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Sleep and Synaptic Homeostasis: Structural Evidence in *Drosophila*
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Science **332**, 1576 (2011);
DOI: 10.1126/science.1202839

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To determine whether sleep plays a positive role in memory consolidation, we induced sleep for 4 hours immediately after a massed training protocol that does not result in the formation of LTM (Fig. 4A). Massed training did not induce LTM in any genotype at 25°C (Fig. 4, B, D, and F). Moreover, activating the EB did not alter LTM, indicating that neither heat exposure nor activating portions of the central complex that do not alter sleep facilitates memory consolidation (Fig. 4C). However, when sleep was induced after massed training by switching flies to 31°C for 4 hours, *C5/+>UAS-TrpA1/+* and *104y-GAL4* flies displayed an LTM (Fig. 4, E and G). Thus, when flies were maintained at 25°C after massed training, no LTM was observed in any genotype. However, when sleep was induced for 4 hours, flies display LTM, even though the training protocol is not sufficient to induce memory consolidation by itself.

An alternative interpretation is that activating neuronal circuits enhances memory consolidation and that LTM would be observed even in the absence of sleep. Therefore, we sleep-deprived *104y/+>UAS-TrpA1/+* flies while they were maintained at 31°C after training. Our sleep deprivation apparatus efficiently kept *104y/+>UAS-TrpA1/+* flies awake, further emphasizing that neuronal activation does not simply result in a paralyzed or comatose-like state (Fig. 4H). Additionally, activation of neuronal activation in the absence of sleep does not result in the formation of LTM after massed training.

We identified a circuit that plays a role in sleep regulation and can be activated on demand to precisely control the timing and duration of sleep. Our data complements previous sleep-deprivation experiments by demonstrating that sleep plays a positive role in both synaptic homeostasis and memory consolidation. Thus, inducing sleep facilitates the formation of LTM even for a training protocol that, by itself, is only able to induce short-term memory. We expect that the ability to acutely control the timing and duration of sleep induction will be critical for elucidating the interaction between sleep and other complex processes and ultimately the function of sleep.

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Acknowledgments: We thank P. Taghert, E. Herzog, P. Gray, L. Seugnet, A. Diantonio, W. Vanderheyden, and B. van Swinderen for discussions. This work was supported by NIH grants R01-NS051305-01A1, NS057105, T32GM008151, and 5F31NS063514-02.

Supporting Online Material

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27 December 2010; accepted 11 May 2011
10.1126/science.1202249

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Sleep and Synaptic Homeostasis: Structural Evidence in *Drosophila*

Daniel Bushey, Giulio Tononi, Chiara Cirelli*

The functions of sleep remain elusive, but a strong link exists between sleep need and neuronal plasticity. We tested the hypothesis that plastic processes during wake lead to a net increase in synaptic strength and sleep is necessary for synaptic renormalization. We found that, in three *Drosophila* neuronal circuits, synapse size or number increases after a few hours of wake and decreases only if flies are allowed to sleep. A richer wake experience resulted in both larger synaptic growth and greater sleep need. Finally, we demonstrate that the gene *Fmr1* (*fragile X mental retardation 1*) plays an important role in sleep-dependent synaptic renormalization.

Sleep is present in every species that has been carefully studied (*1*), including *Drosophila melanogaster* (*2*, *3*), but its functions remain elusive. Increasing evidence points to a link between sleep need and neuronal plasticity (*1*, *4*, *5*). A recent hypothesis (*6*) suggests that a consequence of staying awake is a progressive increase in synaptic strength, as the awake brain learns and adapts to an ever-changing environment mostly through synaptic potentiation (*7*). However, such increase would soon become unsustainable, because stronger synapses consume more energy, occupy more space, require more supplies, and cannot be further potentiated, saturating the ability to learn. Thus, according to the synaptic homeostasis hypothesis, sleep may serve an essential function by promoting a homeostatic reduction in synaptic strength down to sustainable levels. Also, the hypothesis predicts that the more one learns and adapts (i.e., the more intense is the wake experience), the more one needs to sleep. Findings in rodents are consistent with this hypothesis. For instance, molecular and electrophysiological markers of synaptic strength are higher after wake and lower after sleep (*8*, *9*). Moreover, presynaptic terminals of hypocretin neurons in zebrafish larvae undergo both circadian and sleep-wake–dependent structural changes, the latter consistent with sleep-dependent down-regulation (*10*). Finally, in the fly brain, overall levels of synaptic proteins increase after wake and decrease after sleep (*11*), and synaptic structural changes have been described after very long sleep deprivation (*12*). These results suggest that a role for sleep in synaptic homeostasis may hold in phylogenetically distant species and may thus be of general importance.

The evidence in support of the synaptic homeostasis hypothesis is mainly correlative, and thus it is important to seek direct proof that sleep is necessary for synaptic renormalization and to do so at the level of individual synapses. Moreover, the synaptic homeostasis hypothesis predicts that behavioral paradigms that enhance wake-related plasticity in specific neural circuits should increase synaptic strength in those circuits as well as sleep need, but this prediction has never been tested.

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Finally, the cellular mechanisms that underlie synaptic and sleep changes remain unexplored. We exploited the power of *Drosophila* genetics, combined with confocal microscopy and behavioral analysis, to address these questions.

Changes in synaptic strength are often associated with changes in synaptic structure, including synapse number and size, although the link between structural and functional plasticity is complex (*13–15*). In mammals, the diameter and length of synaptic spines correlate with the size of the postsynaptic density and with the magnitude of electric signals transmitted to the dendritic shaft (*16*, *17*). Moreover, the induction of synaptic potentiation leads to growth of synapses and spines, whereas synaptic depression causes synapses and spines to retract or shrink (*13–15*). Similarly, in *Drosophila*, synaptic morphology at the neuromuscular junction changes depending on experience, and these changes correlate with synaptic strength (*18*). Previous in vivo experiments in mammals and flies measured overall changes in electrophysiological and molecular markers of synaptic strength, without cellular resolution, and without direct evidence for morphological changes in synaptic terminals. We selected three specific cell populations in the fly brain and asked whether sleep and wake affect synaptic density and size.

