Sleep in *Drosophila*

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Abstract

The fruit fly, *Drosophila,* has become a popular model organism for the study of sleep. The ability to manipulate circuits and genes in the fly brain has greatly advanced our understanding of the mechanisms that underpin sleep, including how an animal transitions between sleep and wake, how 'sleep pressure' (the need for sleep) accumulates in the brain, and how sleep functions in the brain to optimize behavior. Several key sleep circuits identified in the central fly brain have also been shown to gate the flow of visual information, suggesting a link between perceptual suppression during sleep and attentional processes during wakefulness. Interestingly, it is also becoming clear that sleep in *Drosophila* is not a homogenous 'off' state, but that flies cycle through lighter and deeper stages of sleep. Future studies of fly sleep may therefore reveal evolutionary origins of sleep stages and their associated functions.

key words: sleep, *Drosophila*, fly, behavior, brain, evolution, neuron, circuit, gene,

Introduction

The fruit fly, *Drosophila melanogaster*, has been used for more than 100 years as a genetic model organism to investigate fundamental questions in biology. Since the discovery almost two decades ago that fruit flies sleep (Hendricks et al., 2000; Shaw, Cirelli, Greenspan, & Tononi, 2000), studies in *Drosophila* have helped to advanced our knowledge of the molecular and neural processes that regulate sleep and its functions. This chapter will introduce what is known about sleep in *Drosophila*, and how it has contributed to our understanding of sleep in general.

Sleep in *Drosophila* and humans share many common features (Table 1). The timing of sleep is driven by circadian rhythms; flies are diurnal animals with activity peaks at dusk and dawn. Flies have a 'siesta' in the middle of the day but sleep is more consolidated at night, when they sleep for longer periods and are less easily awoken. Sleep is rapidly reversible (flies can be awoken) and is modulated by homeostatic mechanisms, whereby sleep loss incurs a sleep 'debt' that leads to a compensatory increase in sleep (Hendricks et al., 2000; Huber, Ghilardi, Massimini, & Tononi, 2004; Shaw et al., 2000). Another factor that influences sleep is age. Like humans, flies sleep more at a young age but later in life their sleep becomes reduced and fragmented (Koh, Evans, Hendricks, & Sehgal, 2006; Shaw et al., 2000).

Sleep in flies is also different in some ways to human sleep. Other than quiescence, flies do not show obvious external signs of sleep (like closing their eyes), and although there is anecdotal evidence that flies have a preferred posture (slightly prone, with body close to the ground) (Hendricks et al, 2000), this has not been studied in detail or quantified, probably because it is so difficult to detect. So scientists must rely on measuring several criteria to determine whether flies are asleep. Traditionally, sleep has been identified in flies based on their behavior rather than their brain activity; a fly is inferred to be asleep when it enters a

period of inactivity lasting 5 minutes or more, as this timeframe of quiescence is associated with an increase in arousal thresholds (Hendricks et al., 2000; Shaw et al., 2000). Although it has its limitations, this simple method of measuring fly sleep has proven to be powerful for identifying many molecules involved in sleep (Dubowy & Sehgal, 2017).

Table 1. Sleep criteria in humans and flies

Sleep Criteria	Human	Fly
Immobility	✓	✓
Increased arousal thresholds	✓	✓
Circadian regulation	✓	✓
Homeostasis	✓	✓
Reversibility (can be awoken)	✓	✓
Preferred posture	✓	?
Sleep stages	✓	✓
Neural correlates of sleep	✓	✓
Slow waves, sharp waves, ripples	✓	×

However, since sleep is "by the brain, of the brain and for the brain" (Hobson, 2005), an important feature of sleep is that it should be recognisable through measuring brain activity. Sleep in the fly brain coincides with changes in neural activity that can be observed both at the level of individual neurons and across the whole brain. In individual neurons (specifically 'kenyon cells', which encode olfactory memories in flies)spontaneous activity is dampened and responses to stimuli are attenuated when a fly is asleep (Bushey, Tononi, & Cirelli, 2015). This suggests that sleep is associated with general neural depression and inhibition of sensory processing. Neural activity during sleep has also been measured on a larger scale in *Drosophila* by recording local field potentials (LFPs), which measure activity across hundreds or thousands of neurons at once. Although single cell resolution is lost at this level, one advantage is that LFPs can reveal information about neural oscillations in a way that is more comparable to EEG recordings of human sleep. In *Drosophila*, sleep is accompanied by a general decrease in LFP amplitude (Nitz, van Swinderen, Tononi, &

Greenspan, 2002; van Alphen, Yap, Kirszenblat, Kottler, & van Swinderen, 2013; Bruno van Swinderen & Greenspan, 2003) and an uncoupling of body movement with brain activity (van Swinderen, Nitz, & Greenspan, 2004). Sleep in the fly brain does not immediately resemble sleep in the human brain (for example, slow waves, spindles and sharp wave ripples have not been observed in flies). Nevertheless, flies appear to have distinct sleep stages (discussed at the end of this chapter), which may perform analogous functions to those seen in human sleep.

Measuring sleep in *Drosophila*

To measure sleep in populations of flies, most *Drosophila* sleep labs still use the *Drosophila* Activity Monitor System (DAMS; Trikinetics Inc, Waltham, MA). Flies are placed in individual glass tubes within the DAMS monitors and an infrared beam passes through the centre of each tube, such that when the fly crosses it, the infrared beam is broken and the fly's activity is detected (Figure 1A). This allows activity to be monitored across several days, and periods of inactivity (of 5 minutes or more) for each fly to be identified as sleep (Figure 1B-D).

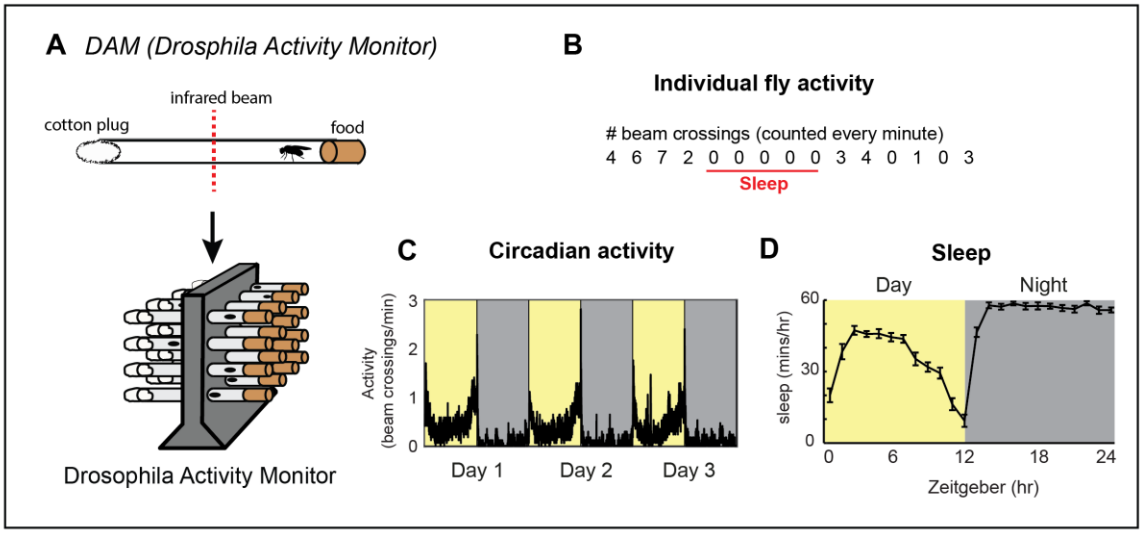

Figure 1. Measuring sleep in *Drosophila*. Sleep is traditionally measured using the *Drosophila* Activity Monitor. A) Flies in individual tubes are placed in a monitor and their activity is recorded by the number of times they cross an infrared beam. B) For each fly, sleep is quantified by cumulative epochs during which the fly is inactive for 5 mins or more. C) Activity is measured for the fly population across the day and the night, here across 3 consecutive days.. n=17 flies. D) Sleep averaged across 24 hrs is quantified for data in (C) using the inactivity metric in (B).

