Neuron, Volume *104*

Supplemental Information

Semaphorin 2b Regulates Sleep-Circuit Formation in the *Drosophila* Central Brain

Xiaojun Xie, Masashi Tabuchi, Abel Corver, Grace Duan, Mark N. Wu, and Alex L. Kolodkin

Figure S1-related to Figure 1

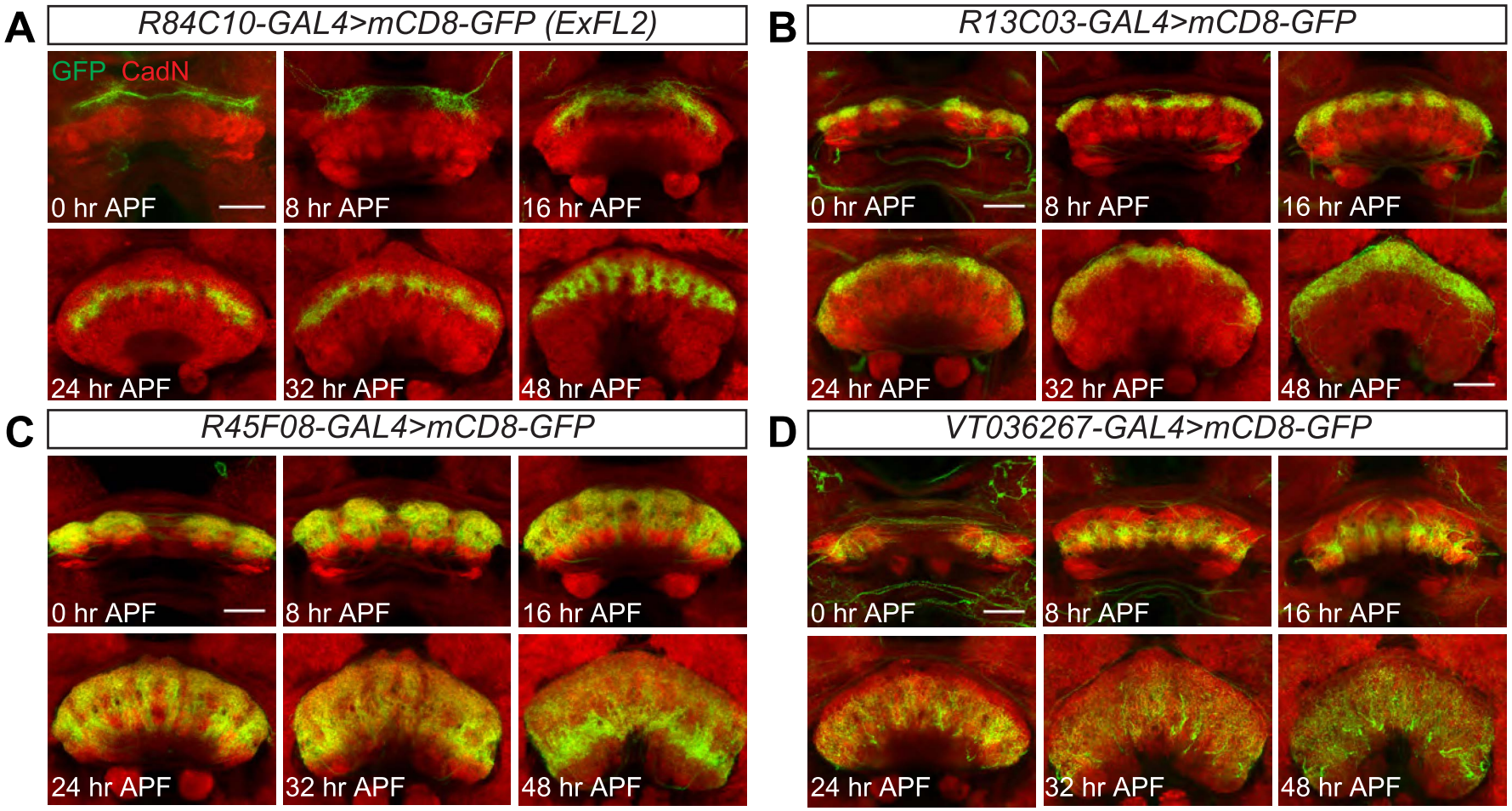


Figure S2-related to Figure 2

