Fig. S1. Changes in BRP and DLG expression in different fly strains. (**A,C**) BRP levels in male *white*^{1118} flies after 6-24h of sleep deprivation (SD). Representative immunoblots and quantification of the gels from 4 independent experiments. SD values are color-coded as in Fig. 1. (**B,D**) Increased DLG expression after 24h SD in male *white*^{1118} and Oregon-R (OR) flies. Representative immunoblots and quantification of the gels from 3 independent experiments. Values in C-D are mean ± standard deviation (n of flies below each bar), expressed as % change relative to sleep (no SD = 0%). *, $p < 0.05$; **, $p < 0.01$ (C, one-way ANOVA followed by Tukey's HSD Post Hoc test; D, t-test).

Fig. S2. Sleep patterns in Canton-S flies. (**A**) Daily sleep pattern in wild type female and male flies (n = 16 / group). (**B**) Females sleep less than males during the day (mean ± standard deviation, in min, 62 ± 54 vs. 237 ± 128) but not during the night (642 ± 42 vs. 598 ± 86).

Fig. S3. Sleep deprivation using the guest/host paradigm. (**A**) Video-based analysis of the time course (30-min bins) of the amount of sleep in male Canton-S flies kept alone (n = 9,

controls) or housed with a guest fly during the 12h dark phase (n = 9, host flies). (**B**) Video-based sleep analysis in a single male Canton-S host fly (upper panel) and a male *white*^{1118} guest fly (lower panel). At dark onset the *white*^{1118} fly was added to the chamber housing the host fly, and was removed at light onset. Note that both flies are awake most of the night, and the host fly shows a sleep rebound during the first 3h of the second light period. (**C**) Locomotor activity (in 1-min bins) of guest and host flies during a ~ 4-h window at night (grey area in B): the 2 flies tend to move or be quiescent (asterisks) at the same time, but their activity is not perfectly overlapping, as movement of one fly normally induces movement of the companion. Only rarely are movements of a fly not followed by a reaction in the companion (black arrow). (**D**) Sleep rebound during the first 3 hours of the light period in male Canton-S flies after 12 hours sleep deprivation by mechanical stimulation (MS) or using the guest/host paradigm (GH), expressed as % increase in sleep duration relative to baseline. Values are mean ± standard deviation (n of flies is below each bar).

Supporting Online Material

Fly stocks and maintenance
Canton-S, white-eye Canton S (w^{1118}) and Oregon-R stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University. Flies were maintained and tested on a 12:12 light:dark cycle at 22°C, 60% humidity, on yeast, dark corn syrup and agar food.

Sleep analysis and sleep deprivation
Experiments usually included 1 day of adaptation, 2 baseline days, 1 sleep deprivation (SD) day and 1-2 recovery days after SD. All flies were 1-2 week old when tested. At the beginning of the experiment, individual flies were placed in the Drosophila Activity Monitor System inside glass tubes with enough food for 1 week of recording. Monitors were housed inside environmental chambers where temperature and humidity were kept constant. Data analysis was performed by a custom-designed software developed in our laboratory (*1*). 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). Based on previous work (*1, 2*), sleep was defined as any period of uninterrupted behavioral immobility (0 counts/min) lasting > 5 min, which is associated with an increase in arousal threshold. The duration of sleep episodes was calculated by counting the number of consecutive 1-min epochs of sleep. Sleep deprivation through mechanical stimulation was performed as before (*1*). CO2 was never used to collect the flies during the whole procedure.

Western Blots. All western blots were carried on protein extracts from dissected entire brains. Flies were immediately frozen after collection and sorted according to their sleep/waking history. Brains were dissected and homogenized in groups of four. One fourth of brain lysate was run for every experiment on a gradient 4-15% gel. Antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa): nc82 (a-BRP) 1:1000; 4F3 (a-DLG) 1:2500; 1G12 (a-CSP) 1:1000; 3C11 (a-Syn) 1:1000; 8C3 (a-Syx1A) 1:1000; a-actin (ImmunO, Mp biochemical, C-4) 1:10000. Western blots and quantifications were performed as previously described (*3*), with some modifications. Specifically, ECL signal intensities were quantified using the ImageQuant software or ImageJ. Optical densities were calculated for each band of interest after performing background-correction (by subtracting the value of the region immediately above the band of interest in the same lane) and normalization (by dividing for the within-lane actin signal used as loading control). The protein/actin ratio of the SD samples was compared to the sleep control lane in the same gel to measure relative increase. Statistical analysis was done fitting the normalized protein/actin ratios in one-way ANOVA, followed by a Tukey-HSD post-hoc test for multiple comparisons, or using Student's t-test for two-groups comparisons.

