Legend to Supplementary Figure 1

S1 (related to Figure 1). Circadian changes in synaptic markers are activity-dependent. (A-C) Changes in total area of vesicles accompanied structural plasticity. (**A**) Representative confocal images of fly brains stained for GFP (white) and PDF (magenta) taken at CT2, CT14 and CT22 during DD4. Scale bar represents 10 μm. **(B-C)** Quantitation of the number of SYT^+ puncta **(B)** and their total area **(C)**. Control *pdf*-GS>syt^{GFP} flies show no significant differences in SYT^+ puncta between CT2, CT14 and CT22, although a tendency to decrease from CT2 to CT22 could be observed. On the contrary the total area covered by SYT^+ puncta changes between subjective day and night and significant differences can be visualized between CT2 and CT14, but not between CT14 and CT22. Same letters indicates no significant differences. Statistical analysis included one way ANOVA (SYT^+ puncta p=0.1886; SYT^+ area p=0.05) with Tukey *post-hoc* test ($p<0.05$, SYT^+ area least significant difference = 11.59 μm^2). **(D-E) Electrical activity modulates cycling of active zones in PDF arborizations. (D)** Effect of hyperpolarization on BRP^+ active zones. Control *pdf*-GS> brp^{RFP} flies displayed more BRP^+ active zones at CT2 compare to CT14 and CT22 while *pdf*-GS>brp^{RFP},*KIR2.1* flies have a reduced and not oscillating number of BRP^+ active zones throughout the day. Statistical analysis included a two way ANOVA (p=0.0006) with Tukey *post-hoc* test ($p<0.05$, BRP^+ active zones least significant difference = 8.56). **(E)** Depolarization impact on BRP^+ active zones. As showed before, *pdf*-GS> brp^{RFP} flies displayed daily significant oscillations in the number of BRP^+ active zones. In contrast, when NaChBac was coexpressed within PDF neurons (*pdf*-GS>brp^{RFP},*NaChBac*) oscillations were obliterated and the number of active zones was similar to the one observed at CT2 in controls. Statistical analysis included a two way ANOVA (p=0.0067) with Tukey *post-hoc* test ($p<0.05$, BRP^+ active zones least significant difference = 3.32). Same letters indicate no significant differences.

Legend to Supplementary Figure 2

S2 (related to Figure 3). (A-B) Dorsal axonal projections of negative controls of the parental lines that show reconstituted GFP signal in Figure 3. A staining against PDF neuropeptide was used to identify the dorsal axonal terminals of sLNv neurons (not shown). **A.** The parental strain *X*-GAL4>UAS-CD4GFP$^{1-10}$ did not exhibit reconstitution of GFP signal at the timepoints were GRASP signal was observed. The negative control for *11-8*-GAL4>UAS-CD4GFP$^{1-10}$ is shown at ZT2 but no signal was observed either at ZT14 or ZT22 (not shown). (**B)** The parental strain *pdf*-lexA>lexAop-CD4::GFP^{11} did not display GFP^{+} signal at any of the ZT analyzed. **(C)** Images represent examples of a negative hit of the screen. *7-49* is expressed in a group of dorsal neurons that project towards the sLNvs axonal terminals. Note the absence of GFP^{+} signal at the 3 timepoints analyzed **(C1-C3)**. **(D)** The relative frequency of brains with reconstituted GFP^{+} signal at the timepoints analyzed is indicated. N.D. stands for Not Determined. **(E-H)** Detailed expression profiles of *3-86* **(E)**, *11-8* **(F)**, *4-59* **(G)** and *OK^{107}* **(H)** neuronal clusters. PDF and GFP signals are shown in magenta and green, respectively. **(E)** Pars Intercerebralis (PI) neurons of the Gal4 line *3-86* send neurites proximal to sLNvs dorsal projections and axons projecting ventrally along the median bundle. Expression is also seen in Mushroom Bodies (MBs) lobes and neurons of the Lateral horn. **(F)** *11-8* is expressed in the calyx of the MBs, in neurons of the suboesophageal ganglion (SOG) **(F1)**, in the PI and, extensively, in structures of the central complex **(F2)**. **(G)** *4-59* is expressed in the MBs (calyx and lobes), in a pair of neurons of the ventral brain **(G1)** and in neurons of the accessory medulla (mAcc). Note the expression in the lLNvs (asterisk) but not sLNvs somas **(G2)**. **(H)** Along with the strong expression in MBs, *OK^{107}* is expressed in neurons of the PI **(H1)** and in PDF negative neurons in the mAcc (**H2 and H2'**). Scale bar represents 10 µm unless otherwise indicated.

