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Tired and Stressed: Examining the Need for Sleep

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Abstract

A key feature of circadian rhythms is the sleep/wake cycle. Sleep causes reduced responsiveness to the environment, which puts animals in a particularly vulnerable state; yet sleep has been conserved throughout evolution, indicating that it fulfills a vital purpose. A core function of sleep across species has not been identified, but substantial advances in sleep research have been made in recent years using the genetically tractable model organism, *Drosophila melanogaster.* This review describes the universality of sleep, the regulation of sleep, and current theories on the function of sleep, highlighting a historical and often overlooked theory called the Free Radical Flux Theory of Sleep. Additionally, we summarize our recent work with short-sleeping *Drosophila* mutants and other genetic and pharmacological tools for manipulating sleep which supports an antioxidant theory of sleep and demonstrates a bi-directional relationship between sleep and oxidative stress.

Keywords

Drosophila; circadian rhythm; sleep; oxidative stress

The sleep/wake cycle is an animal's most obvious physiological manifestation of circadian rhythm. Circadian rhythms, or the oscillation of physiological processes over a 24-hour period, are present in organisms ranging from mammals and invertebrates to bacteria and plants. Driving these oscillations is the circadian clock; while the precise molecular players of the clock vary in different species, the core mechanism of a transcriptional negative feedback loop appears to be highly conserved. A key feature of the circadian clock is its endogenous nature—once the clock has been entrained, or set, by external cues such as sunlight, it is able to maintain its rhythm without external cues from the environment. Thus, animals kept in constant conditions will continue to sleep and wake with approximately 24-hour periodicity.

Though humans have been speculating on the functions of sleep since ancient times, it still remains one of our most mysterious behaviors. Ancient people were intrigued by sleep because its main behavioral features—lack of movement and failure to respond to the environment—parallel death. In Greek mythology, sleep is personified by Hypnos who lives

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in the underworld with his twin brother, Thanatos, the god of death (Lavie, 1998). The idea that sleep represents a death-like state persisted even into the 1800s, with Robert MacNish's "Philosophy of Sleep," in which he explains that "sleep is a temporary metaphysical death" (MacNish, 1830).

Scientific explanations for the cause of sleep arose during antiquity but were largely unquestioned until modern day. The first sleep mechanism was proposed in 6th century BCE by Alcmaeon who believed that sleep occurs when blood retreats to the internal organs (Lavie, 1998). Two centuries later, Aristotle hypothesized that stomach vapors rise to the brain during digestion, and the cooling of these vapors triggers sleep (Lavie, 1998). Remarkably, variations of the stomach vapor theory survived for over two millennia before the birth of neuroscience and the development of new technology led to a surge of sleep research in the 20th century.

The idea of a hypnotoxin, or a sleep-inducing toxin that accumulates in the brain during wake and interferes with neuronal function, emerged in the early 1900s with the work of Ishimori (Ishimori, 1909) and Legendre and Piéron (Legendre & Pieron, 1913). Both groups independently discovered that cerebrospinal fluid extracted from sleep-deprived dogs promptly induces a deep sleep when injected into other dogs. These findings sparked the quest for a sleep-promoting factor that researchers believed would unearth the mechanisms underlying the function and regulation of sleep.

Over the past century, considerable advances have been made in our understanding of sleep biology. The development of the electroencephalogram (EEG) led to the discovery of different sleep states and stages, the discovery of the circadian system shed light onto one branch of sleep regulation (sleep timing), and researchers began to identify genes that influence sleep. Despite this rapid progress in sleep research, the function of sleep and the second branch of sleep regulation (sleep homeostatic mechanisms) still remain unclear.

Since our transition into industrialized society, artificial lighting and 24-hour access to entertainment, food, and stimulants have made insomnia and sleep restriction a growing public health concern (Jean-Louis et al., 2000). Health consequences such as diabetes and heart disease, two of the top ten leading causes of death in the US, have been linked to sleep restriction (Cappuccio et al., 2011; Johnson NB, Hayes LD, Brown K, Hoo EC & (CDC), 2014; Nedeltcheva & Scheer, 2014). Moreover, sleep disturbances are associated with increased risk for development of neurodegenerative diseases such as Alzheimer's disease (Lim et al., 2013), another leading cause of death. Thus, there is a great need to further our understanding of sleep and the role it plays in human health.

In this review, we address the universality of sleep with emphasis on sleep in the fruit fly. We also summarize our current understanding of sleep regulation and present hypotheses on the function of sleep, highlighting a historical hypothesis, The Free Radical Flux Theory of Sleep. Finally, we summarize our recent evidence in support of a bi-directional relationship between sleep and oxidative stress.

The Universality of Sleep

Defining Sleep

While immobility is the most obvious behavioral characteristic of sleep, lack of movement does not distinguish a sleeping animal from one that is simply resting or in another physiological state such as hibernation. Starting with Piéron's observations in 1913, and with several additions over the past century, scientists have developed a set of behavioral criteria that define sleep across animal species. An animal is said to sleep only if it 1) demonstrates reversible immobility, 2) assumes a sleep-specific posture, 3) exhibits an increased arousal threshold, or reduced reaction to a stimulus and 4) shows a sleep rebound, or a recovery period of longer and/or deeper sleep after sleep deprivation (Campbell & Tobler, 1984; Hendricks, Sehgal, et al., 2000). The behavioral criteria of sleep have been helpful in determining whether sleep exists in non-mammalian species.