The ability to use the DAMS system to screen for sleep phenotypes in populations of flies has led to remarkable progress in the identification of sleep-regulating molecules and circuits. However, there are several limitations with this approach. Firstly, flies may still be active on either side of the infrared beam, without any activity being detected, thus reducing the resolution and accuracy of sleep measurements. Secondly, DAMS experiments do not usually take into account arousal thresholds; individual flies that are identified as inactive may be in completely different arousal states, for example they may be feeding, grooming, sleeping, quietly vigilant, or sick. Thirdly, beam-crossing paradigms are not ideal for measuring the dynamics and quality of sleep (although this may be inferred to some degree by the frequency of 'brief awakenings' during sleep (Huber, S. Sean, Holladay, M. Biesiadecki, Tononi, 2004). For this reason, many labs have now adopted video-recording systems (Beckwith, Geissmann, French, & Gilestro, 2017; Faville, Kottler, Goodhill, Shaw, & van Swinderen, 2015; Garbe et al., 2015; Geissmann et al., 2017; Gilestro, 2012; Guo et al., 2016; Guo, Chen, & Rosbash, 2017; Zimmerman, Raizen, Maycock, Maislin, & Pack, 2008). These new approaches for measuring sleep allow high resolution tracking of flies, and in some cases the ability to probe arousal thresholds, in order to more closely examine sleep. One common feature of all of these behavioral sleep paradigms is the ability to monitor sleep in flies on a large scale across populations of flies. Importantly, this has allowed scientists to perform screens to identify genes and neuronal circuits that affect sleep.

Unravelling sleep circuitry in flies

Having brains much smaller and simpler than mammals, flies are an ideal system to understand how sleep circuits work. Recent studies in fruit flies have provided important insights into the components of a sleep circuit. What has become clear is that, although only a handful of neurons in the central fly brain can rapidly put a fly to sleep, there are in fact many neurons that influence sleep and these are widely distributed across the brain. These neurons often have distinct roles in sleep, although sometimes their roles are overlapping. Some neurons act like sleep switches, rapidly transitioning flies between wakefulness and sleep states. Other neurons regulate the timing of sleep. More recently, a new class of neurons has been identified that are involved in sensing sleep pressure – how much a fly needs to sleep. How these distinct groups of 'sleep circuits' operate in the fly brain, and what they might tell us about conserved sleep functions, is discussed below.

The 'sleep switch'

One of the most surprising discoveries about sleep in flies is that a very small cluster of neurons in the central brain – about 20 neurons in total - is sufficient to rapidly induce sleep. These sleep-promoting neurons extend their axons into the dorsal fan-shaped body (dFB), a structure in the central brain of *Drosophila* (Figure 2A), within a structure called 'the central complex'. The central complex has been proposed to have deep homology with the basal ganglia in humans (Strausfeld & Hirth, 2013); it is a core multi-sensory processing unit that gates incoming sensory information and selects appropriate actions. A purported function of the central complex is to generate a rudimentary form of subjective awareness, by integrating self-generated motion with external stimuli to create a neural simulation of an animal's movement through space (Barron & Klein, 2016; Stone et al., 2017). Since sleep is a whole-

brain phenomenon that mostly shuts down our perception and interaction with the outside world, it is thus perhaps not surprising that these fly 'sleep switch' neurons reside in the central complex.

Although sleep appears to shut down the brain and make an animal quiescent, sleep is an *active* and *rapidly reversible* process. In flies, sleep can be rapidly induced by acute genetic activation of the dFB, by expressing light or heat sensitive ion channels in these neurons (Dissel, et al., 2015; Donlea, Thimgan, Suzuki, Gottschalk, & Shaw, 2011; Yap et al., 2017). When flies are awake, the dFB is electrically silent (in the 'off'state), although extended wakefulness increases the likelihood of firing action potentials (Figure 2B). In contrast, sleep coincides with persistent firing of the dFB in the 'on' state (Figure 2B) and increased arousal thresholds to visual and mechanical stimuli (Donlea et al., 2018, 2011; Yap et al., 2017). Yet, like humans and other animals, flies that are asleep can still be rapidly awoken. How does this happen? One crucial ingredient here is dopamine, which inhibits the dorsal fan shaped body's activity (Figure 2B), waking up the fly (Q. Liu, Liu, Kodama, Driscoll, & Wu, 2012; Ueno et al., 2012). This resembles one aspect of the "flip-flop" circuitry that exists in the mammalian brain (Saper, Scammell, & Lu, 2005), namely that the sleep promoting neurons (in mammals, the ventral lateral preoptic nuclei (VLPO) of the hypothalamus) are inhibited by wake promoting neurotransmitters such as dopamine, allowing a rapid transition from sleep to wakefulness.

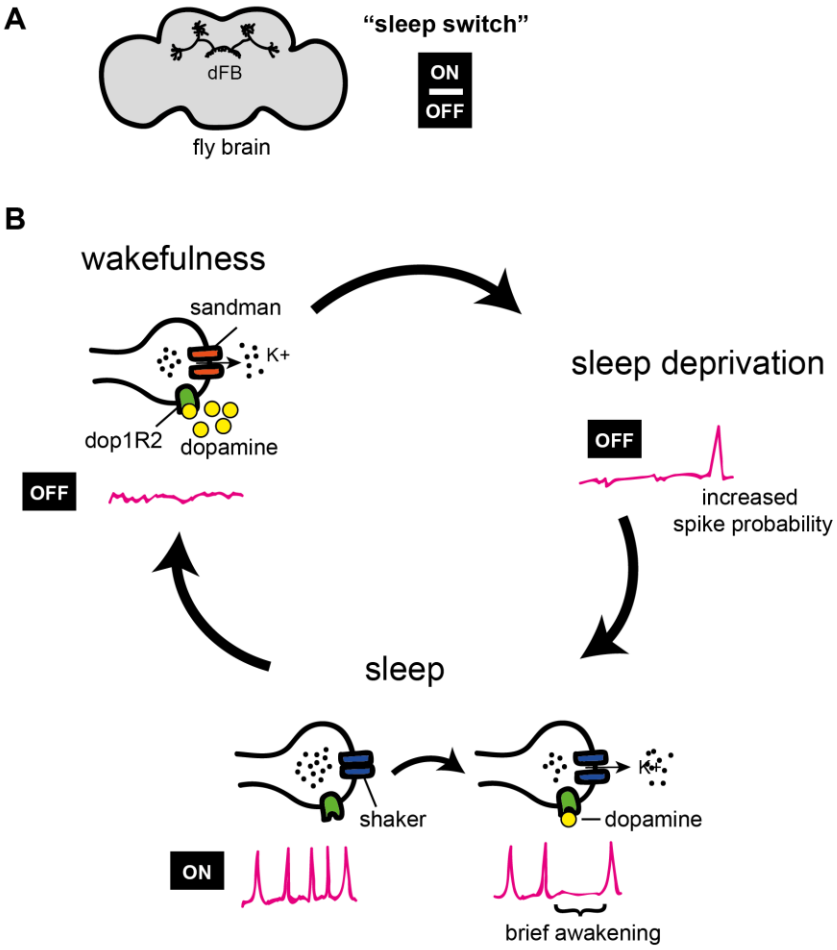

Figure 2. Turning on a 'sleep switch' in flies. A) Neurons in the central brain of the fly, within the dorsal fan-shaped body (dFB) act as a 'sleep switch' to transition a fly from sleep to wakefulness. B) During wakefulness (the 'OFF' state), the wake promoting neurotransmitter, dopamine, is released and binds to the dopamine receptor, dop1R2 (Liu et al., 2012, Ueno et al., 2012, Pimentel et al., 2016). In response, a potassium ion (K+) channel called Sandman is shuttled to the cell surface, where it mediates potassium efflux ('leak conductance') and keeps the cells electrically silent (Pimentel et al., 2016). Following sleep deprivation (extended wakefulness), the dFB neurons become more electrically active, having higher input resistances - as such, they are more likely to produce fire action potentials (spikes) (Donlea et al., 2014). During sleep - the "ON" state - Sandman is internalised (not shown) and a different type of potassium channel on the membrane, most likely Shaker, remains closed, keeping potassium ions within the cells; under these conditions, the dFB neurons are electrically active and spike persistently (Pimentel et al., 2016). However, dFB spiking is inhibited by brief exposure to dopamine, suggesting a mechanism by which an animal can be rapidly aroused from sleep.

