Widespread Changes in Synaptic Markers as a Function of Sleep and Wakefulness in *Drosophila*

Giorgio F. Gilestro, Giulio Tononi,* Chiara Cirelli*

Sleep is universal, strictly regulated, and necessary for cognition. Why this is so remains a mystery, although recent work suggests that sleep, memory, and plasticity are linked. However, little is known about how wakefulness and sleep affect synapses. Using Western blots and confocal microscopy in *Drosophila*, we found that protein levels of key components of central synapses were high after waking and low after sleep. These changes were related to behavioral state rather than time of day and occurred in all major areas of the *Drosophila* brain. The decrease of synaptic markers during sleep was progressive, and sleep was necessary for their decline. Thus, sleep may be involved in maintaining synaptic homeostasis altered by waking activities.

There is increasing evidence for a link between sleep need and neuronal plasticity (*1*). Sleep need increases after learning, and learning tasks that involve local brain regions lead to local changes in sleep intensity. Neural activity during sleep can reactivate neural circuits involved in learning. Moreover, sleep consolidates memories, whereas sleep deprivation interferes with memory acquisition. Presumably, sleep exerts these effects by modifying synapses (*1*). In rats, the number of cortical and hippocampal glutamate AMPA receptors (AMPARs) containing the subunit GluR1 is high after waking and low after sleep, and the slope of cortical evoked responses is steeper after waking relative to sleep (*2*). These data suggest that periods of wakefulness are associated with a net increase in synaptic strength and that periods of sleep are associated with a net decrease. Sleep could therefore play an important role in renormalizing synaptic changes caused by learning during wakefulness (*3*). But how general is this finding, and does it apply to species with brains very different from those of mammals? Both plasticity and sleep are universal across animal species. If the opposing effects of wakefulness and sleep on synaptic markers reflect a fundamental function of sleep, they should also occur in nonmammalian species.

To address this issue, we turned to *Drosophila melanogaster*, whose sleep shares most of the features of mammalian sleep: It consists of long periods of behavioral immobility with increased arousal threshold (*4*, *5*) and is associated with changes in brain electrical activity and gene expression (*1*). In flies as in mammals, the duration and intensity of sleep both increase in proportion to the duration of prior waking, sleep deprivation reduces performance (*6*), and learning and enriched experience increase sleep need and sleep intensity (*7*, *8*). Fly brains are different from mammalian brains, but the central synapses of *Drosophila* and mammals share key pre- and postsynaptic components and use similar mechanisms of synaptic plasticity (*9*, *10*).

Here, we tested whether waking and sleep affect synaptic markers in the *Drosophila* central nervous system (CNS) by measuring changes in protein levels of several pre- and postsynaptic components (Fig. 1A). Bruchpilot (BRP) is an essential constituent of the active zone of all synapses (Fig. 1A), and excitatory synapses lacking BRP show reduced evoked release of glutamate (*11*). In vivo, a strong BRP signal is associated with fully formed synapses (*11*); BRP staining is used to quantify synapse number (*12*, *13*).

Wild-type Canton-S (CS) males were kept awake by an established mechanical method of sleep deprivation that causes a post-deprivation increase in sleep duration and intensity that is proportional to the amount of sleep lost (*6*). Sleep deprivation was performed for 6, 12, and 24 hours and was highly effective (flies lost at least 80% of their baseline sleep) (Fig. 1B). At the end of sleep deprivation, fly brains were dissected and BRP expression measured by Western blots. Sleep-deprived flies showed higher BRP levels relative to controls allowed to sleep ad libitum, and the increase in BRP expression was smaller after 6 hours of forced waking and larger when sleep was prevented for 12 to 24 hours (Fig. 1, C and E). Similar BRP increases were seen after sleep deprivation in males of another strain, *white*^{1118} (fig. S1, A and C), and in CS females (Fig. 1, I and K). In the latter, BRP levels increased similarly after 12 and 24 hours of sleep deprivation (Fig. 1I), consistent with the fact that females concentrate most of their sleep at night (*5*, *6*), whereas males take a nap in the middle of the day (fig. S2).