The first cell group we studied included the small ventral lateral neurons (LNvs), a subset of circadian oscillator neurons that are part of the wake promoting system (*19*) and express the neuropeptide pigment dispersing factor (PDF) (*20*) (Fig. 1A). To visualize changes in presynaptic morphology, we expressed a fusion protein between synaptotagmin and enhanced GFP (syt-eGFP), whose protein product colocalizes with native synaptic vesicles (*21*). We also measured PDF expression, because the latter is another marker of presynaptic boutons in small LNvs (*22*). First, we tested adult females (7 days old) collected either during the light period after 7 hours of mainly (>75%) spontaneous wake or during the dark period after 7 hours of mostly sleep (>80%) or sleep deprivation (>90%) (Fig. 1B). Syt-eGFP and PDF staining were both higher in the presynaptic region of sleep-deprived and spontaneously awake flies relative to sleeping flies (Fig. 1, C and D), whereas no differences were found in the axonal processes extending from the cell bodies to the presynaptic region (P = 0.3 for both syt-eGFP and PDF, Kruskal-Wallis test), suggesting that the changes are independent

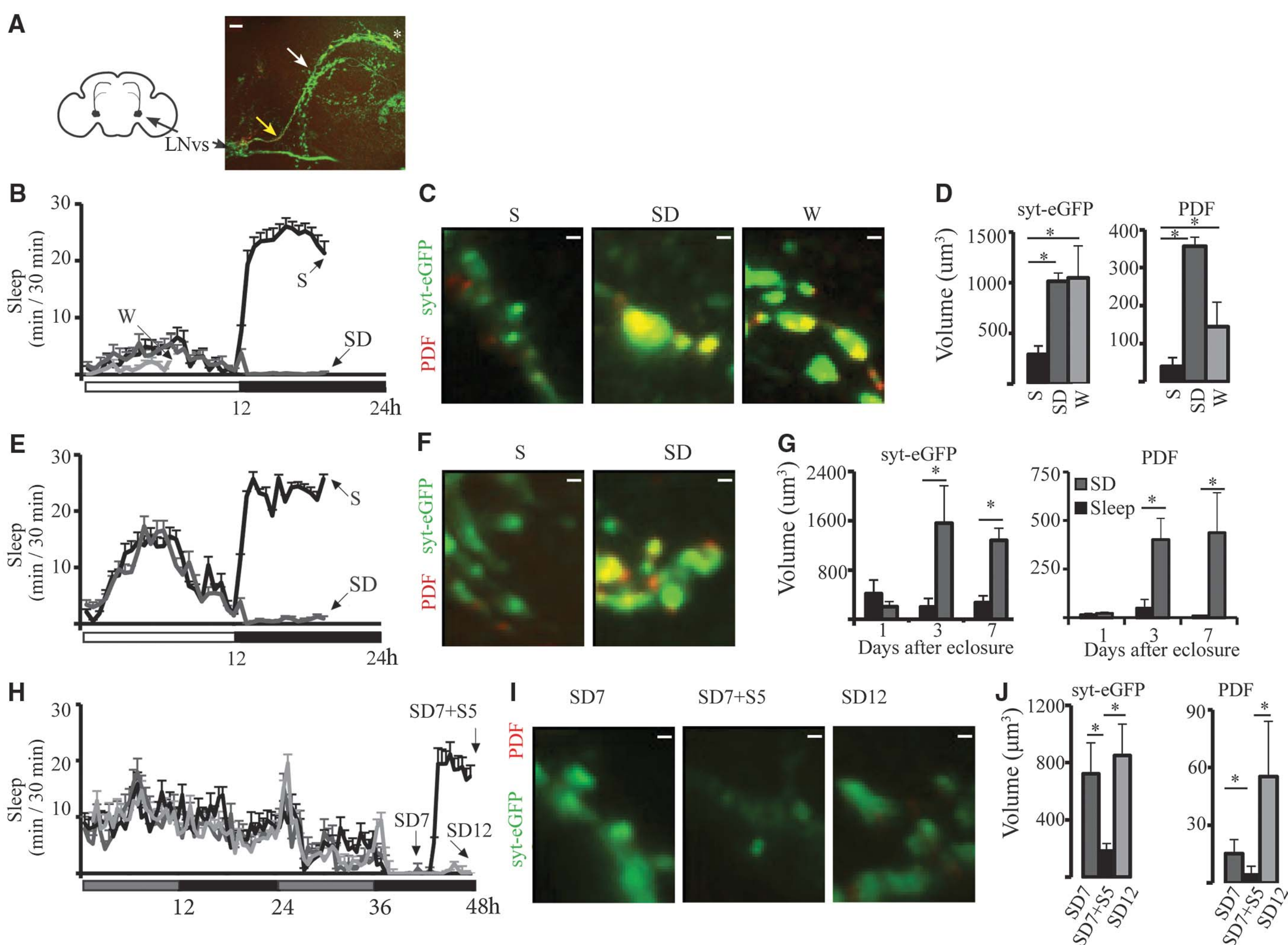

Fig. 1. Sleep/wake presynaptic changes in small LNvs. (**A**) (Left) Schematic frontal section of fly brain with LNvs neurons projecting to the dorsal brain. (Right) Example of small LNvs axonal terminals stained for syt-eGFP (green). Yellow and white arrows point to where LNvs axons leave the posterior optic tract and to the first axonal bifurcation, respectively. Asterisk marks the tip of the terminal region whose volume was measured, as shown in (C), (F), and (I). (**B**) Mean sleep duration in 7-day-old females used for imaging after spontaneous wake (W), sleep deprivation (SD), or sleep (S). Horizontal white and black bars indicate light and dark periods, respectively. (**C**) Examples of small LNvs axonal terminals stained for syt-eGFP (green), PDF (red, overlap yellow), and volume measurements (**D**) in females (S = 9, W = 9, SD = 5). (**E**) Mean sleep duration in 7-day-old males used for imaging. (**F**) Examples of axonal terminals in males. (**G**) Mean volume measurements in males harvested 1, 3, and 7 days after eclosure (N = 5 per time point). (**H**) Mean sleep duration in Per^{01} males kept in constant darkness. At the onset of the second subjective night, flies underwent SD for 7 or 12 hours, or 7 hours of SD followed by 5 hours of sleep. (**I**) Examples of axonal terminals. (**J**) Mean volume measurements (N = 7 per group). Scale bars, 10 μm in (A), 1 μm in (C), (F), and (I). All panels show mean ± SEM.

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of circadian time and specific to the presynaptic terminal. We then tested males; because they have less consolidated wake during the day than females, we only collected flies at night, after sleep or sleep deprivation (Fig. 1E). Sleep-deprived 3- and 7-day-old males consistently showed higher presynaptic syt-eGFP and PDF staining than sleeping flies (Fig. 1, F and G). In contrast, 1-day-old flies showed low syt-eGFP and PDF staining after both sleep and sleep deprivation (Fig. 1, F and G). The lack of PDF staining in very young flies suggests that these neurons are still inactive soon after eclosure. Moreover, because PDF promotes arousal, low PDF staining is consistent with flies being predominantly asleep after eclosure (*3*), even if mechanical stimulation was used to try to keep them awake, consistent with high sleep need and elevated arousal threshold in newborn mammals. Syt-eGFP staining did not change in newly eclosed flies, whose PDF levels were very low. Syt-eGFP and PDF expression were also measured in Per^{01} flies carrying a null mutation of the clock gene *Period*. Because Per^{01} mutants have no spontaneous consolidated sleep, flies were collected immediately after 7 hours of sleep deprivation or after 5 additional hours of either recovery sleep or sleep deprivation (Fig. 1H). Overall, syt-eGFP and PDF staining in presynaptic terminals was reduced in Per^{01} mutants relative to wild-type (WT) flies but was still high after both 7 and 12 hours of sleep deprivation and low after recovery sleep (Fig. 1, I and J).