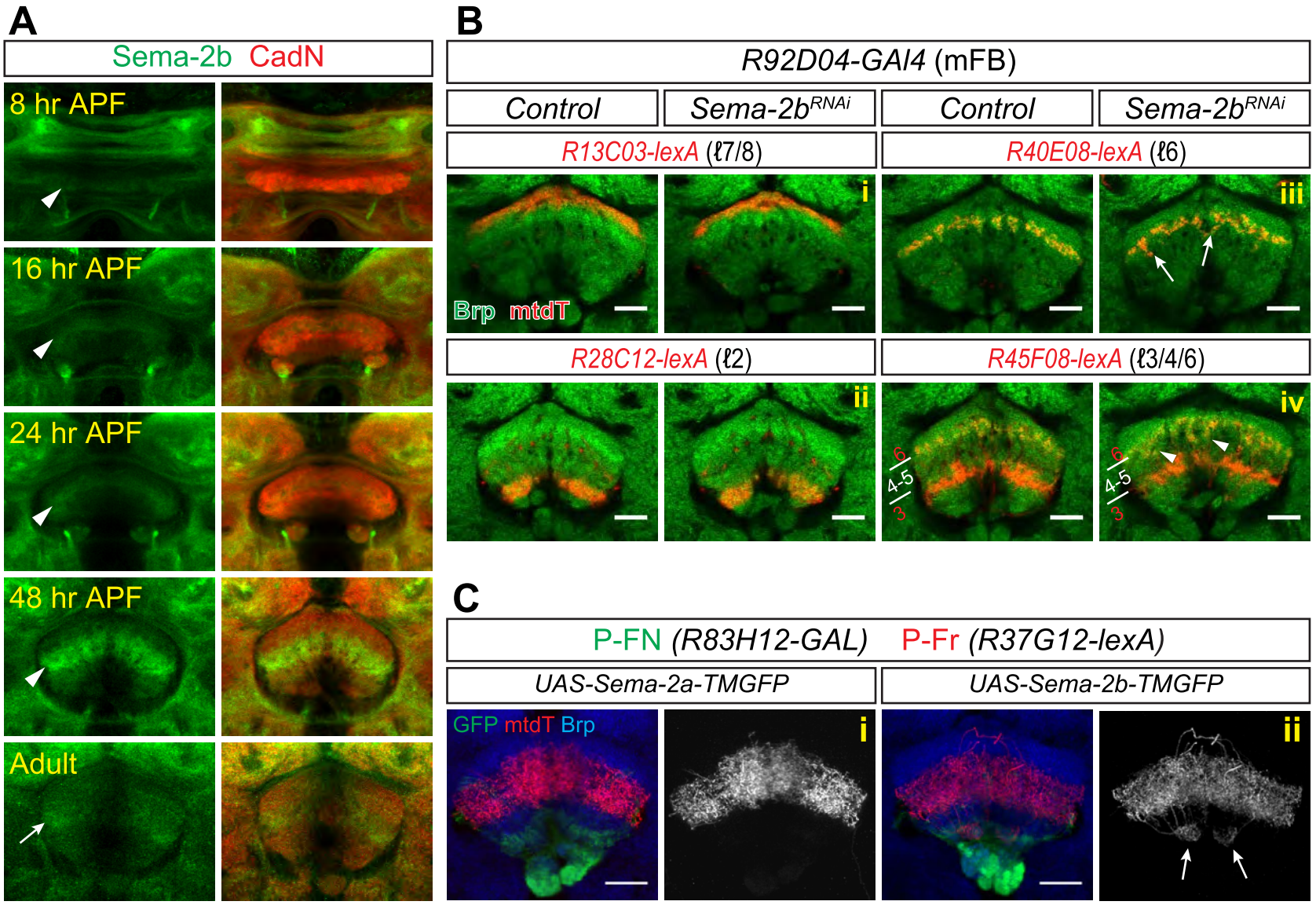


Figure S3-related to Figure 3

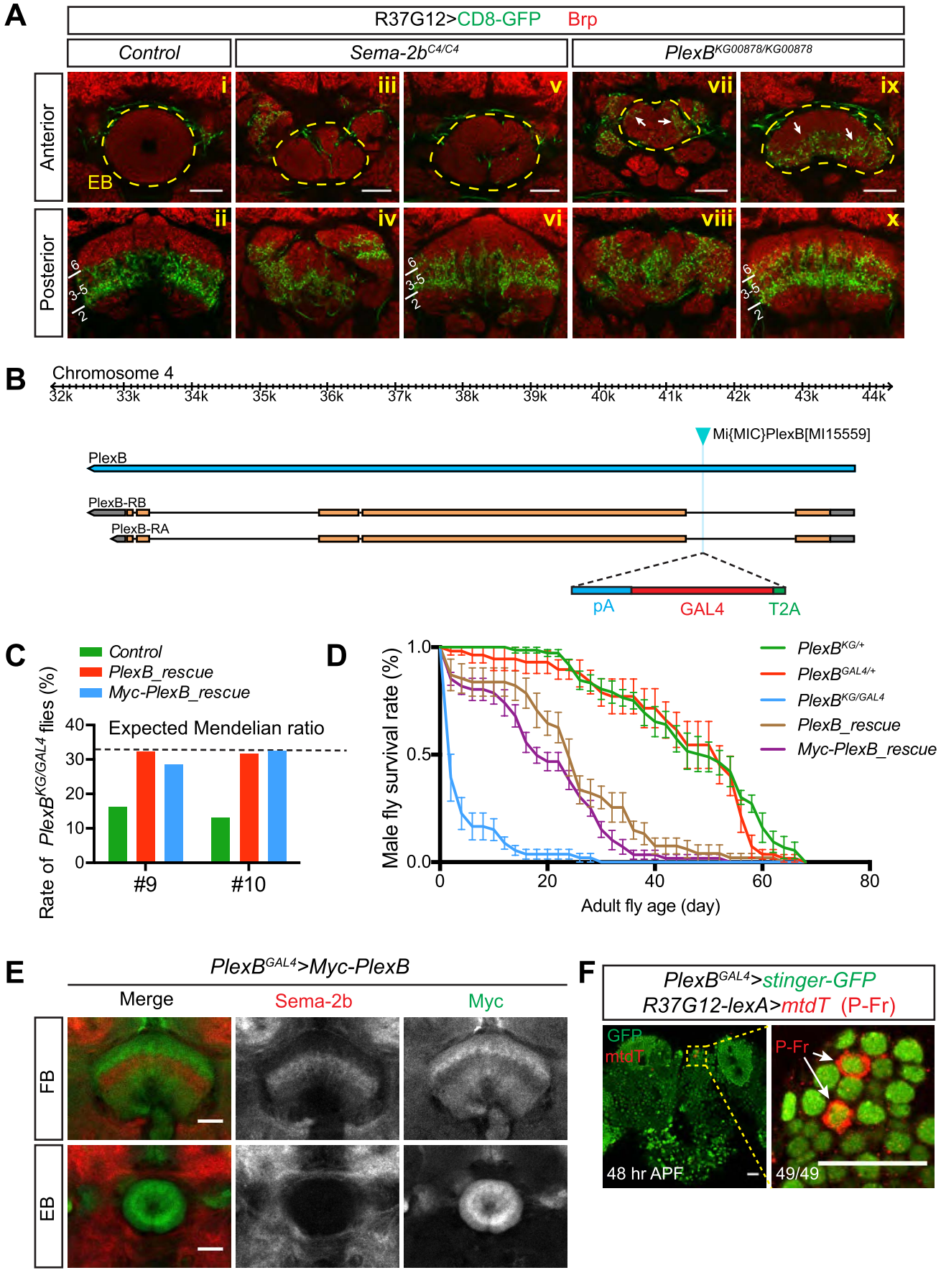


Figure S4-related to Figure 4