Sleep is also associated with electrophysiological changes in the brain. In mammals and birds, sleep has been defined by characteristic changes in brain activity that occur in stages and can be recorded and identified by EEG. For instance, the deepest stage of sleep, called slow wave sleep, occurs during non-rapid eye movement (NREM) sleep and features prominent delta waves in the 0.5-4.5 Hz range (Cirelli, 2009). EEG recordings are difficult or impossible to perform in many species, including invertebrates. However, other correlates of neural activity that vary with sleep have been identified in *Drosophila*, and there is evidence for the existence of distinct sleep stages in the fly (Nitz et al., 2002; van Alphen et al., 2013; Bushey et al., 2015; Yap et al., 2017).

Sleep Across Species

One particularly intriguing feature of sleep is its seemingly ubiquitous presence among animal species. This is especially true when considering how dangerous sleep behavior can be: a sleeping animal is vulnerable to predators and other dangers in its environment for many hours each day. The fact that animals have evolved such a behavior suggests that sleep must fulfill a function that is fundamental to life. This notion supports the idea shared by many, but not all sleep researchers, that there is a core function of sleep across all animal species (reviewed in Cirelli & Tononi, 2008). One major argument against a core function of sleep is the controversy about whether all animals sleep. That is, if sleep truly is crucial to animal life, then we would expect to see sleep behavior in all animal species. Whether or not this is the case is still under some debate; as discussed below, several examples exist of animals that either might not sleep, or that are able to suppress their sleep for extended periods of time with few evident side effects. While these examples are fascinating and certainly warrant further investigation, it is clear that the vast majority of animals tested do exhibit sleep behavior.

Prior to 2000, sleep research focused largely on mammals and birds, despite the fact that sleep behavior had been described in many simple vertebrate and invertebrate species by that time. In 1984, Campbell and Tobler had examined over 200 studies on various species and determined, based on behavioral criteria, that sleep is present in fish, reptiles, amphibians, and invertebrates such as the cockroach (Campbell & Tobler, 1984). Evidence of a sleep

state in bees (Kaiser & Steiner-Kaiser, 1983) and scorpions (Tobler & Stalder, 1988) was also reported. In the last two decades, better technology has provided more extensive evidence of sleep in organisms that were once thought to be sleepless: slow wave sleep has been reported in crayfish (Mendoza-Angeles et al., 2007), a change in brain state during sleep was demonstrated in fruit flies (Nitz et al., 2002), and varying sleep intensity, indicative of sleep stages, has been shown in both honey bees and fruit flies (Eban-Rothschild & Bloch, 2008; van Alphen et al., 2013; Yap et al., 2017). As such, sleep research has now expanded into model organisms such as the zebrafish (Yokogawa et al., 2007), fruit fly (Hendricks, Finn, et al., 2000; Shaw et al., 2000), and the roundworm (Raizen et al., 2008). Sleep states have also been characterized in the sea slug *Aplysia californica* (Vorster et al., 2014) and the jellyfish *Cassiopea* (Nath et al., 2017).

Nonetheless, some researchers have argued against the presence of sleep in all species (Siegel, 2008). The bullfrog is frequently cited as an animal that does not sleep; however, evidence supporting this claim comes from a single study conducted in 1967 in which electric shock was used as an arousal stimulus and an increased arousal threshold during quiescence was not demonstrated (Hobson, 1967). Other animals that have been referenced as proof that sleep is not universal include a species of coral reef fish that engages in “sleep swimming,” which involves continuous movement of the fins while the fish stays in one place (Goldshmid et al., 2004). Sleep swimming may be necessary to prevent hypoxia of the coral colonies within which these fish sleep (Goldshmid et al., 2004). While the fish lack complete immobility during sleep swimming, they are much more likely to be caught by predators during this behavior, suggesting that they do indeed exhibit the increased arousal threshold indicative of a sleep state (Holbrook & Schmitt, 2002). Sleep swimming is also present in the dolphin, another species in which the presence of sleep has been questioned.

Dolphins periodically exhibit circular swimming, a behavior during which they have been shown to be less responsive to stimuli (Ridgway, 2006). Circular swimming coincides with “unihemispheric sleep,” during which only half of the brain produces the slow waves characteristic of deep sleep at one time (Cirelli & Tononi, 2008). This strategy is thought to allow the animals to surface for air during sleep and is shared by other aquatic mammals such as porpoises and whales (Rattenborg et al., 2000). Unihemispheric sleep is also common across numerous avian species in which it likely serves to reduce the risk of predation during sleep (Rattenborg et al., 2000). Behavioral observations suggest that certain reptilian species may also rely on unihemispheric sleep to watch for predators, but only some of these observations have been supported by electrophysiological data (Rattenborg et al., 2000).

Though some have argued against the universal existence of sleep, no group has provided convincing evidence of a species that does not sleep (Cirelli & Tononi, 2008). Sleep in reptiles and amphibians, including the bullfrog, has proven challenging to study, resulting in sparse sleep data on these animals. However, Libourel and Herrel recently reviewed all the available data and concluded that, despite the 1967 bullfrog study, most reptiles and amphibians do fulfill the behavioral criteria of sleep (Libourel & Herrel, 2016). Sleep-swimming in coral reef fish and unihemispheric sleep in dolphins both provide examples of sleep-like behavior occurring in the absence of total immobility. Interestingly, studies of

different populations of the characin fish *Astyanax mexicanus* have shown how adaptation to specific environments can have a dramatic effect on sleep architecture (Duboué et al., 2011, 2012, Jaggard et al., 2017, 2018). The evolution of these particular sleep strategies to meet the unique requirements of these species highlights the necessity of sleep. These examples also suggest that our current behavioral criteria for sleep may require adjustments to account for species that engage in specific movements during sleep. While there are examples of species that may not adhere precisely to our current behavioral criteria of sleep, the general consensus among sleep researchers is that all animals studied have exhibited at least some evidence of sleep behavior (Cirelli & Tononi, 2008).

