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From genetics to structure to function: Exploring sleep in Drosophila

Daniel Bushey and **Chiara Cirelli**
Department of Psychiatry, University of Wisconsin/Madison, Wisconsin, U.S.A.

Outline

Sleep consists of quiescent periods with reduced responsiveness to external stimuli. Despite being maladaptive in that when asleep, animals are less able to respond to dangerous stimuli, sleep behavior is conserved in all animal species studied to date. Thus, sleep must be performing at least one fundamental, conserved function that is necessary, and/ or whose benefits outweigh its maladaptive consequences. Currently, there is no consensus on what that function might be. Over the last 10 years, multiple groups have started to characterize the molecular mechanisms and brain structures necessary for normal sleep in Drosophila melanogaster. These researchers are exploiting genetic tools developed in Drosophila over the past century to identify and manipulate gene expression. Forward genetic screens can identify molecular components in complex biological systems and once identified, these genes can be manipulated within specific brain areas to determine which neuronal groups are important to initiate and maintain sleep. Screening for mutations and brain regions necessary for normal sleep has revealed that several genes that affect sleep are involved in synaptic plasticity and have preferential expression in the mushroom bodies (MB). Moreover, altering MB neuronal activity alters sleep. Previous genetic screens found that the same genes enriched in MB are necessary for learning and memory. Increasing evidence in mammals, including humans, points to a beneficial role for sleep in synaptic plasticity, learning and memory. Thus, results from both flies and mammals suggest a strong link between sleep need and wake plasticity.

Using Drosophila to understand sleep mechanisms and functions

Sleep is conserved across all the animal species that have been carefully studied so far (Cirelli and Tononi 2008), and is necessary to maintain cognitive function and performance (Killgore 2010). Yet, why and how sleep benefits the brain remains unclear. This surprising deficit arises from a simple fact, the brain is extremely complicated and the methods to directly assessing the effects of sleep on brain functioning with high (cellular) resolution are limited and still suffer from many technical limitations. For instance, repeated in vivo two-photon imaging have recently been used in zebrafish (Appelbaum *et al.* 2010) and mice (Maret *et al.* 2011) to study how axonal terminals and single dendritic spines are affected by sleep and wake, but the analysis remains so far confined to few superficial areas of the brain. Most sleep studies have been conducted in mammals, especially rats and mice, and, to a much less extent, in birds (Lesku *et al.* 2011). Sleep in mammals and birds shows electroencephalographic (EEG) patterns similar to those observed in human sleep: during non-rapid eye movement (NREM) sleep, which accounts for most of sleep, large slow waves predominate, while REM sleep is characterized by an “activated” high frequency low voltage pattern similar to that of wake. The rodent brain, however, is still very complex and

Corresponding authors: Daniel Bushey Ph.D. and Chiara Cirelli, M.D., Ph.D., University of Wisconsin/Madison, Department of Psychiatry, 6001 Research Park Blvd, Madison WI 53719, Tel. (608) 263 9236, Fax. (608) 263 9340, bushey@wisc.edu, ccirelli@wisc.edu.

genetic molecular techniques have only recently been developed to probe the cellular mechanisms underlying sleep functions. This is why the use of simpler model organisms, including the zebrafish Danio reiro and the nematode Caenorhabditis elegans, has been of great help (Cirelli and Tononi 2008, Zimmerman *et al.* 2008, Harbison *et al.* 2009, Crocker and Sehgal 2010).

Here we focus on Drosophila melanogaster as a simple system to investigate sleep. Work over the last 30 years has demonstrated that fruit flies are a practical system to explore complex behavior, including circadian rhythms. By using a combination of forward and reverse genetics to isolate fly mutants with abnormal circadian behavior, neurogenetists were able to characterize the complex transcriptional feedback system at the basis of the circadian molecular clock, and to identify mammalian orthologues (Peschel and Helfrich-Forster 2011). Current studies of fly sleep mainly rely on the same infrared-based technology originally designed to study circadian behavior, but with higher temporal resolution (secs/mins rather than hours/days). Sleep/wake are defined based on measures of locomotor activity: sleep is defined as any period of immobility > 5 min, because flies quiescent for > 5 min have a reduced arousal threshold, which is the essential feature that distinguishes sleep from quiet wake (Cirelli *et al.* 2005a, Bushey *et al.* 2007, Bushey *et al.* 2009).

The first papers on sleep/rest in Drosophila confirmed that the rest behavior observed in fruit flies shares most of the features of sleep in humans (Hendricks *et al.* 2000, Shaw *et al.* 2000). Quiescent periods in flies were entrained by the 24-hour circadian cycle, occurring primarily at night. Interfering with these quiescent periods resulted in a homeostatic response with increased rest the following day. Sleep deprivation also resulted in decreased performance. Hypnotics and some stimulants (i.e. caffeine and modafinil) produced similar affects on sleep in Drosophila as they did in mammals. Moreover, quiescent periods were more abundant in young flies than in older flies. Together these results suggested that sleep behavior is present in flies, and may involve at least some of the same biochemical pathways known to affect sleep in mammals (reviewed in (Cirelli 2003, Shaw 2003, Ho and Sehgal 2005)). Based on these encouraging results, several laboratories started genetic screens, using both reverse and forward approaches, searching for fly mutants with either reduced sleep time or with alterations in the homeostatic response to sleep deprivation.

Successful (and less successful) screenings of sleep phenotypes in flies

As we will discuss below, the sleep screens are primarily concentrating on total sleep time over the 24-hour period (Cirelli *et al.* 2005a, Koh *et al.* 2008, Wu *et al.* 2008). Our group has also tried to identify mutations that specifically affect the homeostatic regulation of sleep by studying the response to 24 hours of sleep loss. Flies were allowed to recover starting at light onset, i.e. in the morning when they are normally awake. This is because flies sleep a lot during the night even in baseline conditions, and thus a further increase in sleep after sleep deprivation may not occur due to a ceiling effect. Almost all mutant lines tested so far showed an increase in sleep duration and a decrease in sleep fragmentation after 24 hours of sleep deprivation. As in wild-type flies, the sleep rebound was most pronounced during the first 4–6 hours immediately after the end of the sleep deprivation period, and in most cases did not persist the second day after sleep loss. Similarly to wild-type flies, most mutant lines only recovered a small fraction (10–40%) of the sleep lost. We have over the past several years identified several putative “sleep deprivation” mutants, but in all cases this phenotype was not confirmed after repeated testing. Most likely, this is because even in wild-type flies sleep rebound is extremely variable. Genetic screens work best when variation falls within a very narrow range and thus outliers can be readily identified. When a phenotype is variable and outliers are not easily discernable, false positives cannot be tested

quickly enough to efficiently identify variations due to mutations. So thus far, screening specifically for homeostatic mutants has not been successful (but mutants that are both short sleeping and show abnormal response to sleep loss have been identified, see below).