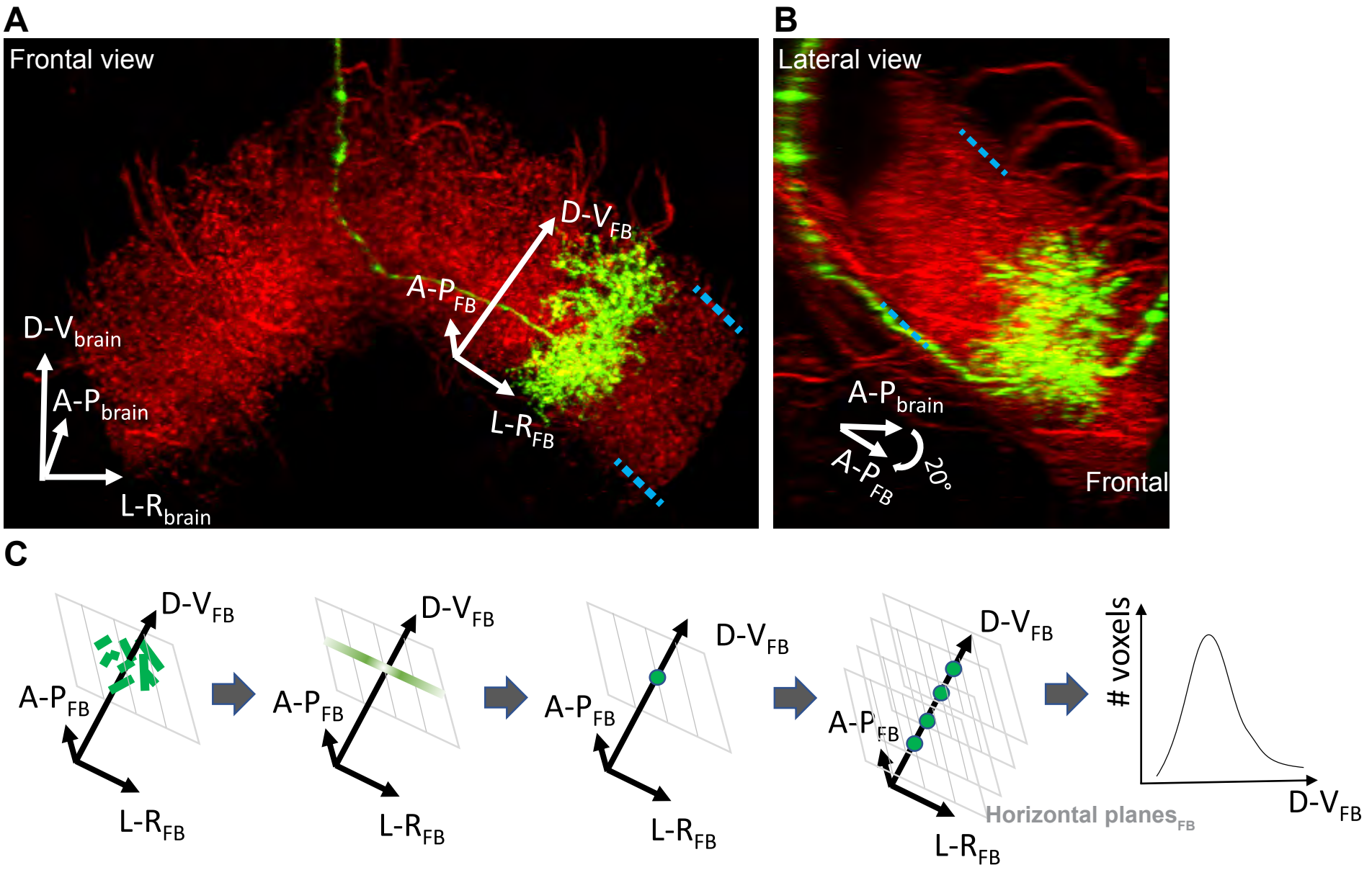


Figure S5-related to Figure 5

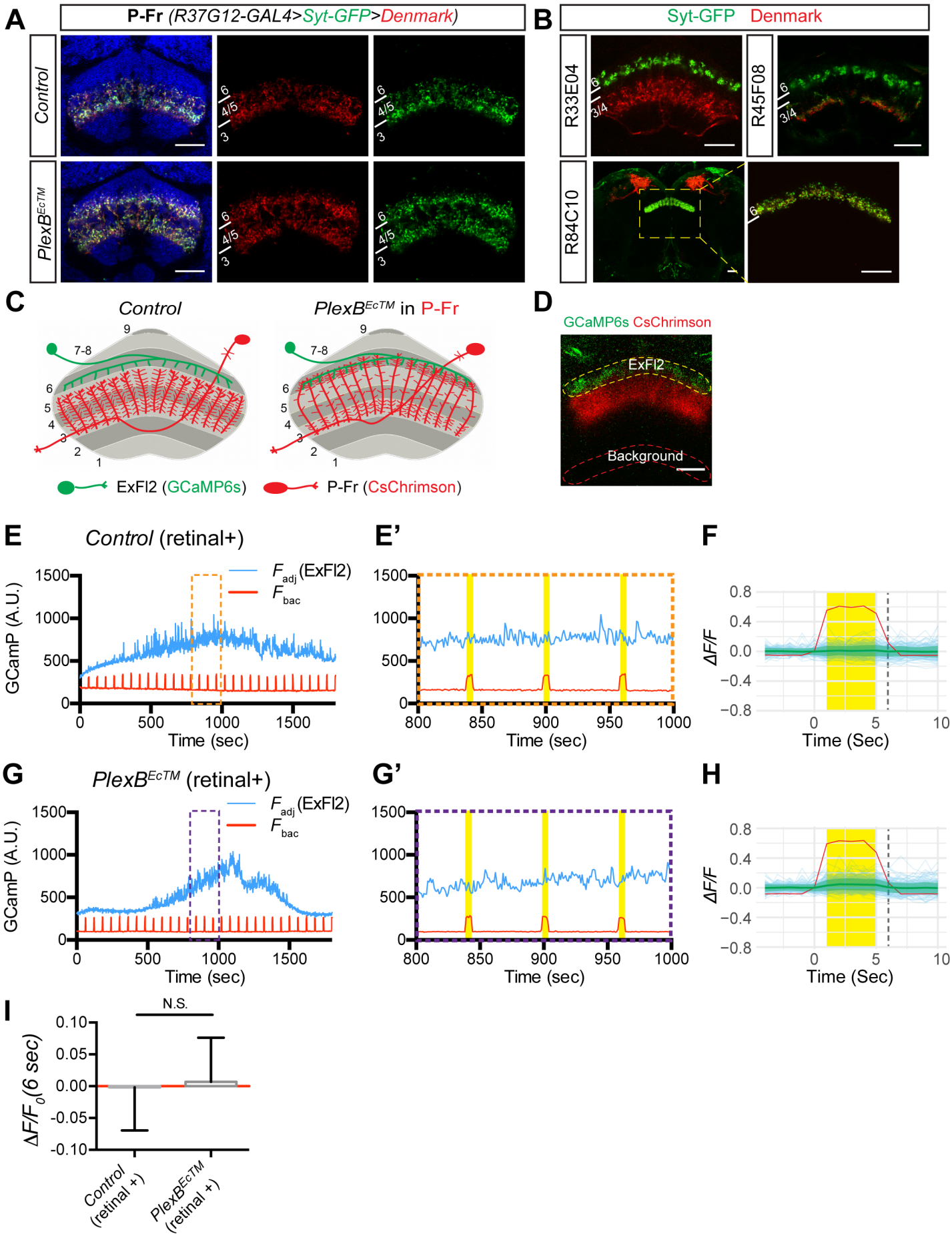


Figure S6-related to Figure 5