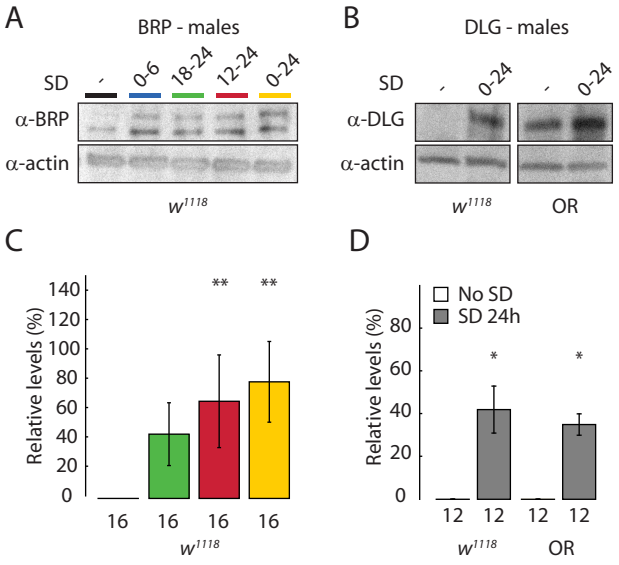
Confocal Imaging. Brains were dissected and stained as previously described (*4*). Signal intensity and volume analysis were performed adapting the protocol in (*5*). The BRP antibody nc82 was used at a concentration of 1:100 and incubated for 72 hours at 4°C. This antibody has been routinely used to quantify synapse number in both larval motoneurons and adult brain projection neurons (e.g. (*6-9*)). Images were taken on a BioRad Radiance 2100 MP Rainbow. For comparative analysis of the expression levels, images were taken in the same confocal session using identical laser and confocal settings. Analysis of the data was performed using ImageJ

(NIH, Bethesda). Selected anatomical regions were manually delimited stack by stack using a pen tablet and analyzed using a customized version of the Measure Stack plugin for ImageJ. The total number of sleeping and sleep deprived flies for results described in the main text: antennal lobes (31, 34), beta lobes of the mushroom bodies (8, 7), ellipsoid body of the central complex (13, 15), central cerebrum (excluding the optic lobes) (31, 24).

References

1. R. Huber *et al.*, *Sleep* **27**, 628 (Jun 15, 2004).
2. P. J. Shaw, C. Cirelli, R. J. Greenspan, G. Tononi, *Science* **287**, 1834 (Mar 10, 2000).
3. V. V. Vyazovskiy, C. Cirelli, M. Pfister-Genskow, U. Faraguna, G. Tononi, *Nat Neurosci* **11**, 200 (Feb, 2008).
4. A. Couto, M. Alenius, B. J. Dickson, *Curr Biol* **15**, 1535 (Sep 6, 2005).
5. P. P. Laissue *et al.*, *J Comp Neurol* **405**, 543 (Mar 22, 1999).
6. H. Bao *et al.*, *J Neurophysiol* **94**, 1888 (Sep, 2005).
7. K. Chen, D. E. Featherstone, *BMC Biol* **3**, 1 (2005).
8. A. Martin-Pena *et al.*, *J Neurosci* **26**, 10199 (Oct 4, 2006).
9. H. Kazama, A. Nose, T. Morimoto-Tanifuji, *Neuroscience* **145**, 1007 (Mar 30, 2007).

Figure S1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BRP - males
SD
-
0-6
18-24
12-24
0-24
α-BRP
α-actin
w1118
B
DLG - males
SD
-
0-24
-
0-24
α-DLG
α-actin
w1118
OR
C
Relative levels (%)
140
120
100
80
60
40
20
0
**
**
16
16
16
16
w1118
D
No SD
SD 24h
Relative levels (%)
80
60
40
20
0
*
*
12
12
12
12
w1118
OR