Another likely reason why screening for sleep deprivation mutants has failed is due to the fact that in flies it is easier to assess sleep quantity than sleep quality (intensity). Even in mammals, the strongest and most consistent effects of sleep loss are not on total sleep duration (which may or may not increase, depending on the duration of sleep deprivation and on the time of day when animals are allowed to recover sleep), but on sleep depth: after sleep deprivation, sleep is more consolidated (with less fragmentation due to brief awakenings), and more "intense". In birds and mammals, this intensity is best measured using slow wave activity (SWA), which is defined as the EEG power spectrum in the 0.5 to 4 Hz range during NREM sleep: the longer and/or more "intense" is wake, the higher is SWA at sleep onset (Achermann and Borbely 2003). Moreover, SWA changes are larger in the brain areas more directly affected by the wake experience. For example, birds watching for the first time a movie with only one eye open show increased SWA in the visual brain controlateral to the open eye (Lesku *et al.* 2011). Also, in humans and rats, learning a motor task increases SWA specifically in the trained cortical area, and does so more than performing a previously learned task (Huber *et al.* 2004a, Hanlon *et al.* 2009). Interestingly, social enrichment increases sleep time also in Drosophila, demonstrating that experience in flies also contributes to sleep need (Ganguly-Fitzgerald *et al.* 2006). In flies, however, we can measure some aspects of sleep depth (e.g. brief awakenings)(Huber *et al.* 2004b), but sleep/wake electrophysiological recordings are invasive and difficult (Nitz *et al.* 2002, van Swinderen *et al.* 2004), and no attempt has been done so far to implement "high-density" recordings as it is done in mammals.

A likely source of variability when studying the sleep deprivation phenotype also stems from the complex interaction between circadian and homeostatic factors, the influence of which can vary to a variable extent across individual flies. When allowing flies to sleep rebound in the morning, the homeostatic process will promote sleep, while the circadian process will oppose it. The influence of the latter can be removed genetically by using mutations affecting key canonical circadian genes. Some of these mutant flies (*tim01, per01, Clk^{jrk}*), which are arrhythmic (they sleep throughout the 24-hour cycle), show prominent (100%, as compared to 30–40% in wild-type canton-S flies) rebound after 12–24 hours of sleep deprivation in dark only conditions (Shaw *et al.* 2002). *cyc^{01}* mutants are unique, in that they show an exaggerated sleep rebound after as little as 3 hours of sleep deprivation, and die after longer periods of sleep loss (Shaw *et al.* 2002). *cyc^{01}* mutants have reduced heat shock protein expression and mutations affecting hsp83, a heat shock chaperone, also result in an exaggerated homeostatic response and death after sleep deprivation, demonstrating an important role for stress response genes in protecting against the lethal effects of sleep loss (Shaw *et al.* 2002).

Sleep can be extremely variable because it is a complex behavior dependent on both genetic factors and experience in both mammals and Drosophila. Genetic background can be controlled for by testing flies in a common genetic background. Over successive generations mutants can be backcrossed into a specific background and compared to wild-type flies from the same background, or comparisons can be made between siblings that inherit a common background. However, it is much harder to control for "quality of wake", i.e. individual experiences. Experience in the small glass tubes where flies are normally housed for sleep recordings (one fly/tube) is theoretically uniform, but the visual/mechanical/acoustic stimuli used during sleep deprivation may introduce a new level of variation. On the other hand, the glass tubes represent a very impoverished environment, where flies can only move a few cm, and cannot fly. As we will see below, compelling evidence in mammals and flies now

shows that sleep is necessary to process/mitigate the effects of experience on neuronal function (e.g. (Huber *et al.* 2004a, Ganguly-Fitzgerald *et al.* 2006, Hanlon *et al.* 2009). If so, then testing in a uniform, impoverished environment may reduce our ability to assess the effects of sleep on brain functions. In line with this, learning mutants do not show increases in sleep time after social experience (Ganguly-Fitzgerald *et al.* 2006). Unfortunately, it is difficult to design an automated fly tracking system that can also monitor multiple flies in complex environment, but an effort in this direction should definitively be made.

A Reverse Genetic Screen Demonstrates the Role of the cAMP/PKA/CREB Pathway in Sleep Regulation

In flies a role in sleep regulation was first demonstrated for the cAMP/PKA/CREB pathway using reverse genetics (Hendricks *et al.* 2001). In reverse genetics a candidate gene is mutated first, and then the effects of the mutation on sleep are assessed (from genotype to phenotype). Candidate genes are chosen based on their known function, which makes them likely to be relevant for sleep. The cAMP/PKA/CREB pathway was a good candidate because it was known that cAMP levels and CREB activity are important for learning and memory (discussed below), and that CREB expression in the fly brain shows a circadian pattern. Furthermore, CREB responsive transcription has a diurnal cycle. Hendricks and colleagues (Hendricks et al. 2001) tested previously identified mutations and found that increasing cAMP or CREB activity decreased both sleep time and sleep rebound after sleep deprivation, while inhibiting cAMP or CREB had the opposite effect (Table 1). Furthermore, they found that sleep deprivation increased CREB driven reporter expression. A later study in mice found that mutations knocking down 2 of the 3 CREB mouse isoforms increased sleep time (Graves *et al.* 2003). Finally, a more recent study in mice found that a few hours of sleep deprivation selectivity impair long-term synaptic potentiation (LTP) in the hippocampus, and do so specifically for the LTP forms that require cAMP and PKA signaling (Vecsey *et al.* 2009). The study also demonstrated that the LTP impairment due to sleep loss resulted from increased nucleotide phosphodiesterase (PDE) activity (which breaks down cAMP), because selective PDE inhibition was able to rescue the LTP deficits, as well as the deficits in a hippocampus-dependent memory task.