Ecological demands can affect sleep amount over extended periods of time and examples of profound sleep suppression have been identified in several animal species. For example, the polygynous nature of pectoral sandpipers creates intense competition among males for mating and males have been shown to suppress sleep in order to increase mating efficiency (Lesku et al., 2012; Kempenaers & Valcu, 2017). For bird species that fly constantly for days at a time, it was often assumed that sleep was accomplished through unihemispheric sleep, allowing the animals to sleep while still maintaining movement. However, recent work using EEG recordings from the great frigatebird *Fregata minor* showed that these birds, which can maintain flight for up to 10 days, accomplish in-flight sleep through both unihemispheric and bihemispheric sleep, but that the duration of this sleep is less than 10% of their normal sleep time on land (Rattenborg et al., 2016). Interestingly, the fur seal, which lives both on land and in seawater, suppresses slow wave sleep when in the water and shows no subsequent slow wave rebound upon returning to land (Lyamin et al., 2018). Taken together, these examples indicate that certain ecological conditions can drive sleep suppression; however, it remains unclear if or how these animals might upregulate other processes to compensate for lost sleep. It should be noted that these exceptions may prove the rule: that is, the vast majority of animals appear to sleep on a daily basis and exceptions to this rule are only made to satisfy specific extreme environmental circumstances.

Sleep in Drosophila

In 2000, Hendricks (Hendricks, Finn, et al., 2000) and Shaw (Shaw et al., 2000) published independent papers establishing *Drosophila melanogaster* as a model system for studying sleep. Hendricks reported that *Drosophila* do indeed assume a specific posture during periods of immobility (Hendricks, Finn, et al., 2000). Using locomotor activity monitors in which single flies are housed in narrow tubes with infrared beams running across to detect movement, Hendricks and Shaw both determined that flies are immobile for nearly half of their day, and that this immobility occurs mostly at night (Hendricks, Finn, et al., 2000; Shaw et al., 2000). During these periods of immobility, flies are less responsive to physical stimuli, indicating an increased arousal threshold (Hendricks, Finn, et al., 2000; Shaw et al., 2000). Following sleep deprivation by constant mechanical stimulation, *Drosophila* experience a sleep rebound in which they sleep longer than their baseline sleep amount (Hendricks, Finn, et al., 2000; Shaw et al., 2000). Thus, fruit flies fulfill the behavioral criteria of sleep.

Sleep in the fruit fly has been shown to have both similarities and differences with mammalian sleep. For instance, sleep in *Drosophila* can be modulated in the same manner by many drugs that affect mammalian sleep. The adenosine A1 agonist cyclohexyladenosine (Hendricks, Finn, et al., 2000) and the antihistamine hydroxyzine (Shaw et al., 2000), both of which are sleep-inducing in mammals, were shown to increase sleep in flies. Other drugs that have been shown to modulate sleep in the fruit fly include the wake-promoting stimulant Modafinil (Hendricks, Kirk, et al., 2003) and the sleep-promoting $GABA_A$ agonist Gaboxadol (Berry et al., 2015; Dissel et al., 2015). Caffeine is well known as a stimulant in mammals and has also been shown to disrupt *Drosophila* sleep (Shaw et al., 2000; Wu et al., 2009; Nall et al., 2016); however, these effects are partially driven by changes in feeding behavior and nutrient intake rather than a direct effect of caffeine itself (Keebaugh et al., 2017). Similar to mammals, *Drosophila* also exhibit a gradual decrease in sleep amount as they age (Shaw et al., 2000), though some work has complicated this finding, perhaps indicating that differences in environmental conditions affect the relationship between aging and sleep (Koh et al., 2006; Bushey et al., 2010). Shaw also investigated whether the expression of genes known to be modulated by sleep in rats are modulated by sleep in the fly. He found that “waking genes” upregulated during spontaneous wake or sleep-deprivation in rats, including the electron transport protein encoding gene *cytochrome oxidase C* and the ER chaperone *BiP*, were also upregulated in awake or sleep-deprived flies. Thus, many specific aspects of *Drosophila* sleep appear to be evolutionarily conserved with mammalian sleep, supporting the expectation that, if sleep has a core function, this function will be conserved between *Drosophila* and mammals.

These seminal papers by Hendricks and Shaw led to a surge in sleep research in the fruit fly. There are numerous advantages to using *Drosophila* as a model system: flies can be grown quickly and in great numbers, they are cheap and easy to maintain, and most importantly, they are genetically tractable. Within just the past two decades, several sleep-related genes have been identified using *Drosophila*, and likewise, a number of genetic tools that allow for the manipulation of sleep in the fruit fly have been developed. While many neuronal molecules have been shown to modulate sleep in both flies and mammals (see Griffith, 2013 or Ly et al., 2018 for excellent review), we focus here on *Drosophila* mutants that we have used in our own investigations of the function of sleep.