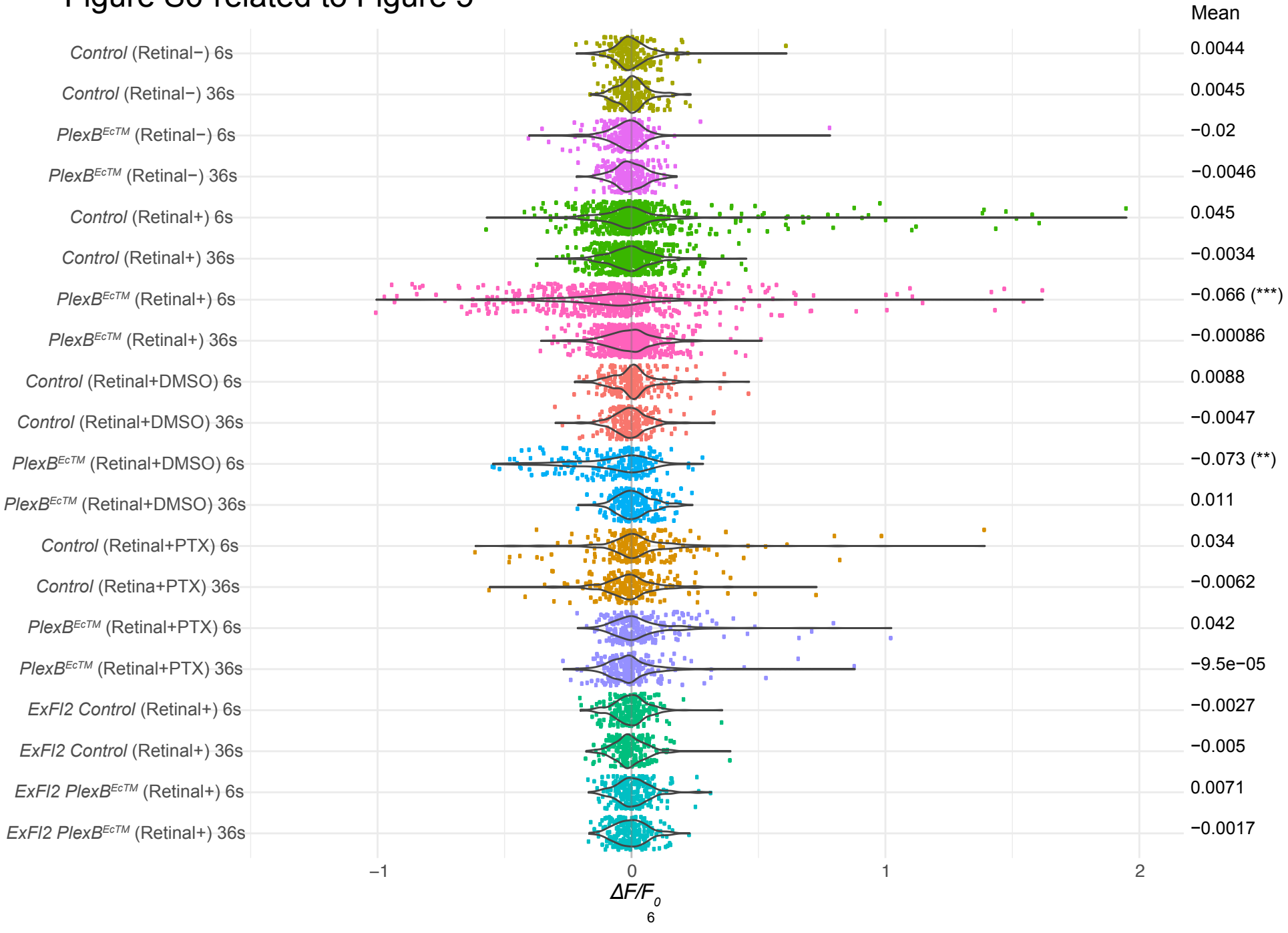


Figure S7-related to Figure 6

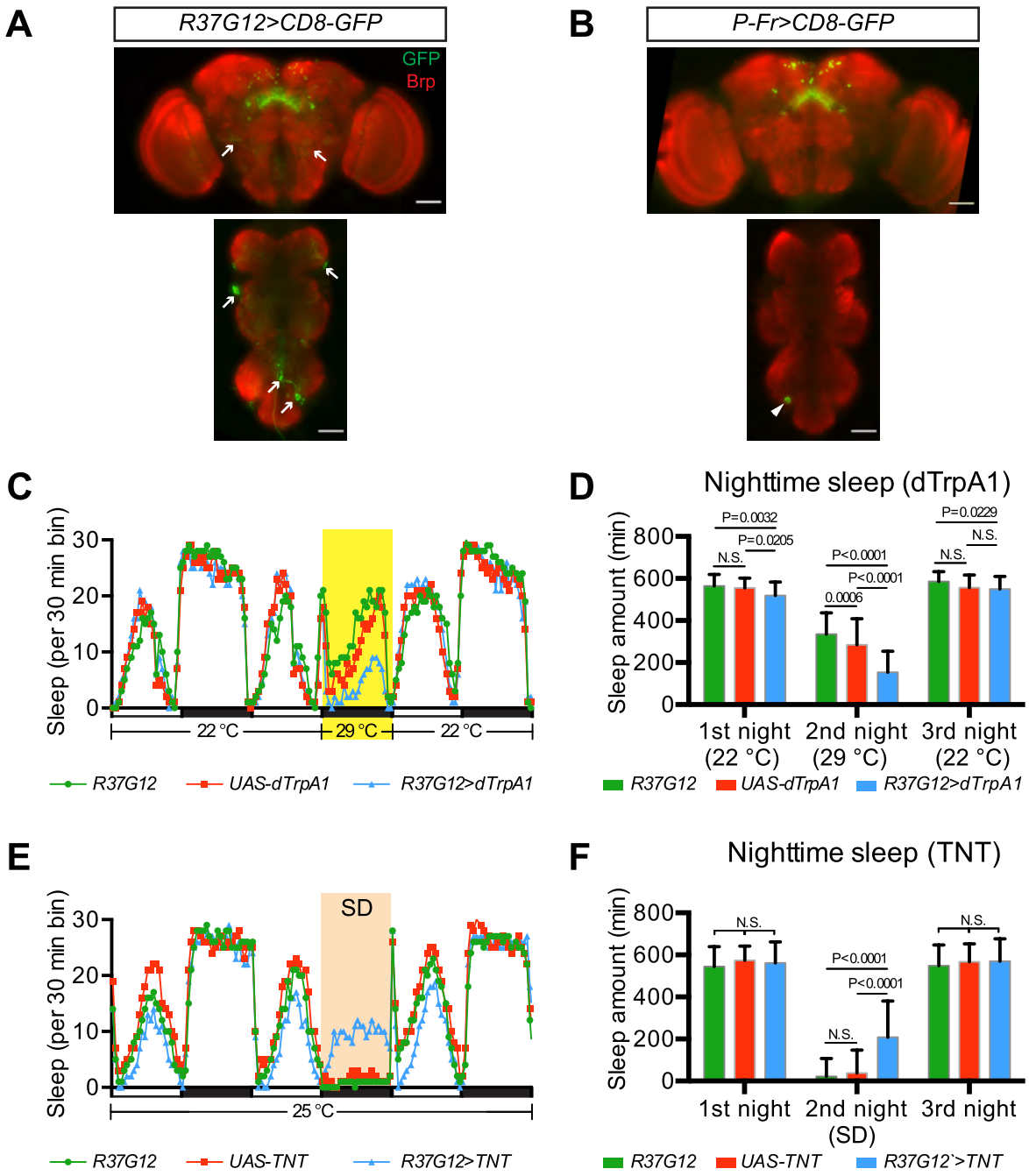
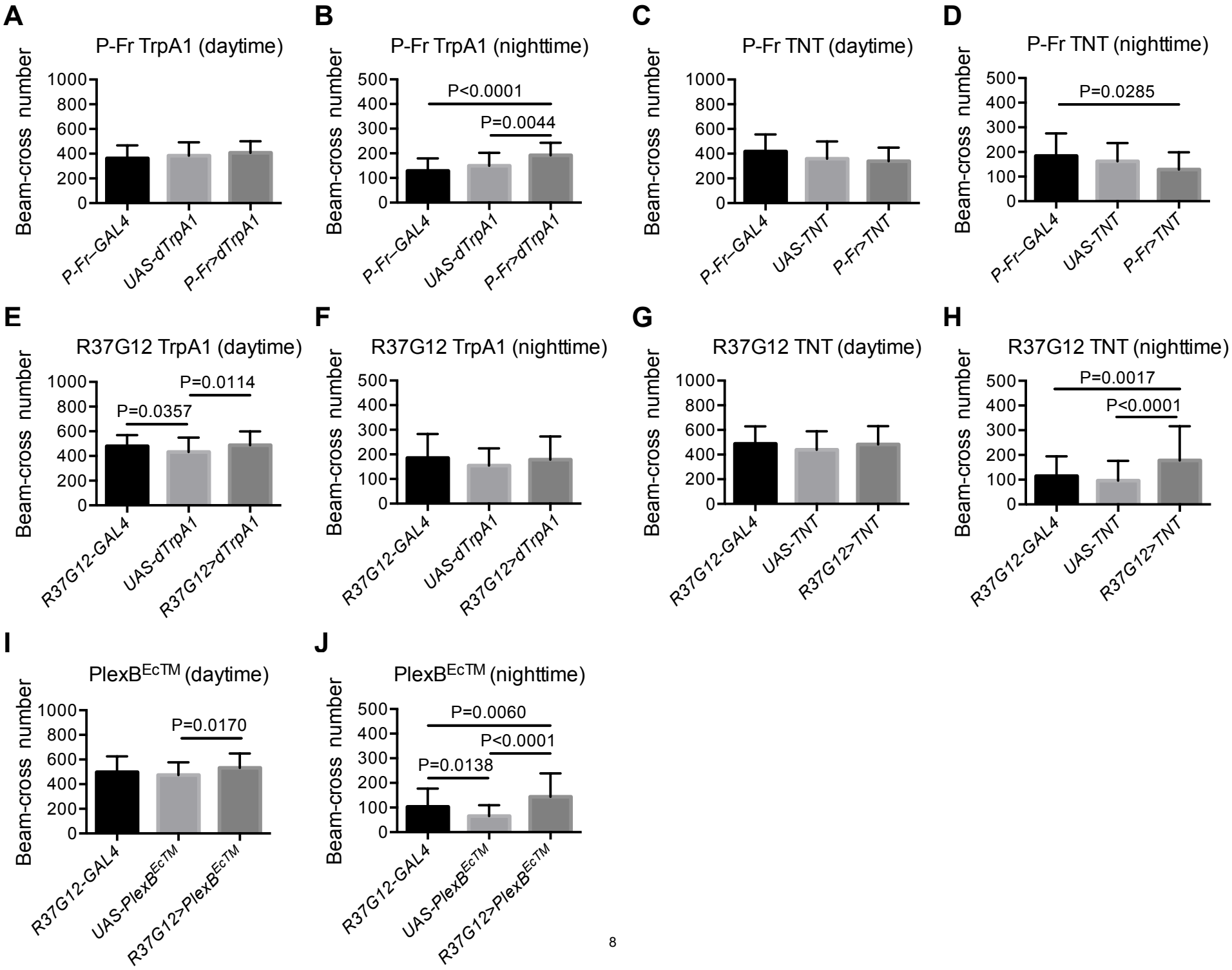