A Forward Genetic Screen Identifies Shaker

A major barrier to studying sleep is the ignorance concerning the specific molecular events occurring during this behavioral state as compared to wake. Forward genetics is an unbiased approach in which prior knowledge of the “important” genes is not required, and novel genes can thus be discovered (Cirelli 2009). The starting point is the phenotype, and a significant part of the work involves going back to the genotype to identify the responsible gene (from phenotype to genotype). Forward genetic methods include quantitative trait loci (QTLs) analysis and mutagenesis screening. In the latter random small mutations are induced over the entire genome, and hundreds/thousands of mutated individuals are screened for the phenotype of interest. Insertional mutagenesis uses transposable elements (in flies) to induce mutations, while chemical mutagenesis uses ethylmethane sulfonate (EMS, in flies) or N-ethyl N-nitrosourea (ENU, in mice).

In our screen mutant flies are continuously recorded for one week, including 2–3 baseline days, 24 hours of sleep deprivation, and 1–3 days of recovery after sleep deprivation. Ten to sixteen flies (4–7 day old at the beginning of the experiment) are tested for each line. This relatively high number of flies is needed because sleep pattern and sleep amount, although consistent across different days in each individual adult fly, may vary among different flies. The analysis of thousand of lines (> 15,000) has confirmed a significant difference between male and female flies: females sleep almost exclusively during the night, while males also

show a long period of sleep in the middle of the day. The daily amount of sleep in the mutant lines tested so far shows a normal distribution, with female flies for most lines sleeping between 400 and 800 min/day, with a mean of ~ 600 min, similar to that of wild-type flies.

Our first EMS screen looked for short sleeping male flies. Males were the first choice because hemizygous (containing only the mutagenized X-chromosome) could be generated and tested. In genetic screens, the likelihood of identifying a mutation depends on the number of individual mutagenized chromosomes that can be tested. This is why Drosophila is so amply used in genetic screens, since hundreds of individuals containing a given mutagenized chromosome can be quickly generated and tested. The first X-chromosome EMS screening identified a short sleeping line carrying *minisleep*, a mutation in *Shaker (Sh)*, a gene coding the alpha subunit of a voltage-dependent potassium channel (Cirelli *et al.* 2005a)(Table 1). The screen used restrictive criteria to select short sleeping mutants, with sleep being required to decrease by at least two standard deviations from the mean of all tested lines. Thus, the screen did not identify other genes on the X chromosome, such as *dunce* and *Hyperkinetic (Hk)*, whose loss also results in a short sleeping phenotype (Hendricks *et al.* 2001, Bushey *et al.* 2007), but not as pronounced as that seen in *Shaker* mutants. Of note, *Hk* encodes the beta (regulatory) subunit of the Shaker channel, and null *Hk* mutations decrease but do not abolish the Shaker current (since the alpha subunit is still functioning). Both *Sh* and *Hk* loss of function (null/hypomorphic) mutants that have a short sleeping phenotype also have short-term memory deficits in the heatbox paradigm, as well as decreased lifespan (Bushey *et al.* 2010). Further studies also found that short sleeping *Sh* mutants were also more resistant to volatile anesthetics (Tinklenberg *et al.* 1991, Weber *et al.* 2009).

Channels homologous to Sh in vertebrates have similar properties and, in both mammals and flies, the Sh current plays a major role in the control of membrane repolarization and transmitter release. Consistent with the results in flies, knocking out the *Sh* orthologue in mice, *Kcna2*, results in reduced NREM sleep (Douglas *et al.* 2007). However, the *Kcna2* short sleeping phenotype is far from being as dramatic as in *Shaker* flies, perhaps because of redundancy - there is one *Shaker* gene in *Drosophila*, but at least 16 genes code for alpha subunits of voltage-dependent potassium channels in mammals (Misonou and Trimmer 2004, Yuan and Chen 2006). Finally in mice, the injection of an antibody against the kv1.2 potassium channel into the central medial thalamus induces arousal from anesthesia (Alkire *et al.* 2009).

After the identification of *minisleep* an independent genetic screen using insertional mutagenesis identified *Sleepless*, a mutation in the quiver (qvr) locus, which also shows very significantly reduced sleep time (to only ~ 2 hours a day, 85% less than controls) (Koh *et al.* 2008). qvr codes a ly-6/neurotoxin family member and its loss reduces Shaker localization, kinetics, and current density (Wu *et al.* 2010). Thus, two independent genetic screens identified a major role for the Shaker current in sleep regulation. There are nevertheless some interesting differences between *Shaker* and *Sleepless* flies, most notably that only the latter show a reduced homeostatic response, as indicated by no changes in sleep duration after sleep deprivation.

Identified sleep mutants suggest a link between sleep and brain plasticity, especially in the mushroom bodies

Table 1 lists most of the sleep mutants identified in either forward or reverse genetic screens, and the distribution of most of the corresponding genes is shown in Figure 1. The isolated genes include those coding for the voltage-dependent potassium channel Shaker,

several neurotransmitters, molecules that are part of the cAMP pathway, steroids, heat shock proteins/chaperones, circadian proteins, and proteins involved in mRNA transport (Fmr1) and chromatin structure. In most cases, previous research had found that these “sleep” genes are also important for learning and/or memory, and many are enriched in the mushroom bodies (MB), a brain area crucial for olfactory learning and memory. A genetic screen that restricted UAS-shi^{TS1} expression within specific neuronal groups, to block synaptic transmission in specific brain regions, found that silencing activity in the MB produced the greatest reduction in sleep time (Pitman *et al.* 2006). Another group independently demonstrated the importance of the MB in sleep regulation after screening GAL4 lines in combination with the UAS-mc* transgene, which expresses a constitutively active PKAc isoform (Joiner *et al.* 2006). Finally, blocking neurotransmission within the MB protects against the impairment in learning caused by sleep deprivation (Li *et al.* 2009).