The first forward genetic screen for short-sleeping *Drosophila* mutants identified the voltage-gated potassium channel gene *Shaker* as a sleep modulating gene (Cirelli et al., 2005). *Shaker* mutants sleep 66% less than wildtype controls and have reduced lifespan (Cirelli et al., 2005). A loss-of-function mutation in *Hyperkinetic*, a regulatory subunit of *Shaker*, also produces a short-sleeping phenotype as well as a learning defect (Bushey et al., 2007). A separate forward genetic screen identified the short-sleeping mutant *sleepless*, an allele of the previously-cloned *quiver* gene (Wang et al., 2000), which sleeps 80% less than wildtype controls and also exhibits a shortened lifespan (Koh et al., 2008). The *quiver* gene encodes a membrane-bound protein that has been shown to regulate the *Shaker* channel (Wu et al., 2010); it was later shown that *quiver* also regulates nicotinic acetylcholine receptors (nAChR) (Wu et al., 2014). While the discovery of these mutants supports a role in sleep for potassium channels, which reduce neuronal excitability by repolarizing the membrane after

an action potential, other short-sleeping mutants have implicated neurotransmitters and even a protein ubiquitination pathway in the regulation of sleep.

The dopamine transporter (DAT) clears excess dopamine, a wake-promoting neurotransmitter, from the synaptic cleft, terminating its activity. It is perhaps unsurprising, then, that a mutation in DAT, named *fumin* (sleepless in Japanese), results in a dramatic decrease in sleep (Kume, 2005). It has been shown that the short-sleeping phenotype in *fumin* mutants is due primarily to dopamine signaling in the dorsal Fan-shaped Body (dFB), a sleep-promoting area of the fly brain (Donlea et al., 2011). Dopamine signaling in the dFB through dopamine receptor 1 (DA1) was shown to promote wake, likely through inhibition of dFB neurons (Ueno et al., 2012; Pimentel et al., 2016). Furthermore, direct activation of the dFB by expression of the sodium bacterial channel construct *NaChBac* or the heat-activated cation channel *TrpA1* under a dFB promoter has been shown to induce sleep (Donlea et al., 2011).

Another sleep mutant with altered neurotransmitter signaling is the short-sleeping mutant *redeye*, which carries a loss-of-function mutation in the nAChR subunit α4 and sleeps 50% less than controls (Shi et al., 2014). Acetylcholine signaling is typically considered wake-promoting; nAChRs are cation channels that excite neurons when activated, and in mammals ACh is known to be released when animals are awake (Platt & Riedel, 2011). Moreover, the Sleepless protein promotes sleep in part by antagonizing nAChRs to reduce excitability in *Drosophila* (Wu et al., 2014). However, since loss of nAChRα4 function promotes wake in *redeye* mutants, this particular nAChR subunit may be enriched in sleep promoting neurons where it normally functions to promote sleep.

Forward genetic screens also identified a short-sleeping mutant named *insomniac* (*inc*), which sleeps 65% less than wildtype (Stavropoulos & Young, 2011). *inc* is thought to encode a BTB-domain adaptor protein for Cullin3, an E3 ubiquitin ligase that is expressed throughout the whole fly (Stavropoulos & Young, 2011). While short-sleeping *inc* nulls have a shortened lifespan, neuron-specific expression of *inc-RNAi* produces a short-sleeping phenotype and a normal lifespan (Stavropoulos & Young, 2011; Pfeiffenberger & Allada, 2012; Hill et al., 2018). Neuron-specific *cullin3* (*cul3*) *RNAi* also results in a similar short-sleeping phenotype (Stavropoulos & Young, 2011). While it is difficult to predict how reduced activity of *inc* or *cul3*, which are implicated in protein ubiquitination, affect sleep, it has been proposed that Inc/Cul3 proteins may impact dopaminergic modulation of sleep (Pfeiffenberger & Allada, 2012).

Overall, the expansion of sleep research to include *Drosophila* has led to a series of advances in our understanding of sleep regulation on the genetic and anatomical level. The characterization of circuits driving sleep-wake behavior in the fly have been reviewed in detail elsewhere, and will not be described here (Artiushin & Sehgal, 2017; Donlea, 2017; Ly et al., 2018). Combining neuroanatomical knowledge with genetic tools has resulted in the development of several methods of sleep modulation. For example, altering the activity of the sleep-promoting dFB can be used to increase or decrease sleep. These tools allow for temporally-restricted, precise control of sleep-wake behavior in the fly.

The Regulation of Sleep

In both mammals and flies, there appear to be two distinct regulatory mechanisms that control sleep: the circadian clock, which regulates the timing of sleep, and a homeostatic mechanism that regulates the amount and quality of sleep (Borbély, 1982; Borbély et al., 2016). While the core components of the circadian clock were identified in *Drosophila* (see below), early evidence for this two process model of sleep regulation came from rodent studies conducted before the fly was established as a model for sleep (Ibuka et al., 1977; Mouret et al., 1978; Mistlberger et al., 1983; Tobler et al., 1983).

Circadian Regulation of Sleep

Years before sleep research in fruit flies became mainstream, *Drosophila* genetics were being harnessed to uncover the components of the circadian clock. The first circadian gene, *period* (*per*), was discovered by Konopka and Benzer in 1971 through a forward genetic screen in *Drosophila* (Konopka & Benzer, 1971). The *per* gene interacts with three other genes—*timeless* (*tim*), *clock* (*clk*), and *cycle* (*cyc*)—to comprise the core autoregulatory feedback loop of the circadian clock in fruit flies. In very simplified terms, the clock functions as follows: Clk and Cyc proteins dimerize and enter the nucleus where they drive transcription of a number of circadian regulated genes, including *per* and *tim;* as Per and Tim proteins accumulate, they too dimerize and enter the nucleus, inhibiting Clk and Cyc and thereby blocking their own transcription; Per and Tim proteins are eventually degraded, lifting the inhibition of their transcription and allowing the cycle to begin again. The timing of the process is tightly controlled by a number of other proteins that influence the stability of Per and Tim proteins through phosphorylation and other post-translational modifications including ubiquitination (Allada & Chung, 2010; Stojkovic et al., 2014). The output of this circadian feedback loop is continuous cycling of thousands of mRNAs and their protein products, resulting in subsequent cycling of the biological processes carried out by these proteins. One major circadian output that can easily be measured in mammals as well as flies is locomotor activity, which has characteristic peaks and troughs throughout the day.