In the fly brain, we can delve even further into understanding the sleep switch, by examining what is happening at the molecular level. A recent study found that sleep/wake transitions

are controlled by distinct types of potassium channels that modulate the firing patterns of the dFB neurons – in particular, two channels called Shaker and Sandman (Pimentel et al., 2016). It was found that during the sleep state, Shaker is important for keeping potassium ions inside the dFB neurons, which keeps them in a repetitive firing state necessary for sleep (Figure 2B). Dopamine signalling through DopR1 receptors in the dFB neurons could rapidly open the Shaker channels, transiently inhibiting their firing and thereby awakening the fly (Figure 2B). More prolonged activation through dopamine could lead to a more stable awake state by shuttling Sandman to the membrane, where it could mediate potassium efflux and chronically inhibit firing of these sleep promoting cells (Figure 2B). This study therefore uncovered a molecular mechanism that could explain two different aspects of this electrical brain switch - how animals can *transition quickly* to an awake state (which must be adaptive for an animal, in case there is a need to escape sudden danger), and how an animal can switch to a more *sustained, awake state*. Another question is how an animal can quickly transition to being asleep. A recent study that found the transition from wake to sleep involves electrical signalling (via gap junctions in the dFB), which help to initiate sleep by decreasing the fly's behavioural responsiveness while it is still awake (Troup et al, 2018). This suggests that the 'sleep switch' uses both electrical signals (gap junctions) and chemical signals (e.g. dopamine) to rapidly transition between wake and sleep. Whether or not a similar molecular mechanisms exists in mammalian sleep circuits is not yet known.

Feeling the pressure to sleep

Switching appropriately between states of wake and sleep is one problem, but how does a brain detect how much it needs to sleep? In mammals, sleep pressure – the need for sleep - is reflected in the amplitude of slow-wave activity, which is greatest immediately following

sleep initiation and dissipates as sleep proceeds. The systems by which the brain detects sleep pressure seem to be complex, involving changes in a number of neuromodulators, such as adenosine, and widespread brain systems that detect sleep pressure at a local and global level (Dissel & Shaw, 2016).

In flies, the sleep homeostat appears to be much simpler, hinging on a few key subsets of neurons that detect sleep pressure in the form of changes in neuronal excitability and synaptic strength. One of these subsets is, again, the sleep-promoting neurons of the dFB (Figure 2A). Compared to well-rested flies, the dFB neurons in sleep deprived flies are more excitable, reflecting an increased need for sleep (Donlea, Pimentel, & Miesenböck, 2014; Figure 2B). This already suggests a simple circuit for feedback control, whereby sleep pressure can be detected in the same neurons that induce sleep. More recently, another component of the sleep homeostat was identified in the ellipsoid body, a donut-shaped structure in the central complex. A subset of ring-like neurons of the ellipsoid body, called the R2 neurons (Figure 3A), specifically appeared to encode sleep pressure, for two reasons (Liu, Liu, Tabuchi, & Wu, 2016). Firstly, activating these neurons dramatically increased sleep specifically *after* activation (resembling sleep rebound that occurs following sleep deprivation) but did not increase sleep *during* activation (unlike the dFB neurons, which rapidly put flies to sleep when switched on). Secondly, 'sleep pressure' was reflected in the levels of calcium activity, synaptic proteins and NMDA receptors of these R2 neurons, which were increased in sleep-deprived flies (Figure 3B). In other words, dFB neurons detect sleep pressure, which is generated by the R2 neurons. This suggests that neuronal excitability, calcium levels, and synaptic strength in these central brain neurons serve as markers for sleep pressure in flies.

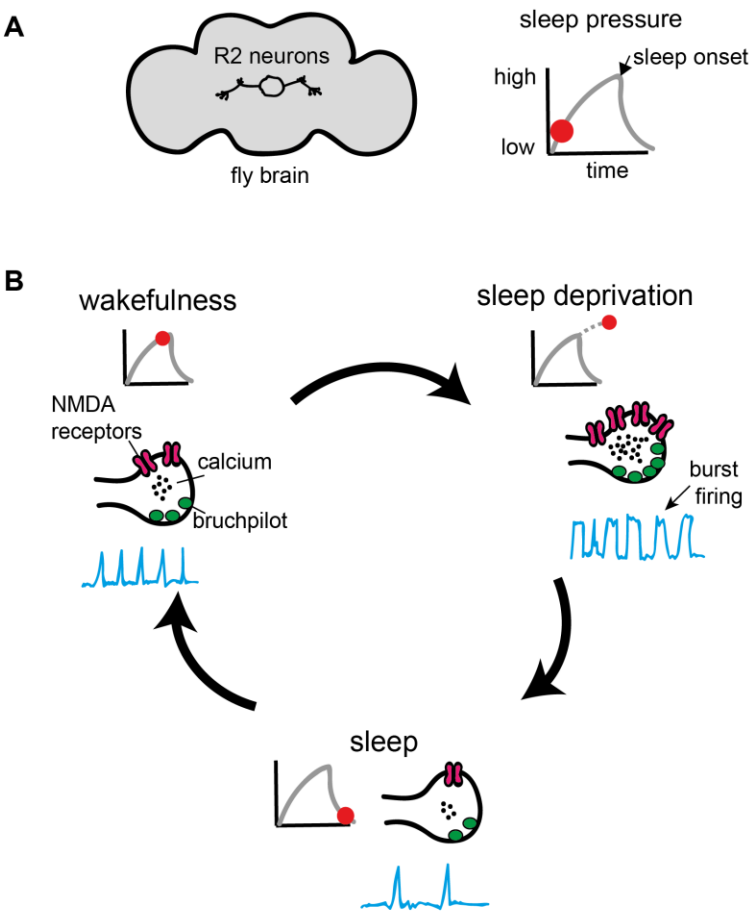

Figure 3. A circuit that signals sleep pressure. A) A group of neurons in the central brain, 'R2 neurons', generate sleep pressure in *Drosophila*. Sleep pressure gradually accrues during wake (sleep pressure level is indicated by the red dot), reaching a threshold at which sleep onset is triggered, after which sleep pressure declines back to baseline levels. B) Changes in sleep pressure are encoded by changes in electrical activity of the R2 neurons, as well as the levels of NMDA receptors, calcium and the presynaptic protein, *bruchpilot* with the cells (Liu et al., 2016). Sleep deprivation leads to an increase in sleep drive, and is associated with increased levels of NMDA receptors, *bruchpilot* and calcium within the R2 cells. In sleep deprived flies, R2 neurons show more frequent action potentials, including bursting firing events. Following sleep, when sleep pressure has dissipated, levels of NMDA receptors, *bruchpilot* and calcium are reduced, and the R2 neurons become less active (having a reduced firing rate).