Figure S8-related to Figure 6



Supplementary Figure Legends

Figure S1-related to Figure 1. Developmental Profile of FB Innervation During Early Pupal Stages

A-D. CadN staining (red) shows changes in FB morphology during early pupal stages. The dorsal-ventral thickness, but not the lateral extent of the FB increases dramatically during the early pupal stages, indicating that FB layers are largely formed during this period.
Different groups of FB-projecting neurons are labeled here using GAL4-expressing fly lines (see Table 1) driving mCD8-GFP expression (green). Some FB neurons send their processes directly to the appropriate laminae under apparent strict spatial and temporal control (A and B). However, the processes of other FB neurons, such as R45F08 and P-Fr neurons (here labeled by using the *VT036427-GAL4* line), undergo extensive refinement of their projections before they are confined to their final target layers (C and D). All scale bars are 20 μm.

Figure S2-related to Figure 2. Sema-2b is Expressed in Medial FB and Manipulations of Sema-2b Expression Affect FB Lamination

A. Antibody staining showing that Sema-2b protein (green) is expressed in the region of the FB that includes laminae mid-way along the dorsal-ventral axis (arrowheads) throughout pupal and adult developmental stages. However, expression is at lower levels in the adult brain (arrow).
B. Anti-Brp staining (green) labels the FB and other neuropil structures in adult fly brain. *R92D04-GAL4* was used to express *Sema-2b-RNAi*, and lexA drivers was used to label different FB neurons that innervate specific FB layers (red). Knockdown of Sema-2b in R92D04 neurons does not change the innervation patterns of R13C03 (i) and R28C12 (ii) neurons in FB layers 7/8 and 2, respectively. Only very minor alterations in bouton distributions of R40E08 (iii, arrows) or R45F08 (iv, arrowheads) neurons are observed when Sema-2b is down-regulated in the FB. Loss of Sema-2b using the *R92D04-GAL4* driver appears to generate modest layer-specific phenotypes that are somewhat stronger in layers closer to where Sema-2b is normally expressed.
C. The *R83H12-GAL4* fly line was used to express membrane-tethered Sema-2a-TMGFP or Sema-2b-TMGFP in P-FN neurons (green). P-Fr neurons (red), labeled using *R37G12-lexA* driving mtdT, innervate the ventral FB when Sema-2b-TMGFP (arrows in panel ‘ii’), but not

Sema-2a-TMGFP, is expressed in P-FN neurons. (N=7 brains for Sema-2a-TMGFP and 16 brains for Sema-2b-TMGFP). Brp staining is in blue.
All scale bars are 20 μm.