Importantly, though the core mammalian homologs differ slightly in name and number, the components of the *Drosophila* molecular clock are largely conserved in mammals. Oscillation of *per* and *tim* occurs in nearly every tissue in the body, but these peripheral clocks are synchronized by the central clock located within a sparse group of pacemaker neurons in the *Drosophila* brain (Allada & Chung, 2010), or in a small region of the hypothalamus called the suprachiasmatic nucleus (SCN) in mammals (Hardin & Panda, 2013). While roughly 150 neurons in the fly brain rhythmically express the molecular components of the circadian clock, a smaller subset of these clock neurons, termed the ventral lateral neurons (LNv), are thought to be essential for behavioral rhythmicity (Allada & Chung, 2010). Synchronization of the peripheral clocks to the central clock is thought to occur by the release of a neuropeptide, Pigment Dispersing Factor (PDF) in flies (Renn et al., 1999) or Vasoactive Intestinal Polypeptide (VIP) in mammals (Aton et al., 2005), from a subset of the central clock neurons.

The circadian clock plays an important role in dictating the timing of sleep. In humans, a mutation in *hPer2*, one of the mammalian homologs of *per*, causes Familial Advanced Sleep

Phase Syndrome (FASPS) (Toh, 2001). Sleep duration is not affected in FASPS patients, but sleep onset occurs about 4 hours earlier than average. Less dramatic changes in time of sleep onset are caused by natural polymorphisms in clock genes, which can determine whether a person is an early-rising "lark," or a late-rising "owl" (Archer et al., 2003; Carpen et al., 2006; Patke et al., 2017). Furthermore, lesions in the mammalian SCN or disruption of the core clock genes in flies and mammals results in locomotor arrhythmicity, or loss of the characteristic daily movement pattern, when animals are kept in constant conditions (without environmental cues) (Konopka & Benzer, 1971; Moore & Eichler, 1972; Stephan & Zucker, 1972; Eastman et al., 1984; Hendricks, Finn, et al., 2000; Shiromani et al., 2004).

In flies, timed secretion of PDF from a subset of the ventral lateral neurons has a wake-promoting effect, resulting in an anticipatory peak of activity just before dawn. Loss-of-function mutations in *pdf* or the gene encoding its receptor *pdfr* result in increased late night sleep (Chung et al., 2009), while constitutive activation of a group of LNv causes decreased nighttime sleep (Sheeba et al., 2008). PDF specifically activates a group of dorsal circadian neurons (DN1s) that induce arousal by releasing the wake-promoting diuretic hormone 31 (DH31) (Kunst et al., 2014). Moreover, the DN1 neurons have been shown to interact with pacemaker neurons in a feedback circuit that governs both the midday siesta and nighttime sleep (Guo et al., 2016). The LNv also express inhibitory $GABA_A$ receptors which suggests a role in GABA signaling in modulating PDF release to promote sleep (Chung et al., 2009). Expression of the circadian gene *wide awake* peaks in clock neurons at dusk, triggering upregulation of $GABA_A$ receptors to promote sleep (Liu et al., 2014). The circadian system has also been shown to gate sleep induction through modulation of sleep-promoting neuronal firing, possibly to prevent sleep induction late in the day when homeostatic sleep drive is high (Cavanaugh et al., 2016).

While the role of the circadian clock in the timing of sleep is clear, the role of the clock in modulation of sleep duration and sleep rebound is somewhat controversial (Franken, 2013). SCN lesions in primates (Edgar et al., 1993) and mice (Easton et al., 2004) have been shown to cause increased sleep duration. However, other evidence in rodents suggests that SCN ablation causes no change in baseline sleep duration with no change in sleep rebound following deprivation (Ibuka et al., 1977; Mouret et al., 1978; Mistlberger et al., 1983; Tobler et al., 1983). Moreover, evidence for the effects of clock gene mutations on sleep is mixed: these mutations can either increase or decrease sleep duration (Naylor et al., 2000; Wisor et al., 2002; Hendricks, Lu, et al., 2003). However, recent work indicates that this may be due to effects of the clock in non-neuronal tissues (Ehlen et al., 2017). Following sleep deprivation, *Drosophila per, tim,* and *clk* mutants all show extended sleep rebound, recovering 100% of sleep lost rather than the 30–40% that is typical of wildtype. For unclear reasons, *cyc* mutants are particularly sensitive to sleep deprivation and start to die after 10 hours (Shaw et al., 2002). Mice with double mutations in the circadian clock genes *cry1* and *cry2* have reduced sleep rebound after sleep deprivation (Wisor et al., 2002). Thus, the circadian clock has a strong influence on the timing of sleep, but likely also interacts with other mechanisms that control sleep homeostasis.

Mechanisms of Sleep Homeostasis

Organisms depend on internal systems to maintain homeostasis, or a context-dependent equilibrium within the body that allows for proper performance of bodily functions. Homeostatic mechanisms typically function through negative feedback, where deviations outside the equilibria are sensed and corrected to restore homeostasis. After sleep deprivation, homeostatic mechanisms promote recovery sleep, characterized by longer duration and/or deeper intensity, to compensate for the lost sleep. Because the processes controlling sleep homeostasis are largely unknown, these mechanisms are often referred to collectively as the sleep homeostat. When Borbély proposed the “two process model of sleep regulation” in 1982, he suggested that the circadian system and the sleep homeostat work together to regulate sleep (Borbély, 1982). In his model, sleep pressure builds continuously while an organism is awake and triggers sleep when it reaches its upper limit (Borbély, 1982; Borbély et al., 2016).