Experimental procedures

Strains and Fly Rearing

For RU experiments, food was mixed with RU486 (mifepristone, Sigma) in 80% ethanol to a final concentration of 200 μg/ml. To avoid any potential direct effects of light (such as masking) all the experiments were performed under constant darkness, with the exception of the screen using GRASP technique (which was performed under LD).

pdf-GS was generated in our laboratory [S1]. *pdf*-GAL4, UAS-mCD8GFP, UAS-mCD8RFP, UAS-myrRFP, *ok*^{107}-GAL4, UAS-*syt*^{GFP}, UAS-*TrpA1* and UAS-*NaChBac-eGFP* were obtained from the Bloomington Stock Center. UAS-*eGFP-Kir2.1* [S2] was provided by J. Blau (New York University, US). UAS-*brp*^{RFP} [S3] was shared by J. Berni (University of Cambridge, UK). LexAop-CD4::spGFP^{11} and UAS-CD4::spGFP$^{1-10}$ provided by K. Scott (UCBerkeley, USA) were previously described [S4]. *pdf*-lexA/Cyo was shared by M. Rosbash [S5]. Stocks *Mai179*-Gal4; *pdf*G80 and *dClock*4.1Gal4 were kindly provided by P. Emery [S6].The GAL4 collection employed for GRASP screening was a generous gift of U. Heberlein (now at HHMI, Janelia Farm, VA).

Brain cultures (extended protocol)

For brain cultures we used the protocol previously described [S7], with minor changes. Briefly, flies reared in LD were anesthetized in ice and washed with 70% ethanol. Brains were quickly dissected in a sterilized petri dish with ice-cold Schneider's *Drosophila* Medium (Invitrogen). After dissection and washing, brains were placed in a drop of medium on a millicell low height culture plate insert (Millipore) that was previously coated with laminin (3.3 μg/ml, BD Biosciences) and polylysine (33.3 μg/ml, Sigma), and placed in Petri dishes with culture medium (about 1.2 ml). Petri dishes were kept in a plastic box in a humidified incubator at 25 °C wrapped in foil (to emulate DD). The

first analysis was made 24 h post dissection (PD). The culture medium was replaced on daily basis. The culture medium was supplemented with 10,000 U/ml penicillin, 10 mg/ml streptomycin, 10% fetal bovine serum (Natocor, Córdoba, Argentina), and 10 mg/ml insulin. To assess prospective correlates of structural plasticity specific morphological features were analyzed. Axonal terminals from sLNvs were carefully evaluated at CT14 on day 2 and CT2 on day 3 in an attempt to identify changes in the degree of fasciculation of axonal bundles, appearance or disappearance (retraction) of neurites as well as positional changes of distinctive boutons/neurites relative to other branches.

Locomotor activity analysis

Data analysis was performed from day 8^{th} through 14^{th} of constant darkness. Period, FFT and rhythmicity were estimated using ClockLab software (Actimetrics). Flies with a single peak over the significance line ($p<0.05$) in $\chi 2$ analysis were scored as rhythmic, which was confirmed by visual inspection of the actograms and flies classified as weakly rhythmic (see [S8]) were not taken into account for average period calculations.