To investigate whether sleep deprivation also increases the expression of postsynaptic proteins, we focused on Discs-large (DLG), the *Drosophila* homolog of the postsynaptic density protein PSD-95/SAP-90 (*14*). DLG is mainly expressed postsynaptically (Fig. 1A) (*15*) and is abundant at the neuromuscular junction and in the CNS neuropil, where its pattern of expression overlaps with that of BRP (*14*, *16*). DLG regulates glutamate release and postsynaptic structure (*17*) and controls the postsynaptic clustering of glutamate receptors (*12*). At the *Drosophila* neuromuscular junction, postsynaptic activation of DLG increases the number and size of the presynaptic active zones (*18*). In mammals, the synaptic delivery and incorporation of AMPARs depend in part on PSD-95 (*19*), and overexpression of PSD-95 enhances the activity of postsynaptic glutamate receptors and the number and size of dendritic spines (*20*). In CS male flies, sleep deprivation increased DLG levels in the CNS, and more so after 24 hours of sleep loss (Fig. 1, D and E). Similar results were observed in CS females (Fig. 1, J and K) and in two other strains (fig. S1, B and D).

We then tested whether sleep loss affects the expression not only of structural proteins such as BRP and DLG, but also of components of the secretory machinery (Fig. 1A): synapsin [Syn (*21*)], syntaxin [Syx (*22*)], and cystein string protein [CSP (*23*)]. After 24 hours of sleep deprivation, all three proteins increased their expression levels by ~30% (Fig. 1, F to H), with CSP showing a significant increase also after 12 hours of continuous waking.

Because both sleeping and sleep-deprived flies were collected at light onset (Fig. 1B), these results cannot be ascribed to circadian differences in the time of collection. However, because flies were stimulated mechanically to keep them awake, it was important to demonstrate that lack of sleep, and not stimulation per se, was responsible for the observed results. To do so, we carried out three sets of experiments. First, sleep loss was enforced for 24 hours via mechanical stimulation as before, but flies were grouped according to the efficiency of sleep deprivation. Even though all flies received the same amount of mechanical stimuli across the 24 hours, the more effective the sleep deprivation, the higher the level of BRP (Fig. 2, A and D), and the correlation was stronger for highly effective sleep deprivation (>70% sleep loss, corresponding to 75% of all flies; $r = 0.65$, $P < 0.001$). In a second set of experiments, we used a different method to keep flies awake. A wild-type (red-eyed) CS male was first kept in standard conditions for at least 2 days, and then a "guest" white-eyed male fly was introduced into the recording tube at dark onset. As confirmed by continuous video recording, both flies were awake most of the night (fig. S3, A to C). As soon as the guest fly was removed the next morning, the host fly showed a sleep rebound, and the increase in sleep duration over the first 3 hours of recovery sleep was similar to that observed after 12 hours of sleep loss by mechanical stimulation (fig. S3D). Relative to flies that slept as usual in single tubes, flies kept awake with this method showed increased levels of BRP, DLG, and CSP (Fig. 2, B and E). Thus, nonspecific factors due to the sleep deprivation methods cannot account for the effects of behavioral state on synaptic markers. Finally, we investigated whether the expression of synaptic proteins also increases after spontaneous waking. BRP levels were measured in flies collected in the middle of the day, after they had been spontaneously

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awake almost continuously for 6 hours, and at the end of the night, after 6 hours spent mainly asleep (Fig. 2C). In both female and male CS flies, BRP levels were again higher after waking than after sleep (Fig. 2F). The increase after 6 hours of spontaneous wakefulness was not as pronounced as after 12 to 24 hours of sleep deprivation.