Though Borbély originally thought that the circadian clock and sleep homeostat had independent influences on sleep, it is now understood that there is some crosstalk between the two processes (Deboer, 2018). As discussed above, altering clock gene expression can impact sleep duration; additionally, prolonged wakefulness can alter the expression of core clock genes (Cedernaes et al., 2015). Interaction between the two processes may allow organisms to stay alert even toward the end of the day, when sleep pressure has accumulated but not yet reached its upper limit (Meerlo et al., 2015).

Sleep deprivation experiments, which typically involve continuous physical stimulation to prolong wake, are most commonly used to study sleep homeostasis. In animals whose brain activity can be measured by EEG, slow wave sleep, characterized by prominent delta waves in the 0.5–4.5 Hz range, is considered the best marker for sleep intensity (Cirelli, 2009). As sleep deprivation is extended, a corresponding increase in slow wave sleep is observed in the subsequent sleep rebound (Tobler & Borbély, 1986; Franken et al., 2001), as is an increased arousal threshold in the sleeping animal (Blake, H.; Gerard, 1937). Additionally, prolonged wakefulness is correlated with increasing theta activity, or waves in the 4–7 Hz range, which is considered a reliable marker for sleep pressure (Vyazovskiy & Tobler, 2005). However, whether there are molecular markers for sleep pressure, and what these markers tell us about the mechanisms of homeostatic sleep regulation, is less clear.

Several molecules have been identified that increase in abundance as sleep pressure builds. For instance, adenosine accumulates in the basal forebrain and cortex during sleep deprivation and is then depleted during recovery sleep (Porkka-Heiskanen et al., 1997). Furthermore, blocking A1 adenosine receptors in the basal forebrain during sleep deprivation prevents recovery sleep from occurring, suggesting that adenosine directly drives sleep rebound (Gass et al., 2009). Blocking ATP synthesis to cause energy depletion results in accumulation of extracellular adenosine and an increase in sleep (Kalinchuk et al., 2003). Thus, it is thought that prolonged wakefulness, which results in increased neuronal firing in both the basal forebrain (Kostin et al., 2010) and the cortex (Vyazovskiy et al., 2009), depletes energy, thereby increasing adenosine concentration and driving sleep.

Nitric oxide (NO), an important signaling molecule, also accumulates in the basal forebrain during sleep deprivation and precedes the accumulation of adenosine (Kalinchuk et al., 2011). Blocking the increase in either NO or adenosine during sleep deprivation prevents subsequent sleep rebound (Kalinchuk, Lu, et al., 2006). Lesions in the cholinergic cells of the basal forebrain prevent accumulation of both adenosine and NO during sleep deprivation (Kalinchuk, Lu, et al., 2006). Thus, it has been proposed that extensive firing of basal forebrain cholinergic cells during prolonged wakefulness induces NO production as a stress signal, causing a subsequent spike in adenosine and promotion of sleep (Porkka-Heiskanen, 2013). Indeed, sleep deprivation has been shown to trigger a number of stress responses including activation of inducible nitric oxide synthase (iNOS) to produce NO (Kalinchuk, Stenberg, et al., 2006), as well as upregulation of the transcription factor nuclear factor-kappa B (NF-κB) (Cirelli et al., 2004, 2006; Williams et al., 2007), which plays a major role in the innate immune response.

Immune molecules are also involved in sleep homeostasis. Increased sleep during illness is a major feature of sickness behavior in mammals (Hart, 1988), and bacterial infection in flies also increases sleep (Kuo et al., 2010). Flies deficient in *Relish,* which encodes an NF-κB protein, do not exhibit an increase in sleep following infection and have reduced baseline sleep (Williams et al., 2007), indicating a role for NF-κB in infection-dependent and -independent sleep regulation. NF-κB controls expression of a number of cytokines, including Interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα), which have both been shown to increase non-rapid eye movement (NREM) sleep in various species (Krueger et al., 2001; Opp, 2005). Likewise, blocking the function of either of these cytokines results in a decrease in NREM sleep as well as a decrease in sleep rebound (Krueger et al., 2001; Opp, 2005). The sleep-inducing effects of these cytokines are likely mediated through complex interactions with other sleep-regulating molecules; for instance, IL-1β promotes the release of NO and adenosine, and also interacts with major neurotransmitters such as serotonin (Imeri & Opp, 2009).

Adenosine, NO, and the cytokines IL-1β and TNFα represent just a few of the numerous molecules that can reliably influence the sleep state of an animal. While these molecules all regulate sleep, they presumably do so in an indirect manner. The components of the sleep homeostat that directly regulate sleep have yet to be determined, but likely share an intimate link with the underlying function of sleep.

Theories on the Function of Sleep

There are currently several prominent hypotheses on the function of sleep: synaptic downscaling, energy restoration, and metabolite clearance. Related to metabolite clearance, we also highlight Reimund's Free Radical Flux Theory of Sleep, which posits an antioxidant function for sleep.