A sleep pressure circuit

A further piece of the puzzle in understanding how a brain 'knows' how much it needs to sleep was uncovered in a recent study, which identified recurrent connections between sleep-inducing dFB neurons and neurons that generate sleep pressure, the R2 neurons. It was found that dFB neurons inhibit a group of neurons called helicon cells, which form excitatory synaptic connections with the R2 neurons (Donlea et al., 2018). Interestingly, helicon cells were found to respond to visual input, which is gated by the dFB neurons, and were also found to be permissive for locomotion. This suggests a mechanism by which both visual responsiveness and locomotor activity can be suppressed or gated during sleep (Figure 4). Furthermore, it suggests that visual stimulation (via neurons such as the helicon cells) and probably other types of neural stimulation, can be transmitted to R2 neurons, where sleep pressure builds up (Figure 4). This system has been likened to a relaxation oscillator in an electronic circuit (Donlea et al., 2018). Here, a build-up of electrical charge in a capacitor (like a build-up of sleep pressure in a neural circuit) reaches a certain threshold, which then suddenly releases the charge through a neon bulb, for example (just as the dFB switches to the 'on' state to promote sleep).

This model of a 'sleep pressure circuit' in the fly brain raises further intriguing questions. How do molecular markers of sleep pressure get converted into an electrical signal that drives sleep? Does sleep pressure accrue in similar ways in the mammalian brain? Is there just one region where sleep pressure accrues in the fly brain, or could there be other pressure points in the brain that drive sleep need? It seems unlikely that this small cluster of R2 neurons in the central brain would be the only neurons that accrue sleep pressure to signal when sleep is needed. In line with this argument, another study found that groups of cholinergic neurons from widespread brain regions also drive sleep need (although this was only studied behaviorally – activating these neurons increased sleep rebound (Seidner et al., 2015). It

remains to be determined whether molecular and electrical signs of sleep pressure build up globally across the fly brain, or whether these signals accrue more specifically in certain types of neural circuits.

The link between sleep and attention in the brain

If there are any particularly vulnerable 'pressure points' in the brain, it is likely to be circuits that are required for selective attention processes. Selective attention requires precise coordination of selection and suppression dynamics to ensure that the appropriate stimulus is attended while irrelevant stimuli are ignored, so any malfunctioning in these circuits would be highly maladaptive to an animal. It has been suggested that sleep is "the price we pay for plasticity" (Tononi & Cirelli, 2013), so it is likely that sleep drives plastic changes in attention circuits to prevent them from functioning sub-optimally. Indeed, attention processes in mammals are particularly vulnerable to sleep deprivation (Lim & Dinges, 2010). In other words, attention circuits may be a 'weak link' in the system that must signal when sleep is needed. It is now clear that flies also have a selective attention, as they can selectively focus on a single visual object while filtering out others (de Bivort & van Swinderen, 2016; Sareen, Wolf, & Heisenberg, 2011; Bruno van Swinderen, 2011). Interestingly, sleep deprivation impairs visual selective attention in flies while leaving basic visual processing intact (Kirszenblat, Ertekin, Goodsell, Zhou, Shaw & van Swinderen, 2018). This suggests that optimizing attention is a conserved function of sleep among different species and that the fly provides a valuable model for studying the interaction between attention and sleep.

Recent evidence in the fly suggests that sleep and attention share some of the same neural substrates. One key brain region in which sleep and attention circuits seem to converge is the central complex. Within this structure, neurons called 'wedge neurons' of the ellipsoid body

have been identified which show a dynamic 'bump' of activity that mapped onto a virtual object that the fly fixated upon behaviorally; the bump could switch between different visual objects, suggesting attention-like dynamics (Seelig & Jayaraman, 2015). Upstream of these wedge neurons, also within the ellipsoid body, are different types of 'ring neurons' that appear to be involved in visual stimulus selection/suppression (Shiozaki & Kazama, 2017; Sun et al., 2017), and have also been shown to be important for behaviors that involve visual memory (Neuser, Triphan, Mronz, Poeck, & Strauss, 2008; Ofstad, Zuker, & Reiser, 2011). These studies suggest that the ellipsoid body is a key structure involved in selective attention. As mentioned earlier, it is also a place where sleep pressure accrues (S.Liu et al., 2016), Figure 3).

Neurons that feed into the ellipsoid body also appear to be involved in both sleep and attention. Interestingly, it was also discovered that a subgroup of neurons upstream of the ellipsoid body, called the tubercular bulbar, or "TuBu" neurons promote sleep, and appear to *relieve* sleep pressure following activation (the flies showed a 'negative rebound' after sleep had been induced via TuBu activation) (Lamaze, Krätschmer, & Jepson, 2018). Supporting evidence came from the lab of Michael Rosbash, who found that activation of a sleep promoting circuit involving TuBu neurons lead to increased arousal thresholds and calcium oscillations in the ellipsoid body (Guo, Holla, Diaz, & Rosbash, 2018). Sleep promoting TuBu neurons project specifically to the *superior* region of a structure called the bulb, where outer ring neurons of the ellipsoid body receive input (Omoto et al., 2017). Interestingly, these neurons are also involved in selecting or suppressing competing visual stimuli, a mechanism that underpins selective attention (Shiozaki & Kazama, 2017; Sun et al., 2017). Furthermore, these neurons were activated during choice behavior (or action selection). Artificial activation of these neurons may therefore induce a sleep state by interfering with

the normal temporal dynamics of activity in these neurons, blocking the processing of stimuli.

The above studies thus suggest a circuit mechanism by which the sleep state could be brought about by disrupting visual processing and action selection (Figure 4). Interestingly, sleep promoting TuBu neurons (which, as described above, are also involved in visual attention processes) are also connected to neurons that regulate sleep pressure - the R2 neurons of the ellipsoid body (Figure 4) (Donlea et al., 2018; Guo et al., 2018; S.Liu et al., 2016). Altogether, this supports the idea that sleep and attention circuits are interconnected, they influence each other, and they likely use common mechanisms to suppress perception (Kirszenblat & van Swinderen, 2015). One interesting avenue for future research would be to examine exactly how sleep maintains attention within an optimal range. For example, one prediction is that synaptic plasticity during sleep alters the dynamics of stimulus selection in the TuBu and ring neurons.

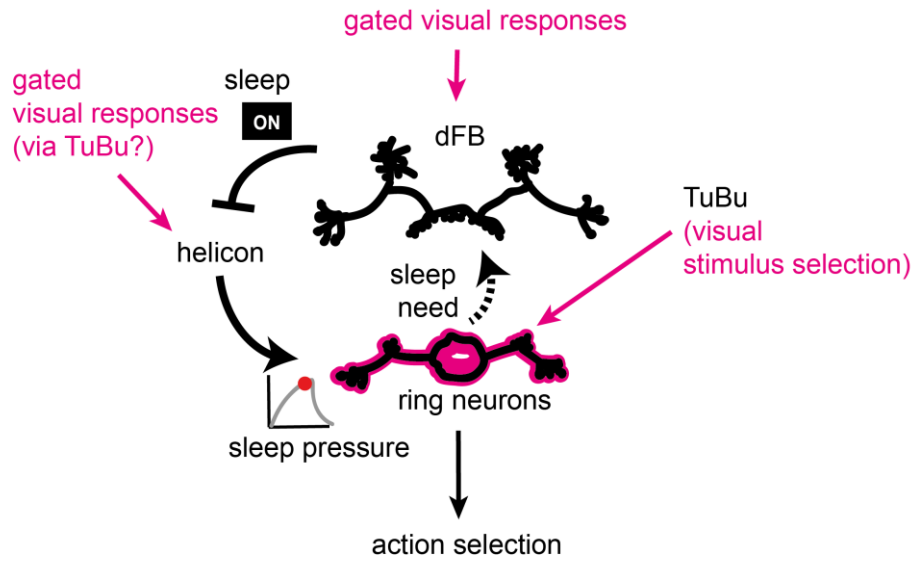

Figure 4. Central brain circuits regulate sleep and gate visual responses. When the dFB 'sleep switch' is active (in the 'on' state) it inhibits the helicon cells (via release of the sleep promoting neuropeptide, Allatostatin); inhibition of helicon cells increases arousal thresholds and reduces sleep pressure (Donlea et al., 2018). When the dFB is silent (i.e. during wakefulness), visual signals flow through helicon cells to the ring neurons of the ellipsoid body (Donlea et al., 2018), where sleep pressure builds up in the R2 neurons (Liu et al., 2016). Sleep pressure is signalled to the dFB by unidentified pathways. These sleep circuits are also involved in guiding the fly's responses to visual stimuli. dFB neurons normally respond to different types of visual input, gated by behavioral state (Weir & Dickinson, 2015; Weir et al., 2014). Visual stimuli are selected and suppressed via the TuBu neurons, which signal to ring neurons in inner and outer layers (Shiozaki & Kazama, 2017; Sun et al., 2017) to guide action selection, but TuBu neurons can also promote sleep (Guo et al., 2018, Lamaze et al., 2018). Helicon cells also respond to visual cues via upstream pathways (potentially the TuBu neurons or parallel pathways), but their responses are suppressed during sleep (Donlea et al., 2018).