The second cell group we analyzed included γ neurons of the mushroom bodies (Fig. 2A, inset), because they can be targeted by mosaic analysis with a repressible cell marker (MARCM) to visualize single cells (*23*), show a relatively simple morphology, and undergo activity-dependent pruning (*24*). Moreover, the mushroom bodies are involved in sleep regulation (*25*, *26*), and mutations altering cyclic adenosine monophosphate–dependent protein kinase signaling or *Fmr1* (*fragile X mental retardation 1*) expression in these brain regions affect both sleep need and experience-dependent structural plasticity (*12*, *27*–*29*). Flies were collected at night after 7 hours of sleep or sleep deprivation (Fig. 2A), and dissected brains were immunostained for GFP-tagged CD8 to visualize neuronal membranes (Fig. 2B). We found that the axonal tips were larger after sleep deprivation than after sleep (Fig. 2, C and D), consistent with an increase in volume of presynaptic terminals. To confirm this result, we generated fly stocks with γ MARCM clones expressing syt-eGFP, and flies were collected after 7 hours of mostly spontaneous wake, or during the dark period after 7 hours of mostly sleep or sleep deprivation (Fig. 2E). As expected, syt-eGFP tended to accumulate in puncta along lightly stained processes (Fig. 2F), in contrast to the diffuse CD8-GFP staining (Fig. 2B). Syt-eGFP puncta were larger in sleep deprived and spontaneously awake flies relative to sleeping flies (Fig. 2, G and H).

Next, we studied whether postsynaptic morphological changes also occur as a function of sleep and wake. To do so, we focused on the first giant tangential neuron of the lobula plate vertical system (VS). This cell (VS1) (Fig. 3A) is unambiguously recognizable, and its stereotyped dendritic tree shows small actin-enriched protrusions morphologically and functionally similar to mammalian dendritic spines (*30*). We compared flies that were spontaneously awake during the day or that slept or were sleep deprived during the first 7 hours of the night (Fig. 3B). Single VS1 spines were visualized using an antibody against actin-GFP and counted in one easily identifiable branch (Fig. 3, A and C) (see Supporting Online Material). The total number of spines was similar in spontaneously awake and sleeping flies but

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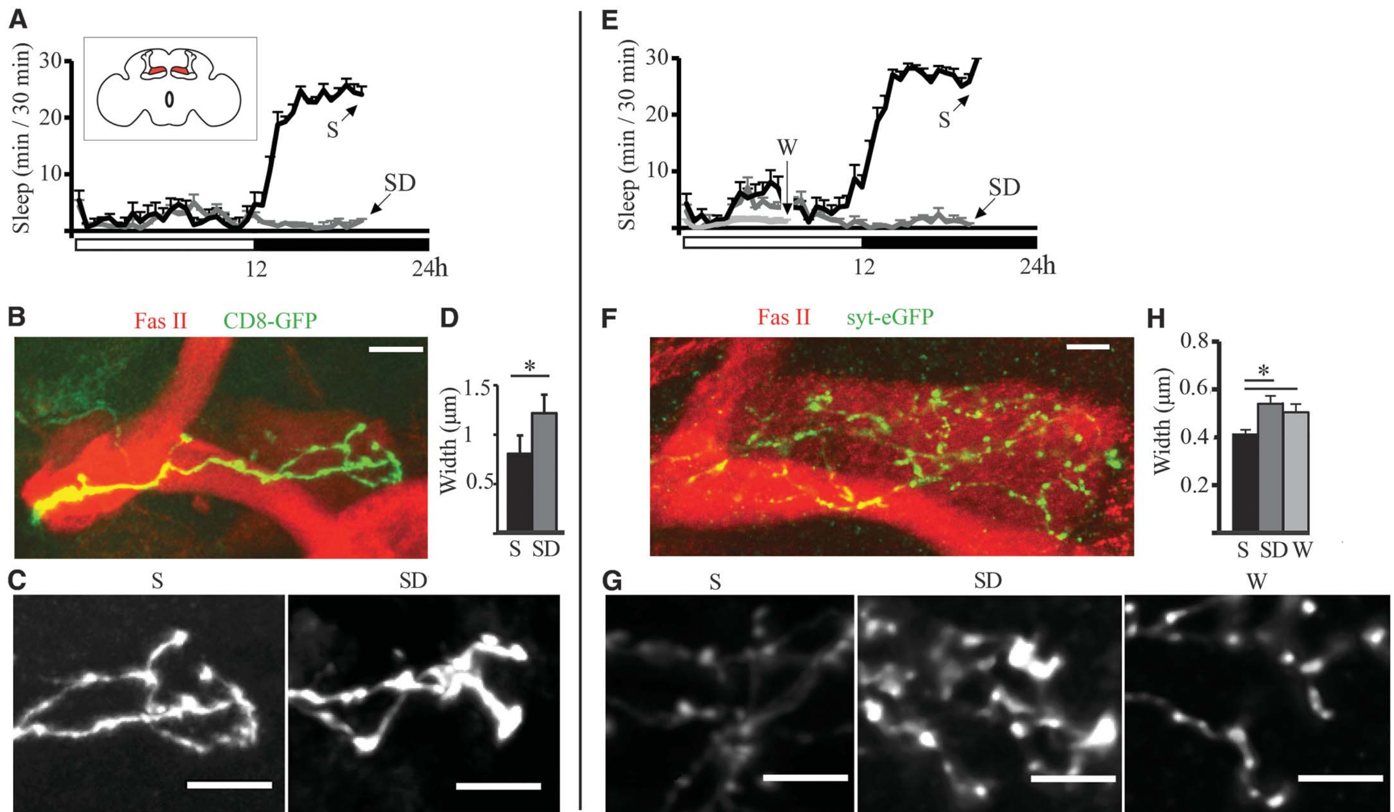

Fig. 2. Sleep/wake presynaptic changes in the gamma lobe of the mushroom bodies. (**A**) Mean sleep duration in female flies used for imaging after 7 hours of S or SD at night. Inset, schematic frontal section of the fly brain showing gamma lobes in red. (**B**) Example of MARCM clones tagged with CD8-GFP (green), which outlines gamma lobe neurons. Fasciclin II (Fas II, red) staining outlines the mushroom bodies. (**C**) Representative images of CD8-GFP clones from S and SD flies. (**D**) Mean width of axonal tips (females, S = 26, SD = 15). (**E**) Mean sleep duration in flies used for imaging after W, SD, or S. (**F**) Representative gamma lobe with two MARCM-generated clones expressing syt-eGFP (green). (**G**) Representative syt-eGFP puncta from S, SD, and W flies. (**H**) Mean puncta width (males and females did not differ and were pooled; S = 34, SD = 26, W = 20). Mean number of tested puncta per lobe per fly was S = 28 ± 2, SD = 29 ± 2, W = 30 ± 2. All scale bars, 10 µm. All panels show mean ± SEM.