Synaptic Downscaling

Various sleep deprivation studies in humans and mammals performed over the last century have demonstrated that sleep deprivation impairs learning and memory (Peigneux et al., 2001). Further insight has been provided by work in fruit flies demonstrating that short-

sleeping mutants have impaired memory (Bushey et al., 2007), and that genetically inducing sleep improves memory (Donlea et al., 2011) and restores the ability to learn in certain memory mutants (Dissel et al., 2015). Additionally, gene expression studies in rats have shown that the wake state induces genes involved in synaptic potentiation, or strengthening of synapses, while the sleep state induces genes involved in synaptic depression, or weakening of synapses (Cirelli et al., 2004). This finding has also been supported by evidence in *Drosophila* of sleep-dependent changes in protein levels of synaptic markers (Gilestro et al., 2009). Thus, a popular theory of sleep function is that sleep is necessary for synaptic downscaling, or weakening of synapses, to maintain synaptic homeostasis (Tononi & Cirelli, 2003).

The synaptic homeostasis hypothesis proposes that learning occurs during wake, resulting in a net increase in synaptic strength that in turn requires more energy, space, and cellular materials for the brain to maintain (Bushey & Cirelli, 2011). Thus, synaptic downscaling occurs during sleep, at a time when most synapses are less active, to globally normalize synaptic strength down to a sustainable level (Bushey & Cirelli, 2011; Maret et al., 2011; Yang & Gan, 2012; De Vivo et al., 2017; Diering et al., 2017). Accordingly, it is proposed that sleep after learning improves memory consolidation because synaptic downscaling increases the signal-to-noise ratio between new memories and old, poorly-integrated ones (Tartar et al., 2006; Tononi & Cirelli, 2014). Moreover, sleep-dependent changes in synaptic strength of sleep-regulatory neurons have been proposed as a possible mechanism linking the synaptic homeostasis model with sleep regulation (Donlea et al., 2009; Liu et al., 2016; Robinson et al., 2016). Structural evidence in flies supports the theory of synaptic downscaling; synapses in three different neuronal circuits increase in size or number during wake and decrease only following sleep (Bushey et al., 2011). However, the system may be more complex in mammals—different observations have been made depending on the type of synapse studied and the type of experience preceding sleep (Frank, 2015).

Energy Restoration

A simple and more cellular theory on the function of sleep is that sleep allows for the restoration of depleted energy stores in the brain. Compared to other organs, the brain has an incredibly high metabolic rate—it makes up only 2% of our body mass but consumes 20% of available oxygen (Siegel et al., 1999). However, positron-emission tomography (PET) studies have shown that the human brain consumes only half as much glucose during deep sleep as it does during wake (Kennedy et al., 1982; Heiss et al., 1985; Buchsbaum et al., 1989). The markedly lower metabolic rate during sleep could provide an opportunity for energy replenishment.

While circulating brain glucose levels do not appear to differ between sleep and wake states (Biston et al., 1996), some evidence suggests that glycogen stores become depleted during the wake state. A study in flies reported that sleep deprivation resulted in decreased glycogen stores in the brain and body during the first 3 hours (Zimmerman et al., 2004). Similar findings were reported in some mammalian studies, but were challenged by other findings (Karnovsky et al., 1983; Gip et al., 2002; Kong et al., 2002; Franken et al., 2003, 2006). These variable results may be a consequence of glycogen sensitivity to dissection

conditions. ATP is another molecule that could serve as a marker for the energy state of the cell, but is incredibly sensitive to oxidation, making direct measurement difficult. An alternative approach is to measure phosphorylation of adenosine monophosphate kinase (AMPK), which occurs when ATP is depleted and adenosine monophosphate (AMP) levels are high. Sleep deprivation in mice does result in increased AMPK phosphorylation in the cerebral cortex, suggesting a state of neuronal energy depletion (Nikonova et al., 2010). However, more direct methods of measuring energy stores are needed to properly investigate this theory.

Metabolite Clearance

The recent discovery in mice of a sleep-activated brain glymphatic system, which parallels the lymphatic system of the body, suggests that sleep may serve to clear harmful metabolites from the brain (Xie et al., 2013). Xie et al. reported a 60% increase in interstitial space in the brain during sleep, allowing for increased convective flow between cerebrospinal fluid and interstitial fluid. The group demonstrated that this sleep-dependent increase in convective flow resulted in better clearance of β-amyloid (Aβ), the protein implicated in the pathogenesis of Alzheimer's disease. Poor sleep quality is a predictor of Alzheimer's disease (Lim et al., 2013), suggesting that inadequate sleep-driven clearance of Aβ may contribute to its aggregation and the development of neurodegeneration. In flies, more recent work uncovered circadian regulation of efflux capacity by the glia that compose the blood brain barrier, which could represent a primitive glymphatic-like clearance mechanism (Zhang et al., 2018). The glymphatic system could also serve to clear reactive oxygen species (ROS), reactive molecules that are produced by incomplete reduction of oxygen during oxidative phosphorylation.

Free Radical Flux Theory of Sleep

Produced in the course of oxidative metabolism, ROS can covalently modify and damage proteins, lipids, and DNA, posing a serious threat to the cell. Due to their high metabolic rate and exposure to additional ROS from neurotransmitter metabolism (Reimund, 1994), neurons are particularly at risk of oxidative damage from ROS. Since the brain is more metabolically active during the wake state than during sleep, it is possible that one purpose of sleep is to allow for the clearance of ROS from the brain. This theory was first proposed in 1994 by Reimund, who termed it the Free Radical Flux Theory of Sleep (Reimund, 1994). Reimund hypothesized that ROS accumulate in the brain during the wake state, and the lower metabolic rate of sleep provides the brain's antioxidant system with the opportunity to catch up, neutralizing neuronal ROS down to baseline levels in preparation for the next day's cycle.