Dissection and Immunofluorescence (extended protocol)

Seven to ten day-old flies were employed. Adult heads were fixed with 4% formaldehyde in 100 mM phosphate buffer pH 7.5 for 45 min at room temperature (RT). Brains were dissected and rinsed three times in PBS with 0.1% Triton X-100 (PT) for 15 min. Samples were blocked in 7% normal goat serum for 1 h in PT and incubated with primary antibody at 4 °C overnight. The primary antibodies employed were rabbit anti-GFP 1:500 (Invitrogen), rabbit anti-RFP 1:500 (Rockland), chicken anti-GFP 1:500 (Upstate), rabbit anti-PDF 1:1500 (custom-made by NeoMPS, France) and homemade rat anti-*Drosophila*-PDF 1:500 [S1]. Samples were washed 4 times for 15

min in PT and incubated with secondary antibody at 1:250 for 2h at RT; then samples were washed 4 times for 15 min in PT and brains were mounted in 80% glycerol in PT. The secondary antibodies used were Cy2- and Cy3-conjugated anti-rabbit, Cy2-conjugated anti-chicken and Cy5- and Cy3-conjugated anti-rat (Jackson ImmunoResearch). Images were taken either on a Zeiss Pascal LSM or a Zeiss LSM 510 Meta confocal microscope. GRASP images from circadian drivers were taken using a Zeiss LSM 710 microscope. After acquisition images were processed employing Fiji, an ImageJ-based image processing environment (downloaded from http://fiji.sc/Fiji).

During the NaChBac and KIR experiments adult induction was performed transferring 3 day-old flies into vials containing standard cornmeal medium supplemented with RU486, which were maintained in LD for 3 days, and then were transferred to DD for 4 days. Adult flies were dissected on DD4.

To analyze structural plasticity and BRP or SYT quantitation at different times in the day, flies were reared in LD and three days after adult emergence they were transferred to DD. Brains were dissected at different time points on DD4.

GRASP analysis (extended protocol)

A GRASP screen was carried for a selection of circadian drivers, and for a subset of the Heberlein's enhancer trap collection [S9]. The analysis was performed at three timepoints (ZT2, 14 and 22). Due to the nature of the signal contacts through GRASP experiments were analyzed by immunohistochemistry in whole-mount brains. Double staining immunohistochemistry was directed to PDF, to visualize the dorsal axonal projections of sLNvs neurons (employing a custom-made rabbit anti-PDF at 1:1500) and to GFP, employing an antibody that specifically recognized the reconstituted GFP molecule. The following anti-GFP antibodies were tested: rabbit anti-GFP

from Abcam (1:800), rabbit anti-GFP from Invitrogen (1:1000), chicken anti-GFP from Upstate (1:500) and mouse anti-GFP from Sigma (1:10. Product number G 6539). In our hands only the mouse monoclonal anti-GFP from Sigma recognized the reconstituted GFP molecule but not the GFP^{1-10} or GFP^{11} alone. The immunohistochemistry protocol was carried out as indicated in the previous section with PBS-Triton 0.6% in all the incubations to ensure penetration of antibodies. Secondary antibodies used were Cy2 (for GRASP with circadian drivers) and Cy2 IgG1 (for GRASP analysis with the different enhancer traps) anti mouse, and Cy3 anti rabbit, both at a 1:250 dilution (Jackson InmunoResearch, USA). Laser and confocal settings were adjusted in each sample to better visualize the GFP signal. After acquisition, a median filter was applied to reduce background noise.

A minimum of 15 brains were analyzed per genotype and timepoint in the GRASP screen. For circadian drivers GRASP experiments at least 7 brains were analyzed per genotype and timepoint in each experiment. A positive GFP signal at a given timepoint was considered only if more than half of the brains presented reconstituted GFP signal. Only in those GAL4 lines that supported GFP reconstitution at some of the timepoints studied we confirmed that parental strains (*pdf*-lexA>lexAop-CD4GFP^{11} and *X*-GAL4>UAS-CD4GFP$^{1-10}$) do not present GFP^{+} signal.

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