To determine the time course of the decline in BRP expression during sleep, we collected flies at the end of the light phase, after they had been mostly awake for several hours, and every 3 hours across the entire dark phase, when they slept. In both males and females, BRP levels declined progressively in the course of sleep, reaching the lowest level toward the end of the night (Fig. 3, A and C). Was sleep per se, or merely the passage of time, responsible for the decline in BRP expression? To answer this question, we conducted an experiment in which two groups of flies were sleep-deprived by mechanical stimulation for 12 hours during the night; one group was then allowed to recover sleep for the first 6 hours of the next day, while the other group was kept awake for the same period of time. BRP, DLG, and CSP levels were ~20 to 40% lower in flies that could sleep relative to those that could not (Fig. 3, B and D). Because BRP levels did not decrease, but rather increased, when flies remained awake spontaneously for 6 hours (Fig. 2F), these results are consistent with the idea that sleep is necessary for down-regulating the expression of synaptic proteins.

All changes described so far were detected using whole-brain homogenates, and thus it remained unclear whether they were restricted to select brain areas. We therefore used confocal microscopy to measure BRP levels in dissected brains of two groups of flies, both collected immediately after light onset. Controls slept undisturbed; the other group was sleep-deprived for the preceding 16 hours. An increase in the level of BRP protein relative to controls could be observed across the whole central brain of sleep-deprived flies (Fig. 4) and was due to sleep loss [two-way analysis of variance (ANOVA), sleep loss, $F = 16.27$, $P < 0.0001$; brain tissue, $F = 0.68$, $P = 0.56$; sleep × tissue interaction, $F = 0.24$, $P = 0.86$]. This increase was similar in four major structures of the fly brain: antennal lobes (mean percentage increase ± SEM, 69 ± 11%), β lobes of the mushroom bodies (40 ± 11%), ellipsoid body of the central complex (67 ± 18%), and entire central brain (76 ± 15%). In flies as in mammals, learning and memory—and activity-dependent structural plasticity in general—involve complex and widespread circuits that span most of the brain (*10*, *24*). Thus, it is perhaps not surprising that forcing flies to stay awake through a complex range of tactile, auditory, and visual stimuli affects synaptic activity in most brain regions.

Finally, we examined whether prolonged sleep loss is also associated with overall changes in the volume of antennal lobes, because activity-dependent plasticity leads to volumetric increases in this structure (*25*). In flies that were sleep-deprived for 16 hours, antennal lobes were slightly but significantly bigger than in flies that had slept (+15%, $P = 0.01$; N of flies, sleeping = 27; sleep-deprived = 19), a result that is compatible with a diffuse increase in either the number or the volume of synaptic connections.

Our main finding is that in *Drosophila*, the expression of several bona fide synaptic markers increases after wakefulness and decreases after sleep, independent of time of day. There are three documented examples of daily structural changes in *Drosophila* neuronal circuits: (i) Neuronal branches at the neuromuscular junction are thicker and carry larger synaptic boutons during the day, when flies are active, than during the night (*26*). (ii) Optic lobe interneurons have larger axons at