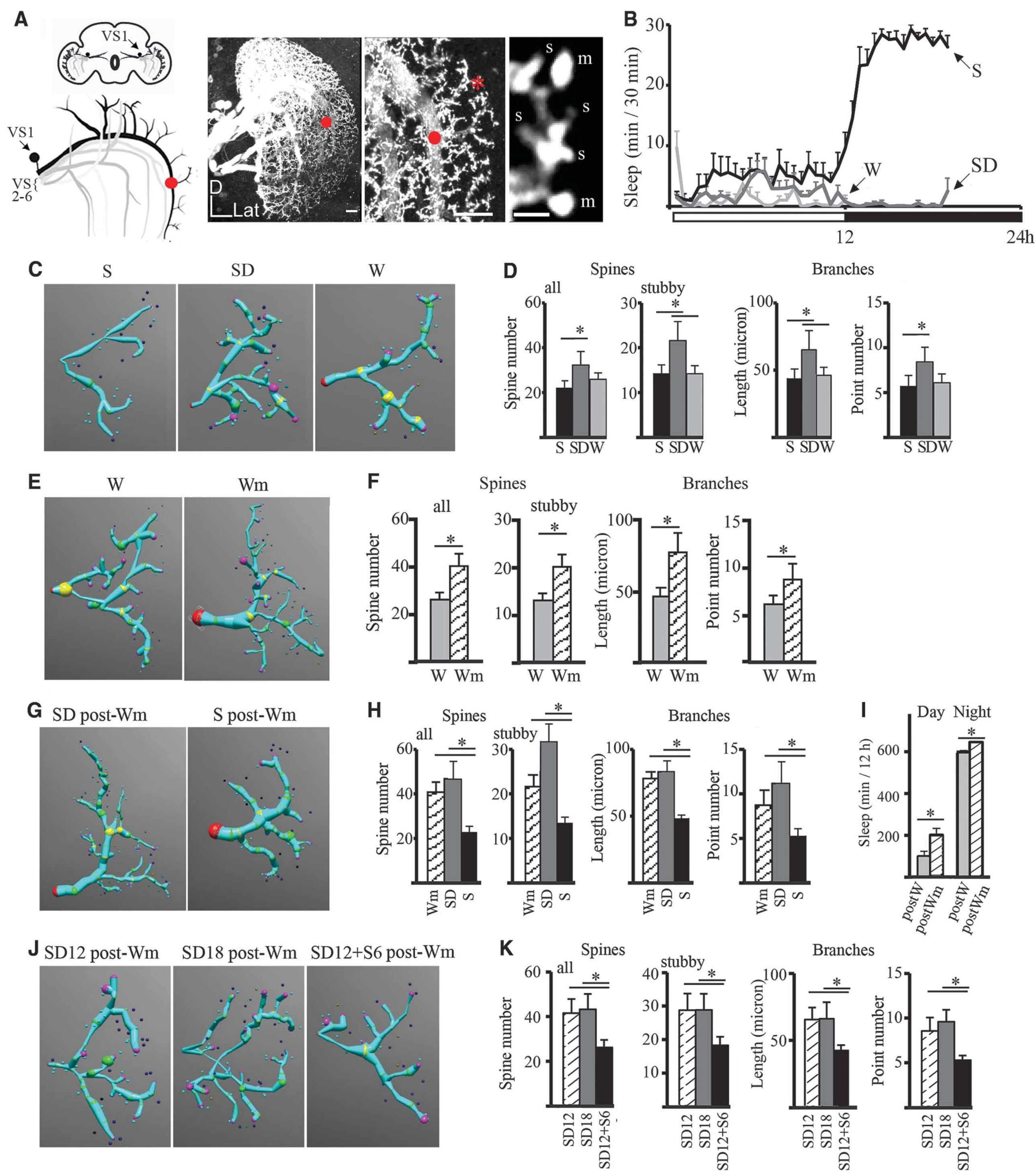

Fig. 3. Sleep/wake postsynaptic changes in VS1. (**A**) (Left) Frontal section of fly brain with VS neurons (branches and cell body are shown only for VS1; trunks are shown for all other VS neurons). (Right) Representative two-dimensional (2D) maximum intensity projections of 3D image stacks of VS neurons (actin-GFP driven by DB331GAL4) at low and medium resolution (left and middle scale bars, 10 μm) and high resolution (right scale bar, 1μm). Red dot indicates the beginning of the scored branch. Red asterisk is above the region shown in the right panel. s, stubby; m, mushroom. (**B**) Mean sleep duration in females used for imaging after W, SD, or S. (**C**) Examples of model neurons [reconstructed using NeuroStudio (*36*)] from S, SD, and W flies. Model shows dendritic processes as blue cylinders connecting user-defined locations on the branch (large spheres) and spines (smaller spheres). (**D**) Mean number of total and stubby spines, branch length, and branch points ($N = 10$ flies per group). (**E** and **F**) Examples of reconstructed neurons from flies awake for 12 hours in single tubes (W, $N = 10$) or in the fly mall (Wm, $N = 12$). (**G** and **H**) Examples of reconstructed neurons from flies allowed to sleep (S postWm, $N = 12$) or sleep deprived (SD postWm, $N = 11$) after 12 hours in the fly mall. [Wm = 12, same flies as in (F)]. (**I**) Sleep time for the 24 hours after 12 hours in the fly mall (postWm, $N = 76$). Control flies (postW, $N = 75$) spent the same 12 hours awake in single tubes. (**J** and **K**) Examples of reconstructed neurons from flies housed for 12 hours during the light period in the fly mall and then sleep deprived for 12 hours at night. Flies were then collected immediately (SD12, $N = 9$), sleep deprived for 6 hours (SD18, $N = 7$), or allowed to sleep for 6 hours (SD12 + S6, $N = 10$). All panels show mean ± SEM.

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increased after sleep deprivation relative to both conditions, mainly because of an increase in stubby spines (which were the majority of scored spines) (Fig. 3D, left). The number of mushroom spines did not change (P = 0.29, Kruskal-Wallis test). The increase in spine number after sleep loss was associated with increased branching and lengthening of the dendritic tree (Fig. 3D), whereas spine density (number of spines divided by branch length) was similar in all conditions (P = 0.20, Kruskal-Wallis test). Because sleep-deprived female flies had been mostly awake during the previous light period, this suggests that these postsynaptic changes may need sustained periods of wake. Another possibility, not mutually exclusive, is that changes in VS1 spines require a wake condition richer than that experienced by flies spontaneously awake alone inside small glass tubes. Indeed, sleep-deprived flies were kept awake using vibratory stimuli, resulting in the flies often falling from the top to the bottom of the tubes. Because visually driven responses in VS neurons are stronger during flight than during nonflight (*31*), it is possible that these cells were activated by the fall.