The interaction between circadian and homeostatic drives to sleep

Like humans and other animals, sleep in flies is regulated by circadian rhythms (see Dubowy & Sehgal, 2017 for a comprehensive review). Patterns of activity and sleep are regulated by the 'clock' neurons. The fly brain has about 150 clock neurons – far fewer than in the mammalian brain - yet they share many physiological and functional similarities (Nitabach & Taghert, 2008; Vansteensel, Michel, & Meijer, 2008).

How does circadian timing of sleep interact with our need for sleep – the sleep homeostat? Studies in *Drosophila* have shown that sleep homeostasis and circadian timing of sleep operate as separate systems (Hendricks et al., 2000; Shaw et al., 2000). For example, we know that mutations in some genes (e.g. *Shaker*; Cirelli et al., 2005) affect the total amount of sleep without affecting circadian rhythms.

However, sleep homeostasis and circadian rhythms also seem to interact with each other. For example, flies with mutations in the circadian gene, *cycle*, show an excessive sleep rebound after only 3 hours of sleep deprivation (Shaw, Tononi, Greenspan, & Robinson, 2002). Furthermore, clock neurons in the fly brain may act as substrates for the accumulation of sleep pressure because there is an increase in synaptic terminals measured after extended

wakefulness or social interaction, and a decrease following sleep (Donlea, Ramanan, & Shaw, 2009; Gilestro, Tononi, & Cirelli, 2009). This suggests that clock neurons may *detect sleep pressure* that accumulates as a result of the fly's waking experience, although it is possible that this is a general phenomenon, not specific for clock neurons. Clock neurons have also been found to signal to circuits involved in sleep homeostasis (specifically, the TuBu and R2 neurons) to control sleep drive and arousal thresholds (Guo et al., 2018; Lamaze et al., 2018). Overall, these studies confirm that sleep need is influenced by circadian processes, supporting what is known from studies in mammals. One problem that arises is where to draw the line in determining where a circadian circuit ends and a sleep circuit begins. Indeed, how do we know what a sleep circuit really is, since so many factors appear to affect sleep?

How can we know what a 'real' sleep circuit is?

As studies of sleep in *Drosophila* gain momentum, more and more neurons are being classified as having a role in regulating sleep. This may in part be due to the fact that sleep has mostly been quantified by examining locomotion – a broad category of behavior that could be affected for many different reasons.

One example of a brain region that has been identified as regulating sleep, but would be potentially misleading to call a 'sleep circuit' is the mushroom bodies. The mushroom bodies were one of the first neuroanatomic structures identified to regulate sleep in flies (Joiner, Crocker, White, & Sehgal, 2006; Pitman, McGill, Keegan, & Allada, 2006). However, recognition for its role in sleep regulation has dwindled somewhat in comparison to the central complex. Activation of the dFB, for example, not only renders flies immobile, but also suppresses behavioral and neural responses to stimuli and is associated with brain

activity distinct to sleep (Donlea et al., 2011; Yap et al., 2017), thus fulfilling several criteria of sleep. In contrast, studies of the mushroom body did not closely examine arousal responses, with the exception of one study that found that neurons of the ellipsoid body, but *not* the mushroom body, promote startle-induced arousal (Lebestky et al., 2009). This suggests that arousal responses and locomotor activity are regulated by distinct systems, and that the mushroom body primarily affects locomotor activity. Interestingly, one study found that the subsets of mushroom body output neurons (MBONs) that are 'sleep-promoting' are the same neurons that promote *attraction* to odors, whereas 'wake promoting' subsets signal *aversion* (Aso et al., 2014). This raises an important issue in the identification of sleep circuits: when we are using inactivity as a readout of sleep, how can we know if manipulation of a particular neuron is actually *waking an animal from sleep* or whether it is simply signalling (to an animal that may already be awake, but immobile) that it should run away from an aversive stimulus?

In other words, it is possible that the mushroom bodies, rather than regulating sleep/wake transitions *per se*, are involved in perceiving aversive or attractive stimuli that guide a fly's decision to move or not. Since sleep switches off perception of the external world, it would therefore be expected that sleep may dampen the ability of the mushroom bodies to perceive odor information. Indeed, a study performed in vivo imaging of mushroom body neurons that encode information about odors ('kenyon cells'), and found that the neurons go 'offline' during sleep, responding less to stimuli such as oxygen and vinegar (Bushey et al., 2015). This suggests that mushroom bodies may be key circuits that are *switched off* during sleep. This may be true for many other behavioral circuits that are only indirectly linked to sleep.

Even more provocatively, we might question whether the dFB should really be called a 'sleep switch'. Fan-shaped body neurons have been shown to be involved in processing visual stimuli (Liu et al., 2006; Weir & Dickinson, 2015; Weir, Schnell, & Dickinson, 2014), so

how could they also drive sleep? One idea is that inducing sleep by unilaterally activating the dFB works by blocking the neurons' normal processing and sorting of information (such as visual stimuli). This is potentially the same phenomenon that may be occurring in the TuBu neurons, as discussed earlier. Could it be that activating the TuBu neurons or the dFB neurons disrupts their normal stimulus processing functions, and that this mimics a transition into sleep (associated with a loss of responsiveness)? Maybe there is no single sleep switch, but rather a number of different brain areas that, when coordinated, guide waking behavior, and when out of joint promote sleep. This would be consistent with the view that one important function of sleep is to maintain synaptic or circuit functions across the brain within an adaptive set point.

One important endeavour for future studies will be to understand how these sleep circuits interact to promote transitions in and out of sleep. Another challenge will be to understand how these circuits promote sleep functions, including memory consolidation and synaptic homeostasis.

What can flies tell us about sleep functions?

Why do we, and most other animals, need sleep? What does sleep accomplish in our brains and bodies? Arguably the most exciting goal of sleep research in flies is to uncover conserved *functions* of sleep. Understanding sleep functions can be achieved by asking different types of questions. We can ask how lack of sleep affects cognition and behavior of an animal. We can then ask how sleep or a lack of it affects the brain, circuits, cells and molecules. Another approach to studying sleep function is to ask what kind of experiences drive the need for sleep. All these questions can easily be addressed in *Drosophila*.

Sleep for learning and cognitive behaviors

It is well known that sleep is important for learning and memory, but there is still little known about the mechanisms involved. *Drosophila* has been successfully used as a model to understand the biological processes of how memories are encoded, stored and retrieved. Surprisingly, however, there has been much less focus on understanding sleep's role in memory, in comparison to the number of studies that have focussed on identifying mutants that are either long and short sleepers. One way to study the effect of sleep on memory is by looking at the effects of sleep deprivation. There are various methods for sleep depriving flies, which essentially rely on mechanically disturbing the flies using automated devices. One limitation with these methods is that it is likely to be stressful for the flies, so various methods have been used to control for stress effects (such as comparison to other forms of stress such as heat or starvation, or to less stressful perturbations such as gentle handling).

Studies in which flies have been sleep deprived have essentially confirmed that sleep deprivation is detrimental to brain functioning. Flies that are sleep deprived perform poorly on a variety of learning tasks (for a review, see Dissel, Melnattur, & Shaw, 2015). For example, sleep deprived flies are worse at courtship learning, in which repeated exposure of a male fly to an unreceptive female leads the male to suppress courtship behavior (Ganguly-Fitzgerald, Donlea, & Shaw, 2006).