Figure S3-Related to Figure 3. PlexB is Expressed in the EB and FB

A. P-Fr neurons were labeled using *R37G12-GAL4* driving mCD8-GFP (green). Brp staining (red) labeled the EB ('Anterior', yellow dashed circles) and the FB ('Posterior') in adult brains. *Sema-2b* and *PlexB* homozygous mutant flies exhibit a spectrum of EB and FB morphological and P-Fr process-innervation defects. Notably, P-Fr processes innervated FB layer 6 (marked by brighter Brp staining) in both *Sema-2b* and *PlexB* mutant flies that also had milder morphological defects (panels 'vi' and 'x'). P-Fr processes are found in the EB only in *PlexB* mutants (arrows in panels 'vii' and 'ix') but not in the control or *Sema-2b* mutants.

B. Schematics, modified from *Flybase.org*, showing the MiMic insertion ($PlexB^{MI15559}$) and the T2A-GAL4 cassette that was used to replace the MiMic within a *PlexB* intron (see Methods for details).

C. Two independent $PlexB^{GAL4}$ lines (#9 and #10) were used for complementation tests. Heterozygous $PlexB^{KG00878/GAL4}$ genotypes exhibited increased early lethality, leading to lower eclosion rates (16.3%, N=870, for #9; 13.1%, N=213, for #10) than the expected Mendelian ratio (33.33%). This early lethality was largely rescued when *UAS-PlexB* (32.3%, N=1055, for #9; 31.6%, N=98, for #10) or *UAS-Myc-PlexB* (28.5%, N=1014, for #9; 32.4%, N=188, for #10) was expressed using $PlexB^{GAL4}$ in heterozygous $PlexB^{KG/GAL4}$ animals. $PlexB^{GAl4}$ (#9) flies were used for all characterizations described in panels D-F.

D. Adult male fly longevity was assessed by counting average survival rates every two days after eclosion. Five groups of flies, each with 10-15 flies, were tested for each genotype. $PlexB^{KG00878/GAL4}$ flies (blue line) that survived to adulthood die shortly after eclosion, in contrast to $PlexB^{KG00878/+}$ (green line) or $PlexB^{GAL4/+}$ (red line) flies. This shortened longevity phenotype was partially rescued by expression of PlexB ('PlexB rescue', brown line) or Myc-PlexB ('Myc-PlexB_rescue', purple line) in $PlexB^{KG00878/GAL4}$ flies. Two-way ANONA with multiple comparisons was performed among different genotypes on different days. $PlexB^{GAl4/+}$ and $PlexB^{KG00878/+}$ groups were found to not be significantly different ($P>0.05$) from each other on all days except for day 58. These two control groups are significantly different ($P<0.05$) from

the other three groups between days 4 and 56. *PlexB*$^{KG00878/GAl4}$ group is significantly different from two rescue groups from days 2 and 28. Error bars are Standard Error of the Mean (SEM).
E. *PlexB*^{GAL4} was used to express Myc-tagged PlexB in pupal brains at 48h APF. Strong Myc immunostaining (green) was observed in the dorsal and ventral FB, and also in the EB; this is complementary to Sema-2b protein expression (red) observed in the medial FB and in other brain regions surrounding the EB.
F. Nuclear-localized GFP (stinger-GFP, green) expression was driven by *PlexB*^{GAL4}, and P-Fr neurons were labeled by *R37G12-lexA* driving mtdT (red), in pupal brains at 48h APF. Note that *R37G12-lexA* is expressed sparsely in P-Fr neurons at this stage. All 49 P-Fr neurons (mtdT positive) from 6 brains labeled by mtdT were positive for stinger-GFP.
All scale bars are 20 μm.

Figure S4-Related to Figure 4. A FB-centric Coordinate System Adapted for Image Quantification.
A and **B.** The FB layers (dashed blue lines) are not perfectly parallel to D-V, L-R or A-P axes of the fly brain, as shown here in frontal (A) and lateral (B) views of all P-Fr processes (in red) and processes from a single P-Fr neuron (in green) in FB layers 3–5. We established a FB-centric coordinate system by adjusting to the FB curvatures.
C. Diagram showing the major steps involved in computing fluorescence intensity voxels as a function of branching measurements along the D-V_{FB} axis (see Methods for details).

Figure S5-Related to Figure 5. P-Fr Neurons do not Affect ExFl2 Neuron Activity When They mis-project Into FB Layer 6
A. P-Fr processes are composed of pre- and post-synaptic terminals in the medial FB, shown here by labeling with synaptotagmin-GFP (syt-GFP, green, axons) and DenMark (red, dendrites). When PlexB^{EcTM} is expressed in P-Fr neurons, both markers are still present in P-Fr processes despite the effect this has on P-Fr lamination in the FB.
B. Syt-GFP (green) and DenMark (red) are expressed by neurons in GAL4 driver strains and label pre- and post-synaptic compartments, respectively, in different FB-projecting neurons.
C. Schematics showing the experimental design used for experiments in panels D-H to examine synaptic connections between P-Fr processes (expressing CsChrimson-mtdT) and

ExFl2 axons (expressing GCaMP6s) in both the *Control* group and in the *PlexB*^{EcTM} group expressing PlexB^{EcTM} in P-Fr neurons.