To test whether a rich wake experience that engages the VS circuit is sufficient to affect VS1 synaptic morphology, we housed up to 100 flies inside a large lighted chamber ("fly mall") for an entire light period (12 hours). In the mall, flies could fly ad libitum, explore, and interact with each other. Flies were collected immediately after the mall experience and compared with flies that, as usual, had remained awake during the day in single tubes. The enriched experience in the mall had profound morphological effects on the VS1 dendritic tree: Total branch length increased because of the addition of more branches with spines (mainly stubby), resulting in an overall increase in spine number (Fig. 3, E and F).

Once experience-dependent synaptic changes have occurred, are they stable? If not, is sleep necessary to bring synaptic morphology back to pre-enrichment levels? To answer these questions, two other groups of flies were moved back to single tubes after 12 hours of mall experience; one group was allowed to sleep for 7 hours, whereas the other was kept awake as before using mechanical stimuli. In flies that were sleep-deprived after enrichment, branch length, branch points, and spine number were at levels similar to those seen in flies collected immediately after enrichment. In contrast, in flies that were allowed to sleep after the mall experience, all morphological parameters reverted to the levels observed in awake flies kept in single tubes (Fig. 3, G and H). Moreover, spine density was negatively correlated with the amount of sleep during the last 7 hours, as well as with the maximal duration of sleep bouts (fig. S1). In another experiment, flies were housed in the mall for 12 hours during the day and then moved back to single tubes to record their sleep. During the 24 hours after the enrichment, flies slept more, both during the day and at night (Fig. 3I). Finally, in the last experiment, flies were housed in the mall for 12 hours during the day, moved back to single tubes and sleep deprived all night (12 hours), and then either collected immediately, allowed to sleep for 6 hours, or kept awake for 6 more hours. Consistent with the previous experiments, decreases in all morphological parameters were seen only in flies that could sleep (Fig. 3, J and K), and spine density was negatively correlated with the amount of sleep during the last 6 hours, as well as with mean and maximal duration of sleep bouts (fig. S2).

Previous experiments suggest that *Fmr1* could mediate at least some of the effects of sleep/wake on synapses. *Fmr1* protein product (FMRP) is present in dendritic spines, and loss of FMRP in flies is associated with overgrown dendritic trees, larger synaptic boutons (*32*), and defects in developmental and activity-dependent pruning (*22*, *24*). Notably, *Fmr1* overexpression results in the opposite phenotype, with dendritic and axonal underbranching and loss of synapse differentiation (*32*). Moreover, *Fmr1* expression is reduced by sensory deprivation in flies (*24*) and increased by sensory stimulation and enrichment in mammals (*33*–*35*).

We recently showed that FMRP levels increase in the adult fly brain during wake relative to sleep, independent of time of day or light (*29*), suggesting that waking experience is sufficient to affect *Fmr1* expression even after the end of development. We also showed that *Fmr1* overexpression in either the whole brain or in the mushroom bodies is associated with an ~30% decrease in sleep duration (*29*), and we hypothesized that this reduced need for sleep occurs because chronically high *Fmr1* levels may allow synaptic pruning to occur at all times, independent of sleep. If so, *Fmr1* overexpressing (OE) flies should fail to show increased spine density after prolonged wake. We thus overexpressed

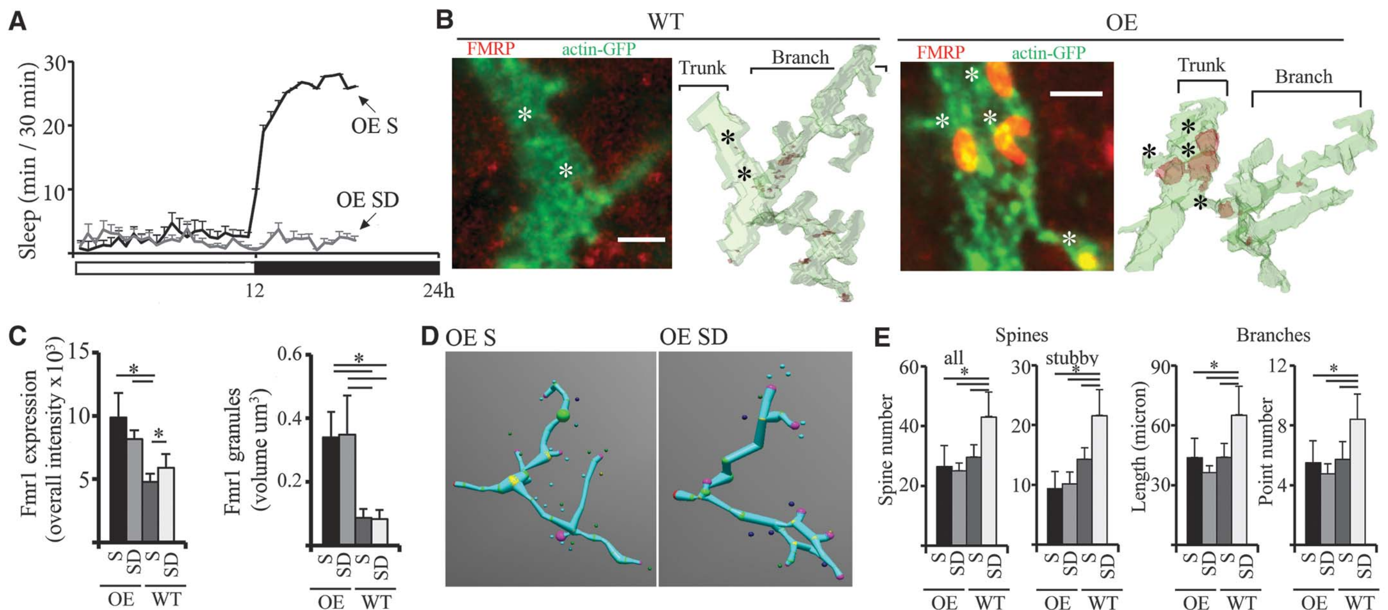

Fig. 4. Effects of *Fmr1* overexpression on synaptic complexity and sleep need. (**A**) Mean sleep duration in OE female flies used for imaging after S and SD. (**B**) (Left) Single confocal images of *Fmr1* WT and OE VS1 neurons stained for FMRP (red) and actin-GFP (green, overlap yellow). (Right) Surface plots generated by segmenting the 3D confocal stacks. FMRP localizes to granules clearly visible in OE but not in WT VS1 neurons. Scale bars, 2 μm. (**C**) Mean overall (trunk+branch+spine regions) FMRP intensity (left), and mean granule volume (right) in WT and OE VS1 neurons. (**D**) Examples of reconstructed neurons (as in Fig. 3C). (**E**) (Left) Total and stubby spine number per branch in OE and WT harvested after S or SD. (Right) Mean branch length and number of branch points. All panels show mean ± SEM. N (flies): OE S = 9; OE SD = 8; WT S = 11; WT S = 8).