Reimund's theory was purely hypothetical, but a handful of groups tested his theory, all using variations on the standard disk over water technique in which rodents are placed on a small platform above water in order to prevent sleep for extended periods of time. This technique was reported to cause an increase in amino-cupric-silver staining, a general indicator of cell damage (Eiland et al., 2002), and increased lipid peroxidation, an indicator of oxidative damage (Silva et al., 2004), in the brains of sleep deprived rodents. Others observed decreased levels of glutathione (D'Almeida et al., 1998) or decreased SOD1

activity (Ramanathan et al., 2002) in the brain, as well as decreased glutathione levels and catalase activity in the liver (Everson et al., 2005) of sleep-deprived rats. However, other groups published contradictory findings, reporting no change in antioxidant activity and no evidence of oxidative damage in the brains of sleep deprived rats (D'Almeida et al., 1997; Cirelli et al., 1999; Gopalakrishnan et al., 2004). Reimund did not speculate on a role for ROS in the regulation of sleep, but it has been reported that oxidized glutathione, extracted from the brains of sleep-deprived rats, induces sleep when injected into control rats (Honda et al., 1994). This finding was supported by a later report that injection of a chemical oxidant into the brains of rats also induces sleep (Ikeda et al., 2005).

Though flies offer a simple system with strong genetic advantages, few groups have investigated the relationship between ROS and sleep in *Drosophila.* It has been observed that feeding flies a low dose of paraquat, an herbicide that catalyzes ROS production, results in sleep fragmentation occurring earlier than is typically observed in aged flies (Koh et al., 2006). Additionally, fragmenting sleep by light cycle interruption results in higher levels of ROS in middle-aged flies and in induction of a number of genes that are upregulated by high ROS levels (Williams et al., 2016).

While the relationship between sleep and ROS remains unclear, several genetic and pharmacological tools have yet to be utilized to investigate this relationship in *Drosophila*. We used a diverse set of short-sleeping *Drosophila* mutants, alongside other genetic and pharmacological methods of sleep manipulation, to investigate the role of ROS in the function and regulation of sleep (Hill et al., 2018).

Bidirectional relationship between sleep and oxidative stress

In order to identify a core function of sleep, we tested a molecularly diverse group of short-sleeping flies for sensitivity to different stressors (Hill et al., 2018). We tested *fumin*, *sleepless*, *redeye*, and *insomniac* null mutants, as well as neuronal RNAi-mediated knockdown of *insomniac* (neuronal *inc-RNAi*). We hypothesized that, if sleep performs a vital function, short-sleeping flies should share a common vulnerability to stresses counteracting that function. We did not identify a consistent phenotype for short-sleeping flies in assays for sensitivity to starvation or infection with an array of different bacterial pathogens. In contrast, all of the short-sleeping flies we tested were sensitive to oxidative stress induced by two different methods. These results suggested that one function of sleep is defense against oxidative stress.

Because we found that decreased sleep lowered resistance to oxidative stress, we hypothesized that sleep promotes oxidative stress resistance. To test this, we increased sleep using two methods—feeding of the sleep-promoting $GABA_A$ agonist Gaboxadol, and genetic activation of the sleep-promoting dorsal Fan-shaped body—and tested for oxidative stress sensitivity. As predicted, increasing sleep with either method caused prolonged survival under oxidative stress.

If sleep is important for oxidative stress defense, then sleep could also function to protect against high ROS incurred by increased neuronal activity during wake. In this case, we

would predict short-sleeping mutants to have a high oxidative load as compared to controls. Due to their highly reactive nature, ROS are notoriously difficult to measure *in vivo*. As a proxy for oxidative burden, we used qRT-PCR to analyze antioxidant and stress response gene expression in neuronal *inc-RNAi* flies. We found that the heads of short-sleeping neuronal *inc-RNAi* flies had increased expression of several antioxidant and stress response genes, including *SOD1*, *GSTS1*, *Hsp60*, and *Pink1*. These results suggest that short sleep in neuronal *inc-RNAi* flies might cause high ROS burden, resulting in homeostatic increases in compensatory antioxidant mechanisms. However, it is possible that neuronal knockdown of *inc* itself induces antioxidant mechanisms, which results in decreased sleep due to decreased sleep need. This would indicate that ROS participate directly in the regulation of sleep itself.

To test whether ROS regulate sleep, we overexpressed the antioxidant enzymes *Catalase*, *SOD1*, and *SOD2* in neurons and measured sleep. We found that neuronal overexpression of *SOD1* or *SOD2* but not *Catalase* reduced sleep, indicating that high ROS in neurons could directly trigger sleep. This work in *Drosophila* establishes a clear relationship between sleep and oxidative stress. While the idea that sleep clears ROS from the brain was proposed over two decades ago, this has never been fully substantiated due to conflicting data in sleep-deprived rats.

Conclusion

Modern society is plagued by chronic sleep restriction, which is associated with various poor health outcomes; thus, a better understanding of the biology of sleep is crucial. Furthermore, aging is associated with decline of both circadian rhythms and sleep health, with a concomitant increased risk for development of many diseases, including cardiovascular and neurodegenerative conditions. Understanding the link between sleep, circadian rhythms, and health will require a more complete understanding of the physiological function(s) of sleep. The genetic tools now available to manipulate sleep in fruit flies offer a powerful new approach to these studies. Using short-sleeping flies as a model for chronic sleep restriction has provided valuable insight into the age-old question of why we sleep. Our work implicates oxidative stress and reactive oxygen species in the function and regulation of sleep behavior. Further work is needed to identify precisely how ROS regulate sleep behavior and to better understand the health implications of the connection between sleep and oxidative stress.

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