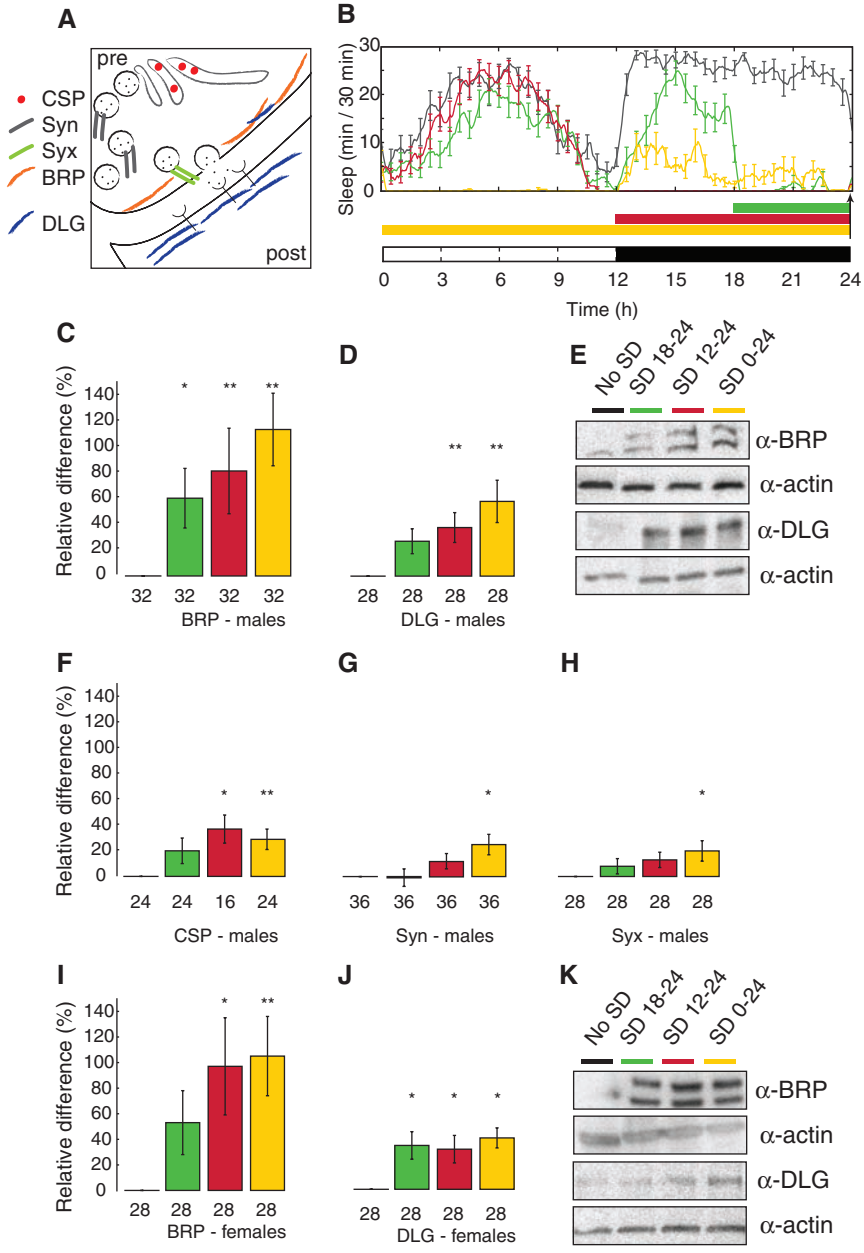

Fig. 1. Levels of synaptic proteins are high after sleep deprivation and low after sleep. (**A**) The *Drosophila* synapse. Abbreviations: pre, presynaptic; post, postsynaptic; BRP, Bruchpilot; CSP, cysteine string protein; DLG, Discs-large; Syn, synapsin; Syx, syntaxin. (**B**) Daily pattern of sleep in Canton-S males sleep-deprived for the last 6 hours of the night (green), 12 hours at night (red), or 24 hours (yellow) and control siblings left undisturbed (no sleep deprivation, black line). Arrow at right shows when flies were collected. White and black bars indicate light and dark periods. Each group includes 12 to 16 flies and represents one of the two or three experiments used for (C) to (H). (**C** to **H**) Representative immunoblots [(E); SD, sleep deprivation] and gel quantification (mean ± standard deviation; *n* of flies below each bar). SD values [color-coded as in (B)] represent percent change relative to sleep (= 0%). (**I** to **K**) Canton-S females. *$P < 0.05$, **$P < 0.01$ [one-way ANOVA followed by Tukey's HSD (honestly significant difference) post hoc test].