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Fmr1 specifically in the vertical and horizontal system of the lobula plate. OE flies were collected at night after 7 hours of either sleep or sleep deprivation (Fig. 4A) and were compared to corresponding sleeping and sleep-deprived WT controls. As expected, *Fmr1* expression was concentrated in granules along the VS1 dendritic tree (Fig. 4B), and overall *Fmr1* levels were higher in sleeping and sleep-deprived OE flies than in their corresponding controls, due to larger *Fmr1* granules in OE flies (Fig. 4C). Crucially, in contrast to WT controls, OE flies showed no increase in either spine number, branch length, or branch points after sleep deprivation relative to sleep (Fig. 4, D and E); all these parameters were similar between the two experimental groups, and their levels were close to those observed in WT flies after sleep (Fig. 4, D and E). Finally, OE flies slept less than their WT controls during baseline (–9.6%; N of flies: WT = 110, OE = 62; $P < 0.05$, Mann-Whitney test) and showed a reduced sleep rebound after 12 hours of sleep deprivation at night (percentage of sleep recovered: OE 39%, WT = 49%; N of flies: OE = 293, WT = 420; $p < 0.05$, Mann-Whitney test; both groups lost >90% sleep during sleep deprivation). Thus, it seems that *Fmr1* overexpression was sufficient to completely abolish the wake-dependent increase in VS1 spine number, whereas the effects on sleep were small. The latter result is not surprising, because sleep need presumably results from the overall amount of synaptic plasticity occurring during wake in many brain areas, whereas *Fmr1* overexpression was restricted to a few VS neurons.

Sleep is perhaps the only major behavior still in search of a function. The results of this study support the hypothesis that plastic processes during wake lead to a net increase in synaptic strength in many brain circuits and that sleep is required for synaptic renormalization. A wake-related increase in synapse number and strength, if unopposed, would lead to a progressive increase in energy expenditure and saturation of learning. A sleep-dependent synaptic homeostasis may explain why sleep is required to maintain cognitive performance (*1*). How sleep would bring about a net decrease in synaptic strength remains unknown, but in mammals, potential mechanisms favoring synaptic depression during non–rapid eye movement sleep may require the repeated sequences of depolarization/synchronous firing and hyperpolarization/silence at ~1Hz observed in corticothalamic cells, as well as the low levels of neuromodulators such as noradrenaline and of plasticity-related molecules such as brain-derived neurotrophic factor (*6*). To what extent such mechanisms may also apply to flies remains to be determined.

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Acknowledgments: The study was supported by National Institute of General Medical Sciences (R01 GM075315 to C.C.), NIH Director's Pioneer award (to G.T.), Army Research Office (DURIP award W911NF-08-1-0169 to C.C.), and Canadian Institutes of Health Research (to D.B.). We thank G. Tavosanis for technical advice.

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13 January 2011; accepted 11 May 2011
10.1126/science.1202839

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Supporting Online Material for

Sleep and Synaptic Homeostasis: Structural Evidence in *Drosophila*

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Published 23 June 2011, *Science* **332**, 1576 (2011)
DOI: 10.1126/science.1202839

This PDF file includes:

Materials and Methods
Figs. S1 and S2
References

Figure S1. After 12h of mall experience during the day (light period) flies were allowed to sleep in the dark period for 7h or kept awake for 7h. Top row: correlations between VS1 spine density and total sleep time for the last 7h, and separately for the first 4h and last 2h of this 7-hour period. Bottom row: correlations between VS1 spine density and duration (mean and max) or number of sleep episodes during the 7-hour period. In addition to the SD post-Wm (n = 11) and S post-Wm (n = 12) flies shown in Figure 3 g-h, these graphs include 3 flies that did not sleep well enough to be selected for the analysis in Fig. 3g-h (sleep was not consolidated and/or lasted <80% of the total recording time).