Another way to study the effects of sleep is to artificially *increase* sleep. As mentioned earlier, it is possible to increase sleep by genetically activating sleep-promoting neurons. Surprisingly, when flies are forced to sleep (using this genetic method) following a courtship learning protocol that was normally not sufficient to induce long term memory, they are able to form long term memories (Donlea et al., 2011). This shows that this genetic form of sleep is a powerful method that can, by itself, drive memory consolidation. Even more

surprisingly, enforced sleep in mutants that have learning and memory deficits, is able to restore normal learning in these flies without fixing their underlying brain problems (Dissel, et al., 2015). This suggests that sleep may allow the brain to find alternative pathways for promoting both synaptic and behavioral plasticity and forming memories.

Apart from its well-studied role in learning and memory, sleep in humans is also thought to be important for the regulation of mood and emotion (Beattie, Kyle, Espie, & Biello, 2015). Although it is difficult to know if flies have emotions, they seem to have building blocks of emotion, called 'emotion primitives' – persistent, internal states that are manifested in particular behaviors, such as aggressive behavior (e.g. fights between male flies) or as fear-like responses (e.g. retreat and escape from a predator) (Anderson & Adolphs, 2014). Interestingly, one study has found that sleep deprivation lowers aggression in male flies, reducing lunges towards other males (Kayser, Mainwaring, Yue, & Sehgal, 2015). Although aggression among humans is often assumed to be bad - being associated with violent and destructive behavior - it is easy to forget that aggression can be a survival mechanism that drives an animal to achieve its goals. In the above study by Kayser et al (2015), sleep deprived males that were less aggressive were also less successful in reproducing with a female when they were placed in competition with a well-rested fly. Understanding how aggressive and fear-like behaviors in flies are modulated by sleep may give clues to evolutionarily ancient mechanisms that underlie the influence of sleep on emotional states.

Sleep for synapses

Of course, the advantage of the fly is that studies of sleep functions need not be limited to observing effects on animal behavior – one can also probe the functions of sleep at the *cellular and molecular level*. One compelling theory of sleep function is the need for

synaptic homeostasis (Tononi & Cirelli, 2013). The essence of the theory is that, during wakefulness, there is a net increase in synaptic strength and that this is countered by sleep, during which synaptic connections are proportionally 'downscaled' or 'renormalized' throughout the brain. This synaptic renormalization process is thought to be important to conserve brain resources, avoid saturation of learning, and to aid memory consolidation. Although the theory is still controversial, several studies in flies as well as other animals have contributed significantly to validating the theory of synaptic homeostasis. Some of the first evidence came from flies, where it was shown that wakefulness is associated with widespread increases in synaptic proteins across the brain, whereas sleep lead to a reduction in synaptic proteins (Bushey, Tononi, & Cirelli, 2011; Donlea et al., 2009; Gilestro et al., 2009).

Sleep for neuronal circuit function

Aside from understanding its more global molecular functions, we can understand sleep by studying its impact on neurons and circuits in the brain. When a fly is sleep deprived, its neurons start to respond less reliably, as though parts of the brain are shutting down (Bushey et al., 2015). This could indicate that the brain is entering what is known as 'local sleep', and may explain why circuits don't function properly and memories can't be formed in sleep deprived flies. It may be that neurons switch off because they need to rest, just like muscles after exercise, in order to maintain their firing properties within a normal range (Vyazovskiy & Harris, 2013). We also know that sleep may alter neuronal activity by altering the neuromodulatory environment. Interestingly, it has been shown that sleep suppresses activity of dopaminergic neurons that are involved in forgetting - in other words, it stops the normal forgetting processes that occur during the day, thereby enhancing memory consolidation (Berry, Cervantes-Sandoval, Chakraborty, & Davis, 2015). This supports the idea that sleep

shuts out the world to prevent 'retroactive interference' (new learning that interferes with previous learning (Mednick, Cai, Shuman, Anagnostaras, & Wixted, 2011)), and as such, allows memory consolidation to occur.

One area of research into sleep function that is lagging in flies is the study of neuronal replay. Neuronal replay is when neurons that fired in a particular pattern are reactivated in the same pattern, often during rest or sleep. Since neuronal replay is crucial for memory processing during sleep in humans and rodents (Atherton, Dupret, & Mellor, 2015; Breton & Robertson, 2013), it seems likely that flies may use a similar mechanism for memory consolidation. However, neural replay has not yet been investigated in flies.

Other functions of sleep

Although many sleep functions are likely linked to brain function, other functions of sleep may be more related to general health of an animal and its ability to respond to environmental stress (see Julie Williams' chapter on sleep and stress). A recent study also found that shorter sleepers are more sensitive to oxidative stress, and increasing sleep improved survival of flies exposed to oxidative stress (Hill et al, 2018). Flies sleep more after stressful situations, such as bacterial infection (Kuo, Pike, Beizaeipour, & Williams, 2010) and exposure to heat (Lenz, Xiong, Nelson, Raizen, & Williams, 2015). Since even the simple nematode, *Caenorhabditis elegans*, shows a sleep-like state following similar types of stressors in the environment (Hill, Mansfield, Lopez, Raizen, & Buskirk, 2014; Nelson et al., 2014), it is likely that stress-related functions of sleep are evolutionarily ancient.

Another potentially ancient function of sleep is metabolite clearance, which is thought to remove toxic proteins from the brain during slow wave sleep (Xie et al., 2013). Slow wave sleep has not been identified in flies, and it is not known whether metabolite clearance occurs

during fly sleep. However, a recent study (Zhang, Yue, Arnold, Artiushin, & Sehgal, 2018) showed circadian changes in blood brain barrier permeability in *Drosophila* – making their brains more permeable to xenobiotic substances at night - suggesting they may have related mechanisms in place. Whether or not homeostatic sleep drive (or a state that resembles mammalian slow wave sleep) influences the blood brain barrier and aids clearance of endogenous toxins in flies is still not known.

What behaviors promote sleep?

Another way to understand sleep function is by seeing what *drives the need to sleep*. The need for sleep in flies seems to be influenced by a number of different cues in their environment. One major factor is a fly's social experience. Flies that are reared socially sleep more than flies raised in isolation (Donlea et al., 2009; Ganguly-Fitzgerald et al., 2006). This is probably because social interactions involve some of the most complex and adaptive behaviors that flies engage in, such as courtship (a surprisingly elaborate process), and fighting between males. These complex interactions require learning and plasticity in the brain, which, according to the synaptic homeostasis theory, should drive the need for more sleep. Indeed, social experience in flies is associated with an increase in synaptic proteins and dendritic growth in the fly brain (Bushey et al., 2011; Donlea et al., 2009), which are downscaled during sleep. Further evidence come from studies of flies with mutations in genes required for learning. Interestingly, learning mutants do *not* show changes in sleep or increases in synapses when grown in social environments (Donlea et al., 2009; Vanderheyden, Gerstner, Tanenhaus, Yin, & Shaw, 2013). These studies are all consistent with the idea that an animal's experiences during wakefulness drive plastic changes in the brain that need to be curated by sleep.