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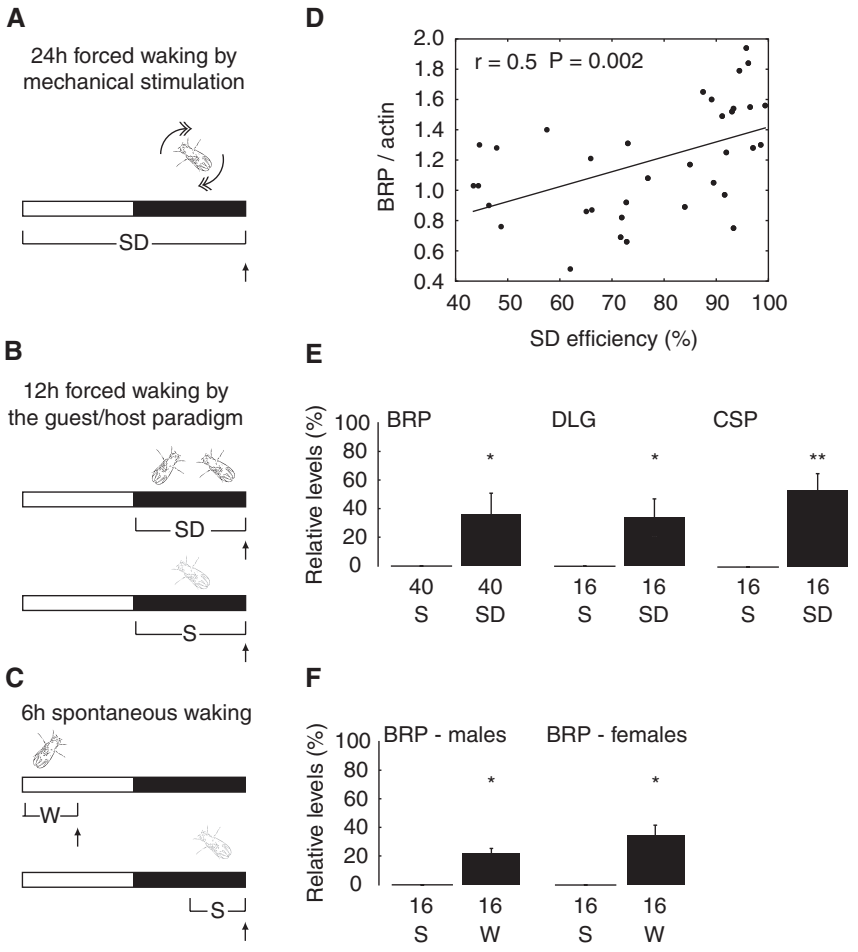

Fig. 2. The expression of synaptic proteins increases as a result of sleep loss. (**A** to **C**) SD, sleep deprivation; S, sleep; W, spontaneous waking. Vertical arrows show when flies were collected. (**D**) Correlation between BRP levels (BRP/actin ratio) and sleep deprivation efficiency during the preceding 24 hours in Canton-S males (Pearson correlation). Each dot represents four flies with similar SD efficiency. (**E** and **F**) Levels of synaptic proteins after SD or W, expressed as percent change relative to sleep (= 0%) (mean ± standard deviation; *n* of flies below each bar). **P* < 0.05, ***P* < 0.01 (Student's *t* test).