For most of these mutations, transgenic rescue experiments confirm that the gene products are indeed necessary within the MB to restore normal learning and memory (Table 1). The dual role in sleep and learning of two neurotransmitters, octopamine and dopamine, and their effects on the MB through activation of the PKA pathway, has been especially well characterized. Octopamine and dopamine are necessary for normal learning in appetitive and aversive learning, respectively (Schwaerzel *et al.* 2003), and PKA may interact with Shaker and quiver to modulate excitability in the MB (Yao and Wu 2001). The fragile x-mental retardation (Fmr1) gene product is also enriched in the MB (Schenck *et al.* 2002). *Fmr1* protein product, FMRP, is present in dendritic spines (Feng *et al.* 1997) and a hallmark of loss of function *Fmr1* mutations, in both flies and mammals, is the failure to remove immature synapses (Hinton *et al.* 1991, Comery *et al.* 1997, Irwin *et al.* 2002, Pan *et al.* 2004, Restivo *et al.* 2005). *Fmr1* overexpression in flies results in the opposite phenotype, with dendritic and axonal underbranching and loss of synapse differentiation (Pan *et al.* 2004, Pan and Broadie 2007). In a previous study we found that sleep duration increases when *Fmr1* function is lost and decreases when *Fmr1* is overexpressed, even when overexpression is confined to the MB of adult flies (Bushey *et al.* 2009).

MB are also closely involved in the temporal and spatial reorganization of cellular memory “traces”, as identified using calcium imaging (Berry *et al.* 2008). Aversive olfactory conditioning results in immediate memory traces (3–6 min) in the antennal lobe, a region necessary for short-term memory (Yu *et al.* 2004), while intermediate memory traces occur in the DPM neuron that innervates the MB 30 min to 1 hour after training (Yu *et al.* 2005). After aversive olfactory conditioning designed to trigger long-term memory, calcium influx occurs in the alpha/beta lobes of the MB within 3 and 9 hours after training, and persists for 24 hours (Yu *et al.* 2006). Of note, neurotransmission from the alpha/beta lobes is also necessary for memory retrieval (Krashes *et al.* 2007). The fact that these memory traces can persist over a 24-hour period suggests that sleep may have a functional role, perhaps to favor the reorganization of memories across different brain circuits, similar to the “reactivation and redistribution of hippocampus-dependent memories to neocortical sites” proposed to occur during NREM sleep, at least in mammals (Diekelmann and Born 2010, Rattenborg *et al.* 2010). Unfortunately, calcium imaging experiments are invasive, and not conducive to analysis of physiological behavioral states. Thus, it remains unknown whether sleep is important for the occurrence/transfer of these memory traces.

A recently characterized general mechanism that acts systemically to control memory formation and sleep need is that of the steroid hormone ecdysone (Table 1). Ecdysone coordinates post-embryonic development and is necessary for neuronal rewiring (Hewes 2008). Administering ecdysone to adult flies increases sleep time in a dose dependent manner, while blocking ecdysone synthesis (DTS-3) or the ecdysone receptor (EcR) reduces sleep time (Ishimoto and Kitamoto 2010). Adding ecdysone during training also enhances

courtship long-term memories, while adding ecdysone after training impairs memory (Ishimoto and Kitamoto 2010). Both experience in the courtship conditioning assay and sleep deprivation increase ecdysone levels, while overexpressing the ecdysone receptor within the MB increases sleep time, and targeted knockdown using RNAi impairs long-term memory. Finally, treatment with ecdysone enhances CREB-dependent expression (Ishimoto and Kitamoto 2010). Overall, these results show that the ecdysone system may represent a systemic mechanism capable not only of controlling brain plasticity in response to environmental stimulation, especially in the MB, but also of directly affecting sleep.

Distinct circadian neurons promote either wake or sleep

Like humans, flies are more active during the day, and show the longest and most consolidated sleep bouts at night, even in constant darkness. Treating flies with a GABA antagonist results in increased sleep latency and decreased total sleep time, while mutations in the Drosophila $GABA_A$ receptor (Rdl) that increase the time the channel remains open result in reduced sleep latency and longer sleep (Agosto *et al.* 2008)(Table 1). The Rdl $GABA_A$ receptors are expressed in the ventral lateral clock neurons (LNvs) (Parisky *et al.* 2008), which express the peptide pigment dispersing factor (PDF). PDF immunoreactivity peaks at the beginning of the light period (Park *et al.* 2000), consistent with a role for PDF in initiating and/or maintaining arousal. In line with this, reducing PDF expression and enhancing neuronal excitability decrease sleep time (Parisky *et al.* 2008). It has been suggested that PDF may play in Drosophila a role similar to that played in mammals by the arousal-promoting peptide orexin/hypocretin (Sakurai 2007).

Drosophila clock neurons have been shown to regulate sleep time in a complex way that is more than a simple reflection of the time of day. For instance, social enrichment in older flies results in longer sleep, but only if the expression of *Rutabaga* and *period* in PDF expressing neurons is normal (Donlea *et al.* 2009). Also, starvation increases wakefulness in Drosophila as it does in mammals, (Keene *et al.* 2010, Thimgan *et al.* 2010), but the clock neurons that do not express PDF (DN1s or LNds neurons) promote sleep during starvation and their loss enhances sleep loss during starvation (Keene *et al.* 2010). Of note, sleep loss due to starvation is not followed by a sleep rebound when flies are placed back on their normal diet (Thimgan *et al.* 2010). Also, in contrast to sleep deprivation due to mechanical or other kinds of stimulation, starvation does not impair performance in an aversive phototaxis memory test (Thimgan *et al.* 2010). Moreover, *lipid storage droplet 2 (Lsd-2)* mutants that show reduced triglycerides storage sleep less on normal media and are not significantly impaired in the aversive phototaxis memory test after sleep deprivation (performance in Lsd2 mutant flies is slightly lower than wild-type controls, but still in the range of wild-type flies) (Thimgan *et al.* 2010). However, starvation in these experiments never lasted more than 12 hours, leaving open the possibility that longer fasting may cause sleep rebound and/or memory impairment. Altogether, these results show that different groups of circadian neurons in Drosophila can either promote wake or sleep, in addition to affect neuronal plasticity in response to social enrichment.