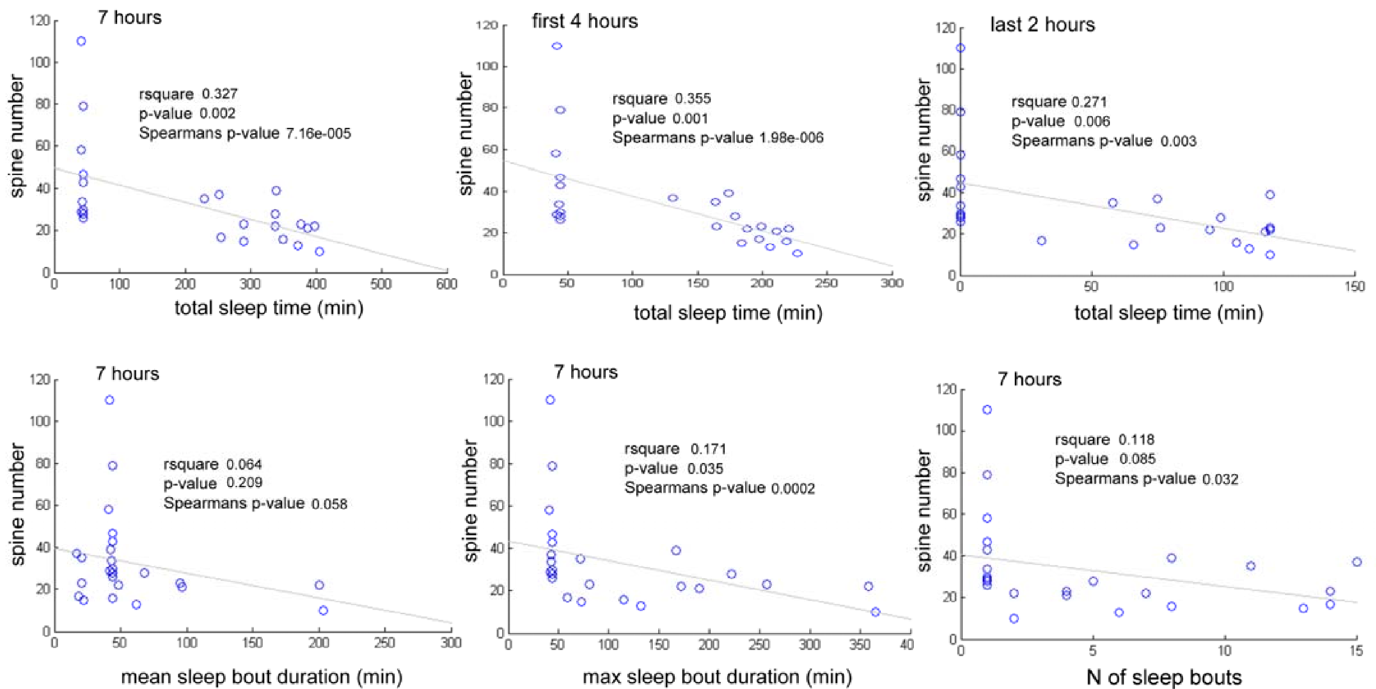
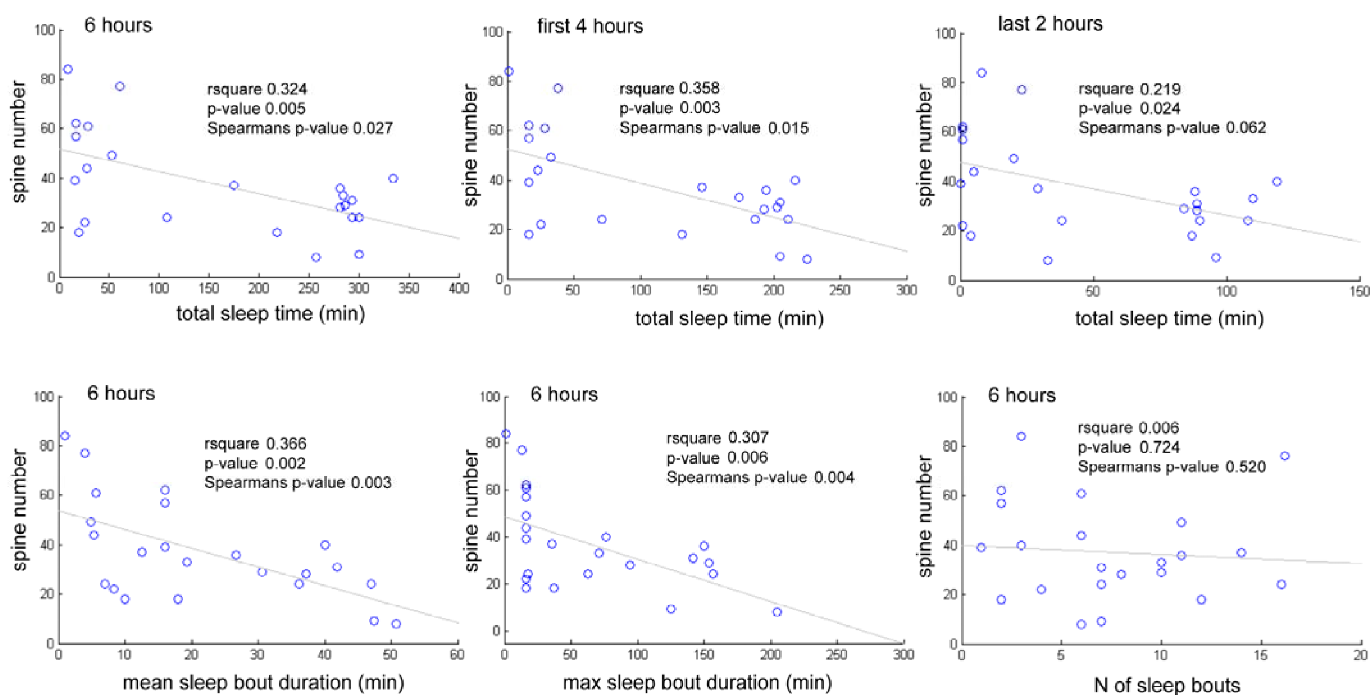


Figure S2. Flies were exposed to 12h of mall experience during the light period and then sleep deprived for 12h during the dark period. Then, the next morning, they were either sleep deprived for 6h or allowed to sleep for 6h. Top row: correlations between VS1 spine density and total sleep time for the last 6h, and separately for the first 4h and last 2h of this 6-hour period. Bottom row: correlations between VS1 spine density and duration (mean and max) or number of sleep episodes during the 6-hour period. In addition to the SD18 (n = 7) and SD12 + S6 (n = 10) flies shown in Figure 3 j-k, these graphs include 3 flies that did not sleep well enough to be selected for the analysis in Fig. 3j-k (sleep was not consolidated and/or lasted <80% of the total recording time).



Materials and Methods

Fly stocks

Flies were cultured and tested at 20°C, 68% humidity, on yeast, dark corn syrup and agar food. Unless otherwise stated genotypes are as described in Flybase (*S1*). Dr. Gaia Tavosanis kindly provided the DB331 GAL4 line (*S2*). MARCM stocks used to generate CD8-GFP clones were kindly provided by Dr. Kendal Broadie (*S3*). All other stocks were ordered from the Bloomington Drosophila Stock Center at Indiana University. *P{UAS-syt.eGFP}1* and *P{UAS-syt.eGFP}2* was derived from 6923 and 6925, *P{UASp-Act5C.T:GFP}1* from 7310, *P{Pdf-GAL4.P2.4}2* from 6900, and *P{UAS-Fmr1.Z}3* from 6931.

Homozygous *P{UAS-syt.eGFP}2 P{Pdf-GAL4.P2.4}2* were used to study the small LNvs neurons. Mushroom body MARCM clones were generated after crossing *FRT82B*/*TM3, Sb*$^{-}$ males to *y w P{hsFLP}1 P{UAS-mCD8::GFP.L}LL4*; *P{neoFRT}82B P{tubP-GAL80}LL3*; *P{GawB}ey*^{OK107} females. Females were allowed to lay for 4h and then progeny were heatshocked 26h later at 38ºC for 90 min. MARCM clones expressing Syt-eGFP were generated similarly except *y w P{hsFLP}1 P{UAS-syt.eGFP}1*; *P{neoFRT}82B P{tubP-GAL80}LL3*; *P{GawB}ey*^{OK107} females were crossed to *FRT82B*/*TM3, Sb*$^{-}$ males. Females *w*^{1118} *P{UASp-Act5C.T:GFP}1 P{DB331-GAL4}* were crossed to male CS (wild-type) flies to generate heterozygous females used for testing flies after spontaneous wake, SD, and fly mall. *Fmr1* transgenic flies were generated by crossing heterozygous *w*^{1118} ; *P{UAS-Fmr1.Z}3 /+* males to *w*^{1118} *P{UASp-Act5C.T:GFP}1 P{DB331-GAL4}* females and separated based on eye color. PCR confirmed genotype. Compared flies were aged matched siblings.