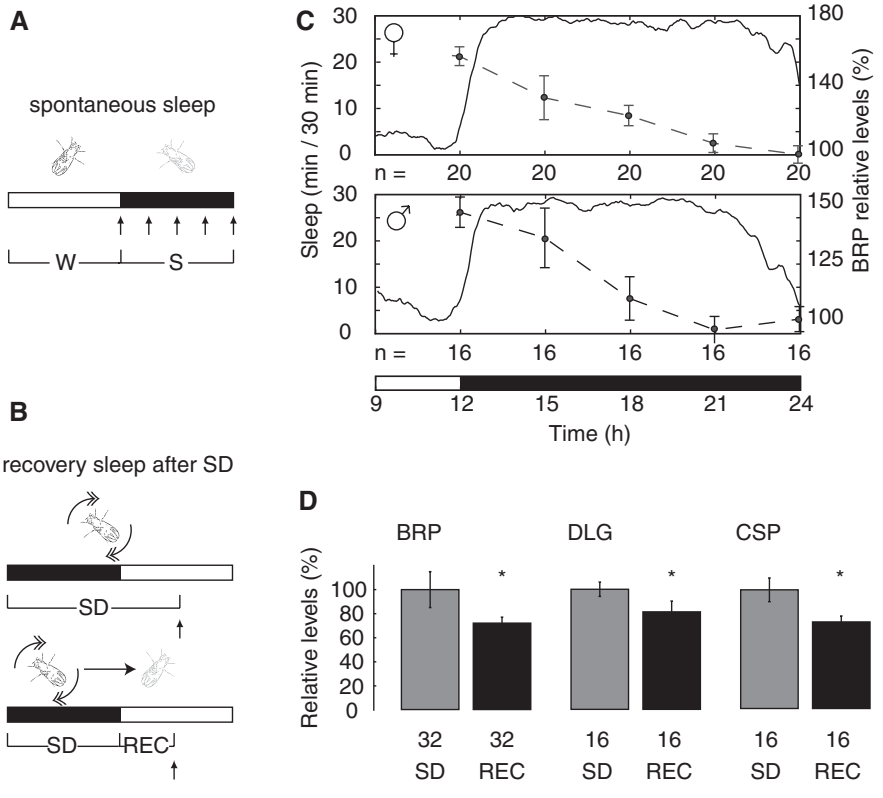

Fig. 3. Synaptic markers decrease during sleep. (**A** and **B**) Rec, recovery sleep after SD; other abbreviations as in Fig. 2. Vertical arrows show when flies were collected. (**C**) BRP levels (data points at 3-hour intervals) are expressed as percent change relative to sleep at the end of the night (= 100%); solid lines indicate mean sleep amount at 30-min intervals. (**D**) Levels of synaptic proteins after recovery sleep, expressed as % change relative to SD (=100%) (mean ± standard deviation; *n* of flies is below each bar). **P* < 0.05 (Student's *t* test).

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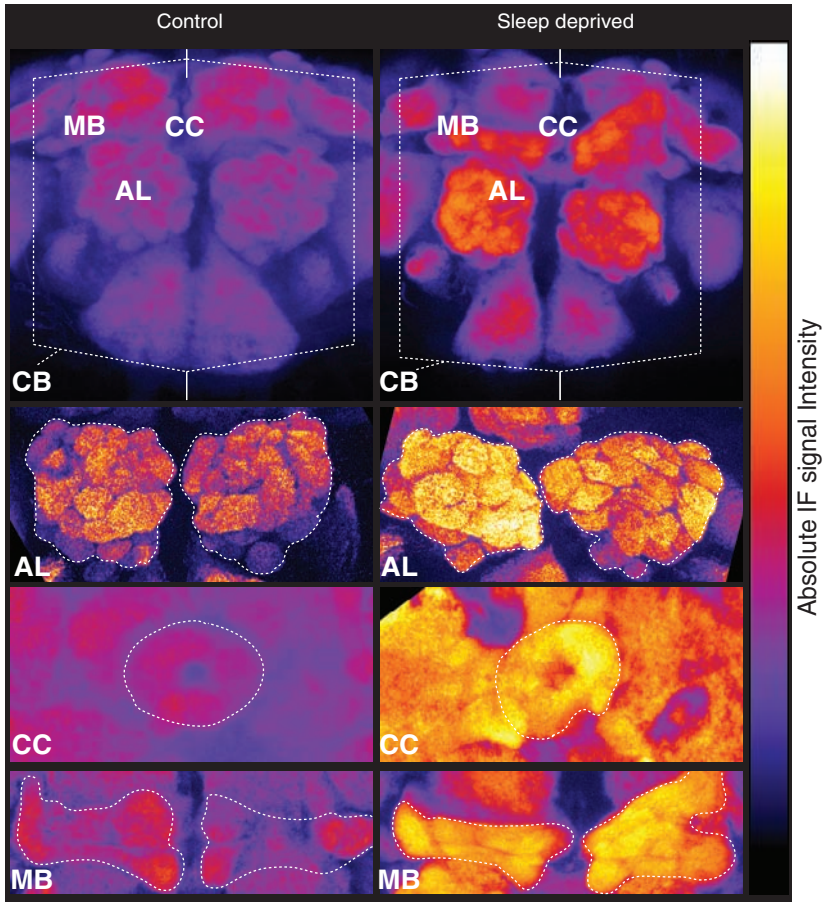