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Fly sleep and hypotheses on sleep functions

Sleep is perhaps the only major behavior still in search of a function. While the entire body certainly benefits from sleep (Knutson *et al.* 2007), most researchers agree that sleep may be especially important for the brain and supply something not provided by quiet wake. There is great uncertainty, however, when it comes to which chemical or molecular pathway in the brain may be depleted during wake and restored during sleep or, alternatively, about which toxic substance might accumulate during wake and dissipate during sleep. For instance, it

was suggested that sleep favors the replenishment of glycogen in glial stores (Benington and Heller 1995), but recent evidence show that this may be the case only in a few brain regions, and not in all strains of mice (Franken *et al.* 2003, Franken *et al.* 2006). The demonstration that flies sleep, and the subsequent identification of several fly sleep mutants (Table 1), have already contributed to the ongoing debate concerning sleep functions, as will be discussed below.

As mentioned above, growing evidence in mammals including humans, birds, and flies points to a link between sleep need and neuronal plasticity, but why and how sleep may benefit the brain by modifying synapses remains unclear. There are three main hypotheses on how sleep could do so, which suggest a role for sleep in synaptic homeostasis (Tononi and Cirelli 2006), macromolecule synthesis (Mackiewicz *et al.* 2009), and memory consolidation (Diekelmann and Born 2010). Importantly, these hypotheses are not mutually exclusive. For instance, there is compelling evidence that sleep benefits several forms of memory (Diekelmann and Born 2010), but the underlying mechanisms remain unclear, and could involve the specific strengthening of a few synapses already potentiated during wake, and/or, as hypothesized by the synaptic homeostasis hypothesis, a more generalized downregulation of synaptic strength during sleep, with subsequent increase in the signal to noise ratio (details in (Tononi and Cirelli 2006)).

Some early clues about the functions of sleep were obtained by considering the extensive changes in brain gene expression that occur between sleep and wake or after sleep deprivation (Cirelli and Tononi 2000b, Terao *et al.* 2003a, Terao *et al.* 2003b, Cirelli *et al.* 2004, Cirelli *et al.* 2005c, Cirelli *et al.* 2006, Terao *et al.* 2006, Zimmerman *et al.* 2006, Mackiewicz *et al.*,Maret *et al.* 2007, Jones *et al.* 2008, Mackiewicz *et al.* 2009, Nikonova *et al.* 2010). In all species studied so far (flies, mice, rats, hamsters, and sparrows) wake leads to the upregulation of transcripts involved in the response to cellular stress and in activity-dependent processes of synaptic potentiation. By contrast, transcripts expressed at higher levels during sleep are involved in synaptic depression, in the synthesis/maintenance of membranes and in lipid metabolism, including the synthesis and transport of cholesterol (Cirelli *et al.* 2004, Mackiewicz *et al.* 2007, Mackiewicz *et al.* 2009). Lesions of the locus coeruleus that deplete the cerebral cortex of noradrenaline, which promotes arousal, attention and the response to novelty (Sara 2009), blunt the homeostatic response to sleep deprivation and abolish the upregulation of a few wake-related genes, mostly plasticity-related genes (Cirelli *et al.* 1996, Cirelli and Tononi 2004, Cirelli *et al.* 2005b). Of note, adrenalectomy has almost opposite effects: sleep homeostasis is not affected, but ~2/3 of all wake-related transcripts are no longer upregulated by sleep deprivation, with the notable exception of some stress response genes including BiP and plasticity-related genes (Mongrain *et al.* 2010). While these results may at first appear disparate, they may also reflect a coherent set of functional changes at the cellular level. One way to make sense of these findings is in terms of plastic processes. Specifically, in the synaptic homeostasis hypothesis we have suggested that during wake there is a net increase in synaptic strength in many brain areas; these plastic changes are a major determinant of sleep need and sleep would be needed to renormalize such changes (Tononi and Cirelli 2003, Tononi and Cirelli 2006). Why would wake result in a net increase in synaptic strength? Because the awake brain is always “learning”, whether it is performing a learning task or simply adapting to an ever-changing environment, and novelty exposure, enrichment and learning mostly occur through synaptic potentiation, not synaptic depression (Nithianantharajah and Hannan 2006, Feldman 2009). Moreover, wake is associated with high levels of acetylcholine and noradrenaline (Jones 2005), which together favor synaptic potentiation (Cirelli *et al.* 1996, Cirelli and Tononi 2000a, Seol *et al.* 2007).

Why would then sleep be needed to revert the net increase of synaptic strength at the end of wake? Because such increase would result in higher energy consumption (Attwell and Laughlin 2001, Rothman *et al.* 2003), in larger synapses that take up precious space (Chklovskii *et al.* 2002), and in the saturation of the capacity to learn. Also, a net strengthening of synapses likely represents a major source of cellular stress, due to the need to synthesize and deliver cellular constituents ranging from mitochondria to synaptic vesicles to various proteins and lipids (Mackiewicz *et al.* 2009). In this view, then, sleep would be necessary to downregulate synapses to a baseline level that is sustainable and ensures cellular homeostasis. Importantly, downregulation would have to occur off-line, i.e. during sleep, because it should affect most synapses, whether or not they are engaged in behavior. How would sleep bring about a net decrease in synaptic strength? One obvious cellular mechanism is the reduced level of neuronal excitability that characterizes most neurons during most of sleep. During NREM sleep, which accounts for 70–80% of all sleep in mammals, neurons are relatively hyperpolarized and fire less as compared to wake (Steriade 2003), including neurons that release noradrenaline, serotonin, hypocretin and histamine (Jones 2005, Saper *et al.* 2010). Synaptic downregulation may be favored by periods of neuronal silence (Kemp and Bashir 2001, Werk and Chapman 2003, Birtoli and Ulrich 2004, Rosanova and Ulrich 2005, Werk *et al.* 2006, Czarnecki *et al.* 2007, Lubenov and Siapas 2008), as well as by the low levels of noradrenaline, serotonin, hypocretin and histamine (Harley 1991, Seol *et al.* 2007). These electrophysiological and biochemical changes across the sleep/wake cycle are well characterized in mammals, but recent evidence shows that they also occur in flies (Crocker and Sehgal 2010). For instance, Drosophila neurons in the medial brain, as well as LNvs neurons, which release the wake-promoting neuropeptide PDF (pigment dispersing factor), are more active in wake than in sleep (Nitz *et al.* 2002, Sheeba *et al.* 2008). Moreover, noradrenaline is wake-promoting in mammals as octopamine, its equivalent in insects, is wake-promoting in flies, an effect mediated in both species by the cAMP/PKA/CREB signaling pathway (Hendricks *et al.* 2001, Graves *et al.* 2003, Crocker and Sehgal 2008, Crocker *et al.* 2010). Specific molecular mechanisms that may mediate synaptic renormalization are also conserved between mammals and flies, including the gene Fmr1 (Fragile X mental retardation 1), for which extensive evidence is available (Hinton *et al.* 1991, Comery *et al.* 1997, Irwin *et al.* 2002, Pan *et al.* 2004, Restivo *et al.* 2005).