Figure S2

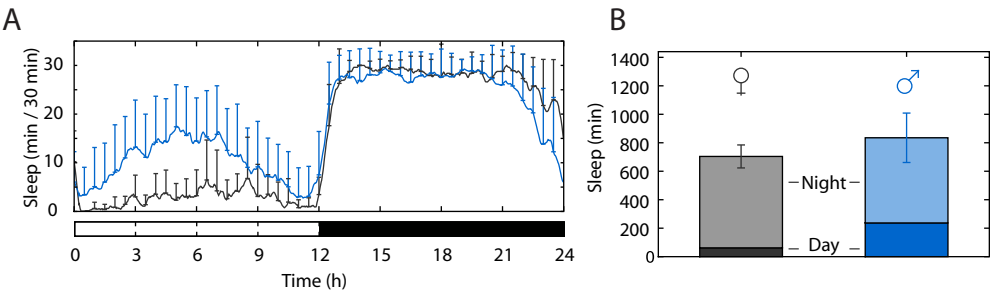
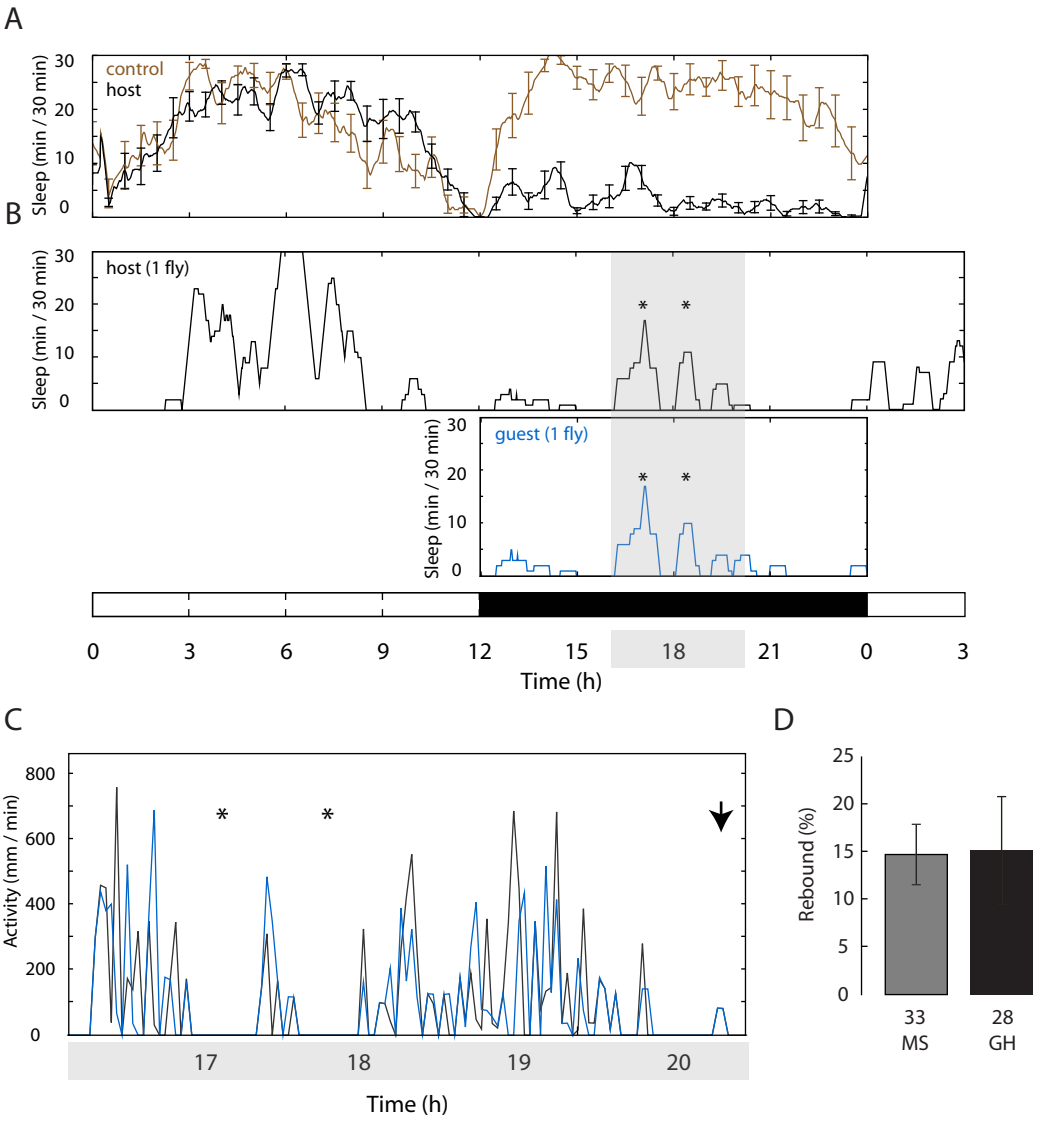

A
Sleep (min / 30 min)
30
20
10
0
0
3
6
9
12
15
18
21
24
Time (h)
B
Sleep (min)
1400
1200
1000
800
600
400
200
0
Night
Day

Figure S3


A
B
C
D
control
host
Sleep (min / 30 min)
host (1 fly)
guest (1 fly)
Time (h)
Activity (mm / min)
Rebound (%)
33
MS
28
GH