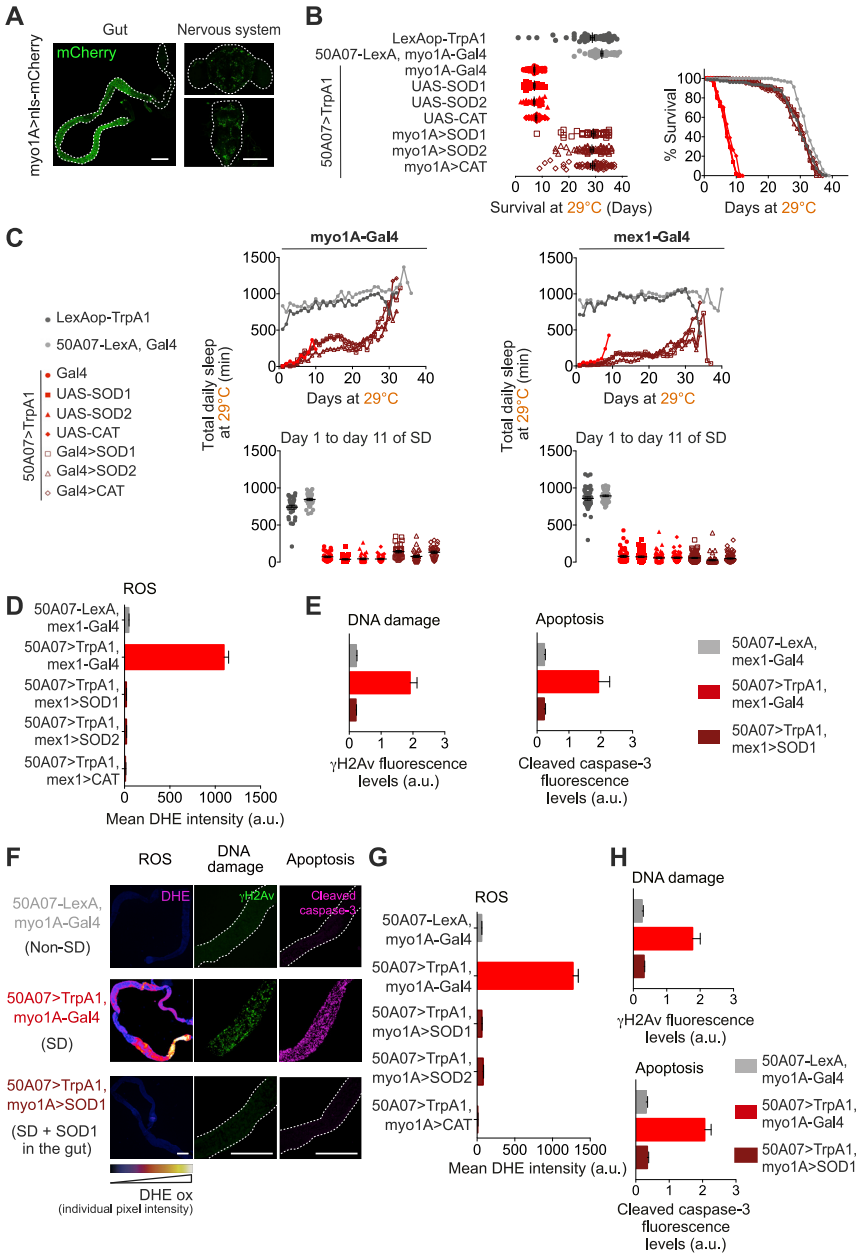

Figure S7. Overexpression of Antioxidant Enzymes in the Gut Prevents Oxidative Damage in This Tissue and Promotes Survival upon Sleep Deprivation, Related to Figure 7

(A) Expression of myo1A-Gal4 in the gut and the central nervous system, visualized with nls-mCherry. (B) Survival of sleep-deprived flies expressing transgenic antioxidant enzymes (SOD1, SOD2 or CAT) in the gut with myo1A-Gal4. Dot plot, survival for individual flies (mean and SEM); curves, population survival (error bars omitted for clarity). Survival experiments were performed at least 3 times. (C) Daily sleep of sleep-deprived flies expressing transgenic antioxidant enzymes (SOD1, SOD2 or CAT) in the gut with myo1A-Gal4 (left) or mex1-Gal4 (right). Sleep is shown either across the entire lifespan (top, error bars omitted for clarity) or during the first 11 days of sleep deprivation (the time it took for the sleep-deprived flies to die if they had no transgenic expression of antioxidant enzymes) (bottom, mean and SEM). (D) ROS accumulation is prevented by transgenic expression of antioxidant enzymes (SOD1, SOD2 or CAT) in the gut with mex1-Gal4. (E) Overexpressing the antioxidant enzyme SOD1 in the gut with mex1-Gal4 (50A07>TrpA1, mex1>SOD1) prevents DNA damage and cell death in this tissue. (F), (G) ROS accumulation is prevented by transgenic expression of antioxidant enzymes (SOD1, SOD2 or CAT) in the gut with myo1A-Gal4. (F), (H) Overexpression of SOD1 in the gut of sleep-deprived flies using myo1A-Gal4 (50A07>TrpA1, myo1A>SOD1) prevents DNA damage and cell death in this tissue. Representative images. Scale bars, 200 μm. Guts outlined in white to help visualization. Mean and SEM. Sample sizes and statistics, Table S1.