The evidence supporting the synaptic homeostasis hypothesis comes from mammals as well as flies. In rats, a recent study examined molecular and electrophysiological markers of synaptic function during sleep and wake (Vyazovskiy *et al.* 2008). It was found that the levels of AMPA receptors in cortical synaptoneurosomes decrease by ~40% after several hours of sleep (Vyazovskiy *et al.* 2008). Phosphorylation changes of AMPA receptors, and of the enzymes CamKII and GSK3β, were also in line with a net decrease in synaptic strength during sleep. Electrophysiologically, it was shown using cortical electrical stimulation and local field potential recordings that both slope and amplitude of cortical evoked responses (classical in vivo measures of synaptic strength) also decrease after sleep (Vyazovskiy *et al.* 2008). Similar observations have been made in humans using transcranial magnetic stimulation and high-density electroencephalogram (EEG) analysis (Bellina *et al.* 2008). Direct evidence for a net decrease in synaptic strength after sleep comes from a recent study in which miniature excitatory postsynaptic currents (mEPSCs) were recorded from frontal cortex slices of mice and rats. Changes in mEPSCs frequency are thought to result from modification of the presynaptic component of synaptic transmission, while amplitude changes indicate alterations in the postsynaptic component (e.g. (Ungless *et al.* 2001)). It was found that, in both rats and mice, the frequency and amplitude of mEPSCs increase after wake and decrease after sleep (Liu *et al.* 2010). Recovery sleep after sleep deprivation also decreases mEPSCs, suggesting that sleep brings about a net decrease in synaptic strength (Liu *et al.* 2010). Finally, mean firing rates in the rat cerebral cortex

increase after periods of wake and decrease after periods of sleep, consistent with a net change in synaptic strength (Vyazovskiy *et al.* 2009). In line with this observation, the levels of glutamate in the rat cortical extrasynaptic space also increase progressively during wake and decrease during NREM sleep (Dash *et al.* 2009). In line with these mammalian studies, we recently found in flies that overall protein levels of both pre- and postsynaptic components of central synapses are high after wake and low after sleep (Gilestro *et al.* 2009). These changes are related to behavioral state rather than time of day and occur in all major areas of the *Drosophila* brain. Moreover, the decrease of synaptic markers during sleep is progressive (Gilestro *et al.* 2009), consistent with the synaptic homeostasis hypothesis. Another study also found morphological evidence, in a specific neural circuit (large LNvs), for synaptic growth in flies that were sleep deprived for 48h after chronic social enrichment, compared to flies that were left undisturbed (Donlea *et al.* 2009). Furthermore, new data from our laboratory show that that number/size of synapses in 3 different Drosophila neural circuits is higher after wake and lower after sleep, and that synaptic renormalization can only occur if flies are allowed to sleep, but not if they are sleep deprived (Bushey *et al.* 2011). Finally, a recent study in zebrafish larvae (Appelbaum *et al.* 2010) found that presynaptic terminals of hypocretin neurons projecting to the pineal gland undergo both circadian and sleep-wake dependent structural changes, the latter consistent with sleep-dependent downregulation. Finally, the findings from fly mutagenesis screens that genes necessary for brain plasticity are also necessary for sleep are in line with the synaptic homeostasis hypothesis.

The results from fly mutagenesis screens, on the other hand, so far do not provide direct evidence that sleep is necessary for the synthesis of macromolecules such as brain lipids (Mackiewicz *et al.* 2009). This, however, may be due to a bias for these screens to focus on short sleeping mutants, while loss of function mutations decreasing lipid synthesis should produce long sleepers. Finally, the finding that in both mammals and flies prolonged wake is associated with increased expression of heat shock proteins and chaperones such as BiP may suggest that sleep loss causes the accumulation of unfolded proteins, which then need to be processed and eliminated during sleep. The fact that Drosophila mutations that affect the expression of BiP and hsp83 result in an exaggerated sleep rebound (Table 1) is consistent with this idea. However, there are no data showing that the overexpression of heat shock proteins reduces sleep time in normal physiological conditions (Shaw *et al.* 2002, Naidoo *et al.* 2007), suggesting perhaps that abnormal accumulation of unfolded proteins occurs only when wake is prolonged beyond its physiological duration. Yet, in both flies and mammals BiP expression also increases during spontaneous wake, and in sleep deprived mice BiP is still induced after adrenalectomy (Mongrain *et al.* 2010), suggesting that BiP induction may not be simply a sign of cellular stress. Long-term sensitization training in Aplysia induces BiP (Kuhl *et al.* 1992), and BiP may be involved in the trafficking of glutamatergic AMPA receptors (Rubio and Wenthold 1999). Moreover, the induction of BiP and of the unfolded protein response promotes the surface expression of GluR1-containing AMPA receptors (Vandenberghe *et al.* 2005). Thus, BiP induction in the context of the unfolded protein response during wake/sleep deprivation may be the result of a physiological increase in glutamatergic signaling and long-term potentiation.