Sleep/wake analysis and sleep deprivation

Within 8h after eclosure from the pupae case, individual flies were placed inside glass tubes with enough food for 1 week of recording using the Drosophila Activity Monitor System. If flies were being monitored for more than one week, they were transferred to fresh tubes on a weekly basis within 3h from lights on. Unless otherwise stated, flies were kept in a 12h:12h light-dark cycle. DAMS monitors were housed inside environmental chambers where temperature and humidity were kept constant. Data analysis was performed using custom-designed software developed in our laboratory and written in Matlab (*S4*). Sleep and wakefulness were determined for consecutive 1-min epochs. Wakefulness was defined as any period of at least 1 min characterized by activity (≥ 1 count/min). Sleep was defined as any period of uninterrupted behavioral immobility (0 counts/min) lasting > 5 min, because previous work (*S4-S6*) has demonstrated that such periods of quiescence are associated with an increase in arousal threshold. Sleep deprivation (SD) was

performed as previously described (*S4*). Briefly, during SD flies remained in the DAMS monitor, which was placed vertically inside a framed box able to rotate along its major axis under the control of a motor. Since locomotor activity during SD was continuously recorded, the extent of sleep loss could be calculated for each individual fly. Only flies that during SD lost > 90% of their baseline sleep were included in the analysis.

Harvesting for confocal microscopy and brain dissection

Flies were selected based on their sleep/wake behavior in the 7-12h before harvesting. During that entire time interval awake flies were required to stay awake >75% of the time (including during the last 4 and 2h before harvesting), while sleeping flies had to sleep for >80% of the entire time (>63% in the last 4 and 2h). Flies were harvested by drowning in 200 uL of 1X Phosphate Buffered Saline Tween-20 (PBST). Drowned flies were dissected, fixed, and labeled using standard procedures (*S7*). An anti-GFP antibody (dilution 1/1000) in combination with a secondary goat anti-rabbit (1/1000) labeled with Alexa Fluor 488 was used to visualize GFP-tagged proteins. Mouse antibodies against FMRP (1/100), FasII (1/50), and PDF (1/100) were detected with a secondary goat anti-mouse antibody labeled with Alexa Fluor 568 (1/1000). Brains were placed in 15 uL VectaShield hard set mounting medium in Secure-Seal spacer wells sandwiched between two coverslips.

Imaging and analysis

All analyses were done blind, with the analyzer unaware of experimental condition. Images were taken with a Prairie Confocal Microscope. Image stacks were taken with a 40X oil objective (NA = 1.30) for small LNvs and gamma lobe neurons, and with a 60X oil objective (NA = 1.42) for VS1 images. Magnification was set according to Nyquist-Shannon sampling theorem for each objective in the X-Y plane. Z steps were taken at 0.33 µm. Laser and PMT settings were kept constant across samples compared to each other. Constant intensity measurements were confirmed using tetraspeck microspheres. After image acquisition, stacks were run through a 3D median filter (3x3x3) array to remove noise and intensity values were normalized between stacks from different brains for visual display and VS1 spine scoring. For small LNvs, the plugin Object 3D counter in ImageJ was used to quantify number of pixels (raw image) above a threshold kept constant across all image stacks in the PDF region of interest (*S8*). Syt-eGFP staining was quantified in the synaptic terminal region that spans from the first axonal bifurcation until the tip of the terminal (from the white arrow to the white asterisk in Fig. 1a), and in the axonal process leading to the presynaptic region, spanning from where the process leaves the posterior optic tract until the first

bifurcation (from the yellow to the white arrow in Fig. 1a). The changes in volume shown in Figure 1 were associated with no or only small changes in the intensity of the syt-eGFP and PDF signal / pixel, suggesting that increases in the syt-eGFP and PDF labeled area were driven mainly by an increase in the number of synaptic vesicles rather than by an increase in the number of synaptic proteins / vesicle. An increase in the number of synaptic vesicles is presumably associated with an overall increase in the volume of the presynaptic bouton, because the two parameters are positively correlated (*S9*). NeuronStudio (*S10*) was used for size measurements in the gamma lobes and to model the branches and count spines on the branches emanating from the VS1 neuron. For gamma lobes analysis, only brains with less than 5 MARCM-generated gamma clones were used, and brains from sleeping, awake and sleep deprived flies were carefully matched based on overall similarity in morphology and in the angle at which clones were imaged. In house software was used for intensity measurements within the VS1 neuron. First, users selected x y z coordinates outlining regions within the confocal stack containing specific structures (e.g. trunk, branches, spines). Second, structures within the user-defined regions of interest were further segmented by setting an automatic threshold defined by the Otsu method. Because out of focus light and scattered light from nearby objects increased intensity levels, users confirmed and adjusted the threshold so that pixels within the intended structure were included, while other structures or out of focus light were excluded. Because actin-GFP does not diffusely stain the neuron, segmentation was augmented by closing gaps within 6 pixels (sphere) and filling holes to make a continuous structure. Thresholds to segment FMRP staining were set to twice the average FMRP staining within the actin-GFP stained region. This threshold accurately counted granules in the OE neurons and consistently pinpointed regions with increased FMRP staining in WT neurons. For quantification, the index generated from the segmentation was then applied to quantify the original intensity values. Overall FMRP intensity was measured in trunk+branch+spine regions of VS1, whose dendritic tree is highly stereotyped. Trunk is the main shaft, extending from the cell body to the last branch, and does not have spines. Branches emanate from the trunk and contain spine protrusions. We characterized the first branch emanating laterally after the trunk turns ventrally. Spines < 2 μm long were classified as either branched, stubby, or mushroom, whereas spines > 2 μm were designated as either thick or thin. Branched spines ended asymmetrically. Mushroom spines had a constriction between the base and the end of the spine while continuous spines were designated as stubby. Thin and thick spines were differentiated based on actin-eGFP intensity, with thin spines being less intense and thick spines being more intense than the branch of origin. Images shown in Figures 1-4 are maximum intensity projections.

Fly mall (FM) experiment

In the DAM monitor each fly is housed inside a small glass tube (6.5 cm long, 0.5 cm in diameter), which is too small for flight. In contrast, 30-60 flies can be housed in the FM, which is a tube large enough (70 cm long, 10 cm in diameter) to allow flight and social interactions, as confirmed with videorecordings. The FM was lit with LED lights shining through a polypropylene white colour sheeting, with paper siding printed with rings every 1.5 to 2 cm radiating around the tube. The FM was corked with a small Petri dish containing standard food. During the experiment, flies kept in regular DAM monitor tubes were removed from the incubators and placed in the same room with the FM. The FM was inverted and knocked every 30 min, to encourage flight in the tube. Flies were removed from the FM by aspiration, and moved back to DAM monitor tubes after the FM experience. Video recordings to confirm behavior took place in the same FM without printed rings so as not to obscure the flies.

Statistical analysis

Differences across multiple groups were assessed by Kruskal-Wallis test ($P < 0.05$), followed by Mann-Whitney rank sum test for post-hoc analysis ($p < 0.05$).

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