D. A live imaging snapshot shows CsChrimson (red) expression in P-Fr processes and GCaMP6s (green) in ExFl2 axons in the FB. The average GCaMP6s fluorescence intensity was measured in two regions: one inside the FB (within yellow dashed line) for GCaMP fluorescence in ExFl2 axons (***F***) and the second outside of the FB (within red dashed line) for background calibration (***F***$_{bac}$). The difference between yellow and red regions reveals the "adjusted" CCaMP6s fluorescence in ExFl2 axons (***F***$_{adj}$***=F-F***$_{bac}$).

E-I. Similar approaches as in Figures 5F-5K were used to assess GCaMP fluorescence changes in ExFl2 axons following optical stimulation of P-Fr neurons (see legend to Figure 5 for details of these experiments). Note that GCaMP fluorescence in the ExFl2 axons are also dynamic, and that there is no correlation between GCaMP fluorescence levels following LED stimulations in either the *Control* or the *PlexB*^{EcTM}–expressing groups even when GCaMP fluorescence peaks above the baseline. Eight brains are imaged for each condition, and 208 quantified LED stimulation events are included for quantification for *Control* and 235 events for *PlexB*^{EcTM} groups. A T-test was performed for panel I.

All scale bars are 20 μm.

Figure S6-related to Figure 5. Summary of Functional Image Data in Violin plots and the Statistical Analyses Using a Mixed Effect Model

All the $\Delta F/F_0$ (6 sec) data points shown in Figures 5J, 5K and S5I (labeled as *ExFl2* here) are summarized in one Violin plot. In addition, $\Delta F/F_0$ (36 sec) data points, an artificial 30-sec shift of original measurement, are treated as internal LED-off controls and are shown here for comparison. The data are subjected to additional statistic analyses using a linear mixed effect model that different parameters are considered as fixed (i.e. genotypes, solution, LED) or random (i.e. trials, samples) and therefore it can minimize the peudoreplication effect caused by using multiple trials from a single brain (see Methods for details). Unlike the data shown in Figure 6, no F_0 threshold is used to exclude any data points here. Note that a significant interaction effect of LED stimulation, genotype and solution (Retinal+) is marked by: **, $P<0.01$; ***, $P<0.001$.

Figure S7-related to Figure 6. Comparisons of two P-Fr Neuron Drivers

A and **B.** Comparisons of *R37G12-GAL4* (A) and *P-Fr–GAL4* (a split GAL4: *R37G12-p65AD; VT036267-GAL4BD*) (B) driving *UAS-mCD8-GFP* expression in the adult fly central nervous system. Note that R37G12>GFP is detected in P-Fr neurons and a few other interneurons in the brain and the ventral nerve cord (VNC) (panel A, arrows), however P-Fr >GFP expression is restricted to P-Fr neurons in the brain and 1-2 interneurons in the VNC (panel B, arrowhead). Scale bars are 50 μm.

C. Sleep profiles of *R37G12–GAL4/+* (green line), *UAS-dTrpA1/+* (red line) and *R37G12>dTrpA1* (blue line) flies during a three-day recording period. This is similar to Figure 6A except that the yellow column indicates 12-hr dTrpA1 activation at 29°C.

D. Quantification of nighttime sleep amount in the 1st, 2nd and 3rd 12-hr–dark periods shown in panel C. Note that the temperature increase (from 22 °C to 29 °C) reduces sleep overall but that *R37G12>TrpA1* flies exhibit significantly more sleep loss than the two control groups. A total of 62-64 flies were tested for each genotype.

E. Sleep profiles of *R37G12-GAL4/+* (green line), *UAS-TNT/+* (red line) and *R37G12-GAL4>TNT* (blue line) flies during a three-day recording at 25 °C. Sleep was mechanically deprived during the 2nd night (SD, pink box).

F. Quantification of sleep time per fly in the 1st, 2nd and 3rd 12-hr–dark periods shown in panel E. *R37G12-GAL4/+*: N=121 flies; *UAS-TNT/+*: N=64 flies; *R37G12>TNT*: N=62 flies. Note that the mechanical stimulation largely attenuates fly sleep overall, but *R37G12-GAL4>TNT* flies exhibit significantly less sleep loss than the two control groups.

Figure S8-related to Figure 6. Locomotion Measurements of Different Fly Lines Used in Sleep Assays

Fly locomotion was measured by counting the number of beam crossings during the 12-hr light (daytime) or dark (nighttime) periods of the first day of three-day sleep assays that are shown in other figures: Panels A and B corresponding to day-1 daytime and nighttime measurements, respectively, in Figure 6A; Panels C&D to Figure 6C; Panels E&F to Figure S7C; Panels G&H to Figure S7E; Panels I&J to Figure 7A. For the TrpA1 experiments, daytime or nighttime

locomotion of TrpA1-expressing flies is increased but this increase is not consistent between two drivers (A vs E or B vs F). For the TNT experiments, nighttime locomotion is changed, but in opposite directions, by using the two drivers. For the PlexB^{EcTM} experiments, both daytime and nighttime locomotion of experimental group is increased. However this does not explain why these flies have elevated arousal threshold during sleep.