Conclusion

A decade of screening and studying sleep in Drosophila has provided insight into the molecular pathways involved in both sleep and wake. Consistent with data in mammals including humans, studies in flies show that sleep need is a function of brain plasticity, since genes necessary for neuronal plasticity and brain regions where this plasticity occurs are directly involved in sleep homeostasis. Thus, any hypothesis about sleep functions must explain the strong link between sleep need and plastic changes.

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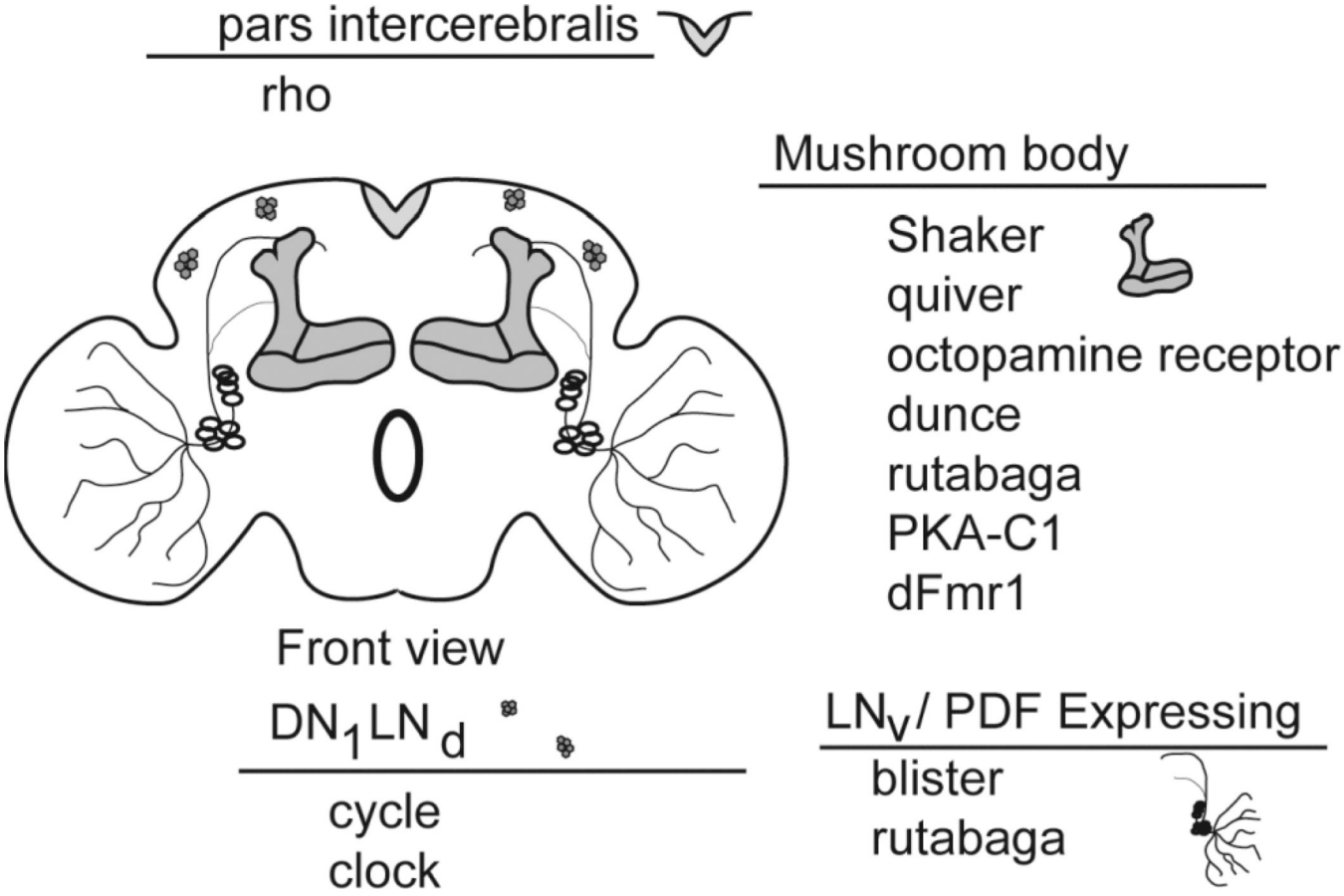

Figure 1.
"Sleep" genes and their distribution in the fly brain.

Table 1

utations affecting Drosophila sleep, and their effects on learning and memory.

	Total sleep time	Arousal Threshold	Response to sleep deprivation (homeostatic response)	References (effects on sleep)	Learning/memory	References (effects on learning/memory)	Brain areas with high expression	Reference	Demonstrated ability to rescue memory deficits	References
otassium Channels and genes ffecting Shaker currents										
haker	Reduced sleep (LOF)	Normal	Normal	(Cirelli *et al*. 2005a)	Impaired courtship conditioning; Impaired aversive olfactory learning; Impaired short-term memory	(Bushey *et al.* 2007) (Cowan and Siegel 1984) (Cowan and Siegel 1986)	MB	(Rogero *et al*. 1997) (Schwarz *et al.* 1990)		
uiver/sleepless	Reduced sleep (LOF)		No response to sleep deprivation	(Koh *et al.* 2008)			MB	(Wu *et al*. 2010)		
yperkinetic	Reduced sleep (LOF)	Normal	Normal	(Bushey *et al*. 2007)	Impaired short-term memory	(Bushey *et al.* 2007)				
eurotransmitters										
opamine transporter (DAT)	Reduced sleep (fumin)	Reduced at low stimulation	Normal	(Kume *et al*. 2005) (Wu *et al*. 2008)	Poor memory retention	(Zhang *et al*. 2008) (Seugnet *et al*. 2008) (Waddell 2010) (Schwaerzel *et al*. 2003)			D1 receptor expression	(Seugnet *et al.* 2008)
ctopamine biosynthesis: yrosine decarboxylase 2 Tdc2) / Tyramine β hydroxylase Tbh)	LOF mutations increase sleep; feeding flies octopamine decreases sleep	Increased		(Crocker and Sehgal 2008)	Impaired appetitive olfactory memory	(Schwaerzel *et al*. 2003)	Octopamine receptor enriched in MB	(Han *et al.* 1998)		
5-HT1A serotonin receptor	Reduced sleep time Increasing serotonin levels increases sleep			(Yuan *et al*. 2006)						
AMP\PKA\CREB pathway										
unce (3',5'-cyclic-AMP hosphodiesterase activity)	Reduced sleep (LOF)			(Hendricks *et al*. 2001)	Impaired aversive olfactory memory	(Dudai *et al.* 1976) (Qiu and Davis 1993)	MB	(Qiu and Davis 1993)		
utabaga (adenylate cyclase ctivity)	Increased sleep (LOF); LOF mutations prevent experience dependent increases in sleep			(Hendricks *et al*. 2001, Donlea *et al*. 2009)	Impaired memory	(Schwaerzel *et al*. 2003) (Blum *et al*. 2009)	MB	(Han *et al.* 1992)	MB, PDF	(Schwaerzel *et al*. 2003) (Blum *et al*. 2009, Donlea *et al.* 2009) blist