Changes in sleep need may also be reflected in the quality of sleep. Interestingly, social experience also make flies sleep more deeply, specifically in males (van Alphen et al., 2013). This suggests that there is something about male behavior – engagement in courtship and fighting – that drives the need for deeper sleep (how sleep intensity is measured in flies is discussed below). It follows that sleep must be important for these behaviors; indeed, as mentioned earlier, sleep is required for aggression in male adults (Kayser et al., 2015), for courtship learning in adults (Seugnet, Galvin, Suzuki, Gottschalk, & Shaw, 2009; Seugnet, Suzuki, Vine, Gottschalk, & Shaw, 2008) and for development of normal courtship behavior in young flies (Kayser, Yue, & Sehgal, 2014). One interesting aspect of courtship and fighting is that they involve situations in which a fly must detect valence (whether an event or object is 'good' or 'bad') – for example the attractiveness of a potential mate during courtship, or the threat of a male competitor. It is therefore possible that an important function of sleep is to regulate valence in flies, by modifying valence circuits in the brain that control signals of reward or punishment. One finding that supports this idea is that sleep related changes in aggression are signalled through octopamine (Kayser et al., 2015), a neurotransmitter related to norepinephrine that signals reward (Schwaerzel et al., 2003). This suggests that future studies in flies should examine plasticity of valence circuits and how they are altered during sleep.

Although flies may sleep more after certain experiences, reflecting a need for more sleep, the opposite can also occur: some environmental cues can suppress sleep. For example, male flies that have been sleep deprived normally need to 'catch up' with a nap afterwards, but this need for extra sleep can be suppressed by exposing them to a female pheromone that raises their sexual arousal (Beckwith et al., 2017). A recent study dissected the neural pathways underlying this effect, showing that P1 neurons (which encode increased sexual arousal) inhibit sleep promoting DN neurons (Chen et al, 2017). In the same study it was shown that

sleep deprivation could inhibit P1 neuron activity, revealing a bi-directional relationship between sleep and sexual behaviour. Another condition that can also suppress sleep in flies is starvation (Keene et al., 2010), and there is some evidence that this may occur via upregulation of the amino-acid, serine in the brains of starved flies (Sonn et al, 2018). Overall, these studies suggest that sleep may have to compete with other survival needs like food and sexual reproduction. Nevertheless, skipping sleep in these cases may not be ideal in the long term and may still come at the price of learning and plasticity (Tononi & Cirelli, 2013).

In summary, sleep achieves a large number of different functions, many of which appear to be evolutionarily ancient. It is possible that through evolution, some of these functions got 'packaged' into distinct stages of sleep. For example, in humans and other mammals, there is evidence that rapid-eye movement (REM) sleep is important for regulation of mood and emotional memories (Beattie et al., 2015), whereas slow-wave sleep is thought to be important for metabolite clearance (Xie et al., 2013), synaptic downscaling (Tononi & Cirelli, 2013) and consolidation of declarative memories (Diekelmann & Born, 2010). What are the evolutionary roots of these different types of sleep? One approach is to find out whether flies also have different kinds of sleep, and whether this may provide clues to ancient functions of sleep.

Do flies have sleep stages?

'Sleep' is often used as an all-encompassing word to describe a behavioral state which, in reality, is actually a series of very different brain states that appear to have distinct functions. These different brain states – which include slow wave sleep and REM sleep - are not specific to humans; they have been found in many other animals, including all other

mammals, most birds (Vorster & Born, 2014), and possibly even some reptiles (Shein-Idelson, Ondracek, Liaw, Reiter, & Laurent, 2016), suggesting they evolved early in evolution. However, it is still not clear whether flies or other insects display slow wave sleep, or whether they might use alternative forms of sleep to perform similar functions.

Deep sleep in flies

Until recently, sleep in *Drosophila* had been treated as a homogenous 'ON' or 'OFF' state – a fly was considered simply to be either sleeping or awake. As discussed earlier, this was partly due to the limitations of the tools used to measure fly sleep, which was achieved mostly by measuring a fly's locomotor activity. The first investigation into whether flies might have different sleep stages came from a study by van Alphen et al. (2013). This was made possible by a new behavioral platform that continuously filmed the flies' behavior while regularly probing their behavioral responsiveness to mechanical stimuli (Figure 5).

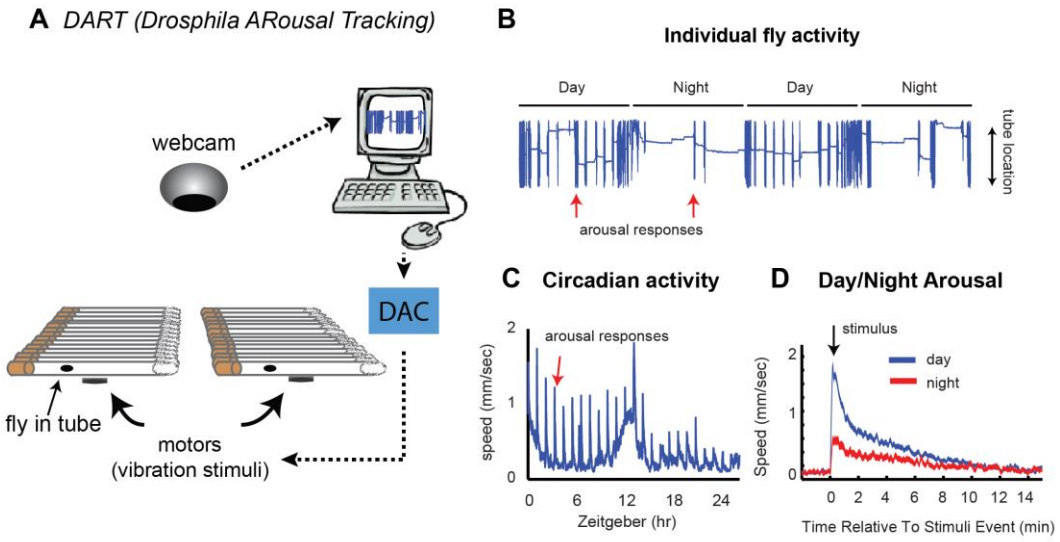

Figure 5. Measuring arousal in *Drosophila*. The *Drosophila* ARousal Tracking (DART) system measures arousal by probing responses to mechanical vibrations (Faville et al., 2015). A) In the DART set up, flies in tubes are filmed from above, while a computer and digital analogue converter (DAC) control motors beneath the flies, which vibrate every hour to measure arousal responses during sleep. B) Example activity trace of an individual fly across the day and night. Flies are more often aroused during the day than the night (red arrows). C) Activity is quantified as the average speed of the population, with arousal responses evident every hour as spikes in activity. D) The amplitude and dynamics of day time and night time arousal responses can be measured.

This new paradigm allowed the following question to be asked: do flies have different levels of behavioral responsiveness that could indicate lighter or deeper sleep? In humans, sleep cycles through different stages, with deep sleep occurring in the beginning of the night (Figure 6A). Interestingly, van Alphen et al (2013) found that arousal thresholds in flies were also highest at the beginning of the night, and appeared to cycle through different levels of arousal (Figure 6B). When looking across the population of flies, van Alphen et al (2013) found that flies showed different levels of sleep intensity depending on how long they had been immobile, with flies being the least reactive (and hence judged to be having more intense sleep) after ~20 minutes of sustained immobility (Figure 6C). This study provided clear evidence that fly sleep is not a homogenous state, but is dynamic and shows stages of lighter and deeper sleep. Interestingly, sleep intensity was not necessarily correlated with time immobile: sleeping flies could become relatively more responsive at various times during a prolonged bout of immobility, especially after 40 minutes in the Canton S wild-type strain. The finding that sleep duration doesn't necessarily correlate with sleep depth is interesting and suggests that simply using immobility as a sleep measurement may not reveal the full picture about sleep quality. To gain a better understanding of sleep quality, electrophysiological readouts of sleep stages are needed. The combination of behavioral and electrophysiological readouts of different types of sleep in flies should provide a platform for

understanding the functions of sleep stages, shedding light on how and why different sleep stages evolved.