Fig. 4. Widespread BRP increase after sleep loss, as shown by representative examples of BRP immunofluorescence (IF) in controls and flies sleep-deprived for 16 hours (sleep loss >80%) ending at light onset (sum of selected optical stacks, false-colored on a quantitative scale). Immunoreactivity levels were measured in antennal lobes (AL), β lobes of the mushroom bodies (MB), ellipsoid body of the central complex (CC), and central cerebrum excluding the optic lobes (CB).

the beginning of the night than at the beginning of the day, and their dendritic trees also undergo daily morphological changes (*27*). (iii) The dorsal axonal projections of the small ventral lateral neurons, which are involved in rhythmic behavior, show a higher degree of arborization in the morning than at night (*28*). It is thought that these forms of structural plasticity are controlled by the circadian clock, because they are largely maintained in constant darkness and are abolished in flies carrying mutations of circadian genes (*26*, *28*, *29*). However, the sleep/waking cycle is an endogenous circadian rhythm; it persists in constant darkness and is disrupted by mutations of circadian genes. Thus, structural changes in neural circuits occurring between day and night may be due, at least in part, to changes in behavioral state. Consistent with this, the number of synaptic terminals in ventral lateral neurons is reduced during sleep, and this decline is prevented by sleep deprivation (*30*).

What could be the functional importance of the large, widespread decrease in synaptic markers that takes place during sleep? Processes of synaptic potentiation and depression, which are often associated with structural changes, are usually assumed to occur concurrently so as to maintain an overall synaptic balance (*3*). However, the increase in synaptic markers observed after periods of wakefulness in rats (*2*) and now in flies suggests that this balance may not be fully preserved. An increase of synaptic strength by the end of the waking day would result in higher energy consumption, larger synapses that take up precious space, and saturation of the capacity to learn (*3*). Also, a net strengthening of synapses likely represents a major source of cellular stress, resulting from the need to synthesize and deliver cellular constituents as varied as mitochondria and synaptic vesicles. Sleep may thus play an important role in renormalizing synapses to a baseline level that is sustainable and ensures cellular homeostasis. Because similar changes appear to occur in phylogenetically distant species with different brain organizations, synaptic homeostasis may represent a cellular correlate of wakefulness and sleep that is conserved across evolution.

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31. Authors' contributions: G.F.G. performed the experiments; G.F.G., G.T., and C.C. designed the experiments; G.T. and C.C. conceived and coordinated the project. All authors wrote the manuscript and read and approved the final version. We thank D. Paasch, D. Denucci, and M. Uy for technical assistance with fly work; M. Pfister-Genskow for help with Western blot analysis; L. Rodenkirch and the UW Keck Laboratory for Biological Imaging for assistance in confocal microscopy; and D. Bushey for discussion and comments on the manuscript. Supported by National Institute of General Medical Sciences grant R01 GM075315 (C.C.) and an NIH Director's Pioneer award (G.T.).

Supporting Online Material

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Materials and Methods
Figs. S1 to S3
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