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	Total sleep time	Arousal Threshold	Response to sleep deprivation (homeostatic response)	References (effects on sleep)	Learning/memory	References (effects on learning/memory)	Brain areas with high expression	Reference	Demonstrated ability to rescue memory deficits	References
Cyclic-AMP response element binding protein 2 (Creb2)	Increased/decreased sleep		Increased sleep rebound after sleep loss	(Hendricks *et al*. 2001)	Impaired (OE enhances long-term memory?)	(Yin *et al.* 1995) (Perazzona *et al*. 2004) (Sakai *et al*. 2004)	Enriched in cell bodies, not in neuropil	(Yin *et al.* 1995)		
AMP-dependent protein kinase (PKA-C1)	Expression of a constitutively active form reduces sleep			(Joiner *et al*. 2006)	Impaired learning; Heterozygotes have normal memory and are resistant to age-related memory impairment	(Skoulakis *et al.* 1993) (Yamazaki *et al*. 2007)	MB	(Skoulakis *et al.* 1993)		
Epidermal growth factor										
pidermal growth factor receptor EGFR)	LOF mutations prevent experience dependent increases in sleep			(Donlea *et al*. 2009)						
homboid (Rho)	OE increases sleep (in combination with star); LOF mutations reduce sleep		Dominant negative mutants recover less sleep after sleep deprivation	(Foltenyi *et al*. 2007)			Pars Intercerebralis	(Foltenyi *et al.* 2007)		
tar	OE increases sleep (in combination with rho)			(Foltenyi *et al*. 2007)						
-Spitz	OE mutations increase sleep			(Foltenyi *et al*. 2007)						
teroids										
cdysone receptor (EcR)	LOF mutations reduce sleep		LOF mutations reduce change in sleep bout duration after sleep loss	(Ishimoto and Kitamoto 2010)	Impaired courtship	(Ishimoto *et al*. 2009)			OE in MB increases sleep; LOF in MB impairs long-term memory	(Ishimoto *et al.* 2009)
nolting defective (mld, DTS-3)	LOF mutations reduce sleep		LOF mutations reduce change in sleep bout duration after sleep loss	(Ishimoto and Kitamoto 2010)						
mRNA transport and control										
Fmr1	LOF mutations increase sleep; OE decreases sleep	LOF or OE increase	LOF or OE reduce sleep rebound	(Bushey *et al*. 2009)	Olfactory learning and memory impaired, Impaired courtship	(Bolduc *et al.* 2008)	MB	(Schenck *et al.* 2002)	Greatest effect on sleep is in MB	(Bushey *et al.* 2009)

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	Total sleep time	Arousal Threshold	Response to sleep deprivation (homeostatic response)	References (effects on sleep)	Learning/memory	References (effects on learning/memory)	Brain areas with high expression	Reference	Demonstrated ability to rescue memory deficits	References
		arousal threshold				(McBride *et al.* 2005)				
Heat shock proteins/chaperones										
Binding immunoglobulin protein (BiP)	No effect		OE increases sleep rebound, LOF mutations reduce sleep rebound	(Naidoo *et al.* 2007)						
Hsp83	No effect		LOF mutations increase sleep rebound	(Shaw *et al.* 2002)						
Circadian genes/genes expressed in circadian neurons										
Cycle	LOF mutations increases sleep during starvation		LOF mutations show high mortality rate after sleep deprivation and increased sleep rebound	(Shaw *et al.* 2002) (Keene *et al.* 2010)						
Period	Necessary for experience dependent increases in sleep	Normal		(Donlea *et al.* 2009) (Shaw *et al.* 2002) (Thimgan *et al.* 2010)	Impaired long-term memory and courtship (OE enhances it)	(Sakai *et al.* 2004)				
Clock	LOF mutations increases sleep during starvation			(Keene *et al.* 2010)						
Blister (bs, serum response factor)	LOF mutations show no increase in sleep after social enrichment			(Donlea *et al.* 2009)			Expressed throughout the brain but rescue in sLNvs	(Donlea *et al.* 2009)	Expressed throughout brain but rescue in sLNvs	(Donlea *et al.* 2009)
Pigment-dispersing factor (Pdf)	LOF mutations increase sleep			(Parisky *et al.* 2008)						
Resistant to dieldrin (Rdl)	Reduced sleep (LOF); increased sleep (GOF)			(Parisky *et al.* 2008)						

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	Total sleep time	Arousal Threshold	Response to sleep deprivation (homeostatic response)	References (effects on sleep)	Learning/memory	References (effects on learning/memory)	Brain areas with high expression	Reference	Demonstrated ability to rescue memory deficits	References
				(Agosto *et al*. 2008)						
Chromatin Structure/Microtubule dynamics										
Elongator Protein 3	LOF mutations reduce sleep			(Singh *et al*. 2010)	(no studies on memory) Increases bouton number at NMJ	(Singh *et al.* 2010)				
Triglyceride Storage										
Lipid Storage Droplet 2 (Lsd-2)	LOF mutations reduce sleep		LOF mutations show no sleep rebound	(Thimgan *et al*. 2010)			Fat Body	(Gronke *et al.* 2003)		
Drummer	No effect		LOF mutations show increased sleep rebound	(Thimgan *et al*. 2010)			Fat Body	(Gronke *et al.* 2005)		

LOF, loss of function; GOF, gain of function; OE, overexpression

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