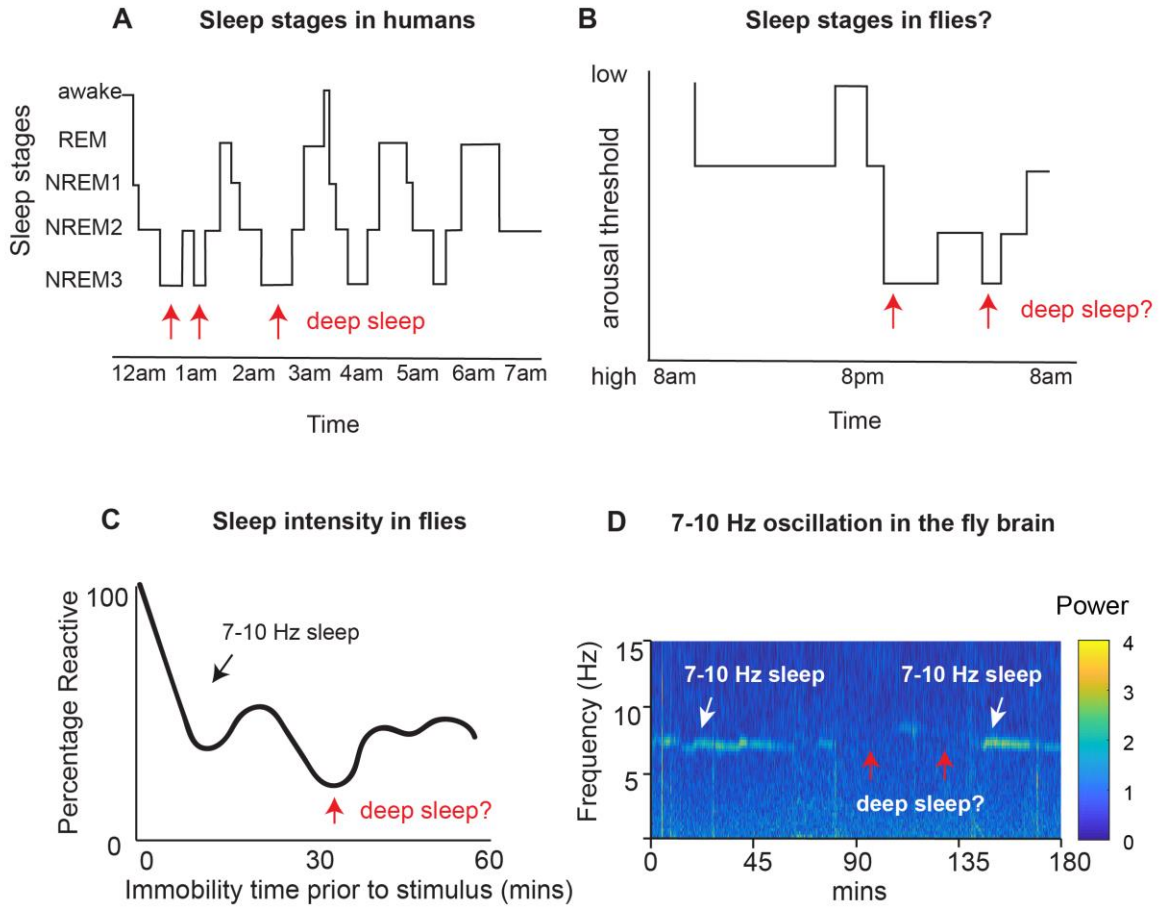

Figure 6. Light and deep sleep stages in flies and humans. A) Humans cycle through different sleep stages, with the deepest sleep stage (NREM3) characterised by lower arousal and increased slow-wave activity. B) Flies alternate between light and deep sleep (van Alphen et al., 2013) depending on the time of day or night, as measured by arousal threshold to mechanical stimuli of different strengths, and C) how long they have been immobile prior to an arousing stimulus (here, sleep intensity is quantified by the percentage of flies reactive to a mechanical stimulus) (van Alphen et al., 2013). C) Flies show different levels of arousal, with deepest sleep (when they are least reactive to a stimulus) occurring at approximately 30 minutes after sleep onset in wild type flies. 7-10 Hz sleep, as shown in (D), is highest following sleep onset and just prior to waking, suggesting flies may have distinct stages of sleep (Yap et al., 2017). D) Representative brain recordings in an individual fly show differences in oscillatory brain activity during sleep. A 7-10 Hz oscillation is sometimes present (white arrows) due to an increase in power within the 7-10 Hz frequency range, while other epochs lack this oscillation (possibly indicating deep sleep).

Brain activity linked to sleep stages in flies

Although the above study showed that flies have light and deep sleep, it did not identify electrical signatures of sleep stages, as has been done in humans. In humans, slow wave activity predominates during stage 3 of NREM sleep (Carskadon & Dement, 2011), while wake-like brain activity is indicative of REM sleep. However, until recently, it was not known whether flies display different kinds of brain activity during sleep. This question was addressed by a recent study in which electrodes were implanted into the brain of the fly to record local field potentials (LFPs) during sleep and wakefulness (Yap et al., 2017). Interestingly, while sleep was found to be associated with an overall decrease in the amplitude of LFP activity, confirming previous results (Nitz et al., 2002; van Alphen et al., 2013; van Swinderen et al., 2004), Yap et al. also observed increased oscillatory activity in the central brain of a number of flies, in the frequency range of 7-10 Hz (Figure 6D). Importantly, this 7-10 Hz oscillation was only observed in sleeping flies (not flies that were awake and immobile), and seemed to wax and wane during sleep. The 7-10 Hz oscillation predominated immediately after a fly fell asleep, and just before it woke up, and was comparatively less evident in the middle of a sleep bout – suggesting that it may define a transitional sleep stage.

Distinct forms of induced sleep in flies

In humans, anti-insomnia drugs can be used to promote specific sleep stages – for example, the GABA agonist, gaboxadol has been shown to increase slow wave sleep (Walsh, Deacon, Dijk, & Lundahl, 2007). This raised an intriguing question: could inducing fly sleep using similar drugs produce equivalent sleep stages in flies? If the same drug that could induce slow wave sleep in humans could induce the 7-10 Hz oscillation in flies, this might suggest

that these sleep stages could be analogous and performing similar functions. However, when Yap et al. induced sleep with the anti-insomnia drug gaboxadol, they observed primarily a decrease in LFP amplitude, without the enhanced 7-10 Hz oscillation, suggesting this was not the case. Instead, they saw that the 7-10 Hz oscillation was increased with *genetic* induction of sleep – when the dorsal fan shaped body (the 'sleep switch'; Figure 2A) was activated. Intriguingly, both gaboxadol and genetically induced sleep have been shown to enhance learning and plasticity in memory mutants (Dissel, et al., 2015). This suggests that the global reduction in LFPs may have more to do with this sleep function, whereas the 7-10 Hz oscillation (which was only observed in genetically-induced sleep) may serve a different function altogether. Another explanation is that, considering that gaboxadol enhances slow wave sleep in humans, it is possible that the global reduction in LFPs seen in flies exposed to gaboxadol may serve a similar function to slow wave sleep in humans. Slow wave sleep essentially reflects synchronous 'down states' (periods of inactivity) of large populations of neurons, that may allow brain cells to rest (Vyazovskiy & Harris, 2013) and undergo synaptic homeostasis (Tononi & Cirelli, 2013). It is possible that gaboxadol-induced decreases in LFP achieve the same effect in flies, and may therefore accomplish key slow wave sleep functions. In contrast, the 7-10 Hz sleep stage might serve a different purpose that is more related to transitioning between wake and sleep, by blocking information processing and thereby allowing a fly to suppress responsiveness to the external world.

Summary

Sleep is a complex behavioral phenomenon that involves the whole brain. Yet one of the major discoveries in fruit flies is that only a handful of neurons in the central brain can act as a 'sleep switch' – quickly transitioning animals between sleep and wakefulness. Emerging

evidence suggests that this 'sleep switch' is connected to central brain circuits that generate sleep pressure, telling a fly when it needs to sleep. Intriguingly, many of these key sleep circuits are also crucial for coordinating behavior during wakefulness, by regulating the selection, suppression and integration of sensory stimuli in order to select appropriate actions. One important endeavour for future studies will be to understand the electrical, molecular and circuit mechanisms underpinning this correspondence between wake and sleep.

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