Figure S1

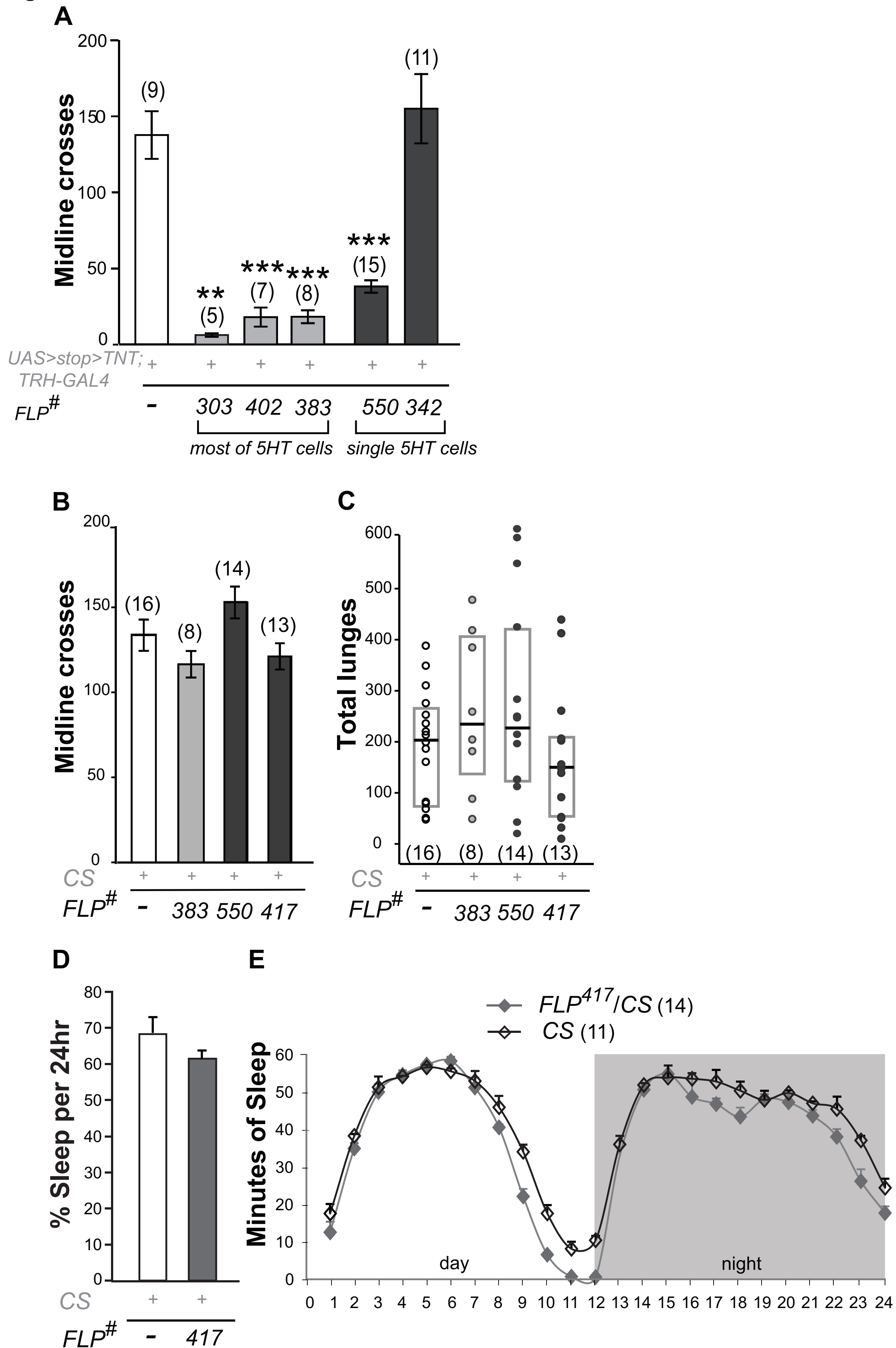

Figure S1, related to main Figures 1-2.
TNT inactivation of isolated 5HT neurons has selective effects on behavior.

A. Inactivation of large populations of 5HT neurons (light-gray bars) results in very low levels of locomotion, while inactivation of restricted 5HT neurons (dark-gray bars) produces varying effects. Data presented as means ± SEM, number of animals is indicated in parenthesis. ** - $p<0.01$; *** - $p<0.001$ vs. corresponding control (white bar), analyzed by nonparametric two-independent-sample Mann-Whitney U-test.

B. Chromosomal placement of FLP transgenes does not alter locomotion**.** Males carrying a single copy of various et-FLP transgenes (light and dark gray bars) have similar levels of locomotion as wild-type Canton-S males (white bar). Data are presented as means ± SEM, number of animals is indicated in parenthesis.

C. Chromosomal placement of FLP transgenes does not alter aggression**.** Numbers of lunges between pairs of males carrying a single copy of various FLP transgens are the same as in pairs of wild-type Canton-S males. Each dot represents the lunge count for an individual pair of flies. Number of tested pairs is indicated in parenthesis. The data are presented as boxplots with a median line. The lower and upper parts of the boxes are 25th and 75th percentiles, respectively.

D-E. Chromosomal placement of FLP^{417} transgene does not alter the average percentage of sleep **(D)** or the distribution of sleep **(E).** Gray line - males carrying a single copy of FLP^{417} transgene (FLP^{417} /CS), black line - wild-type Canton-S males (CS). Data are presented as means ± SEM.

Figure S2

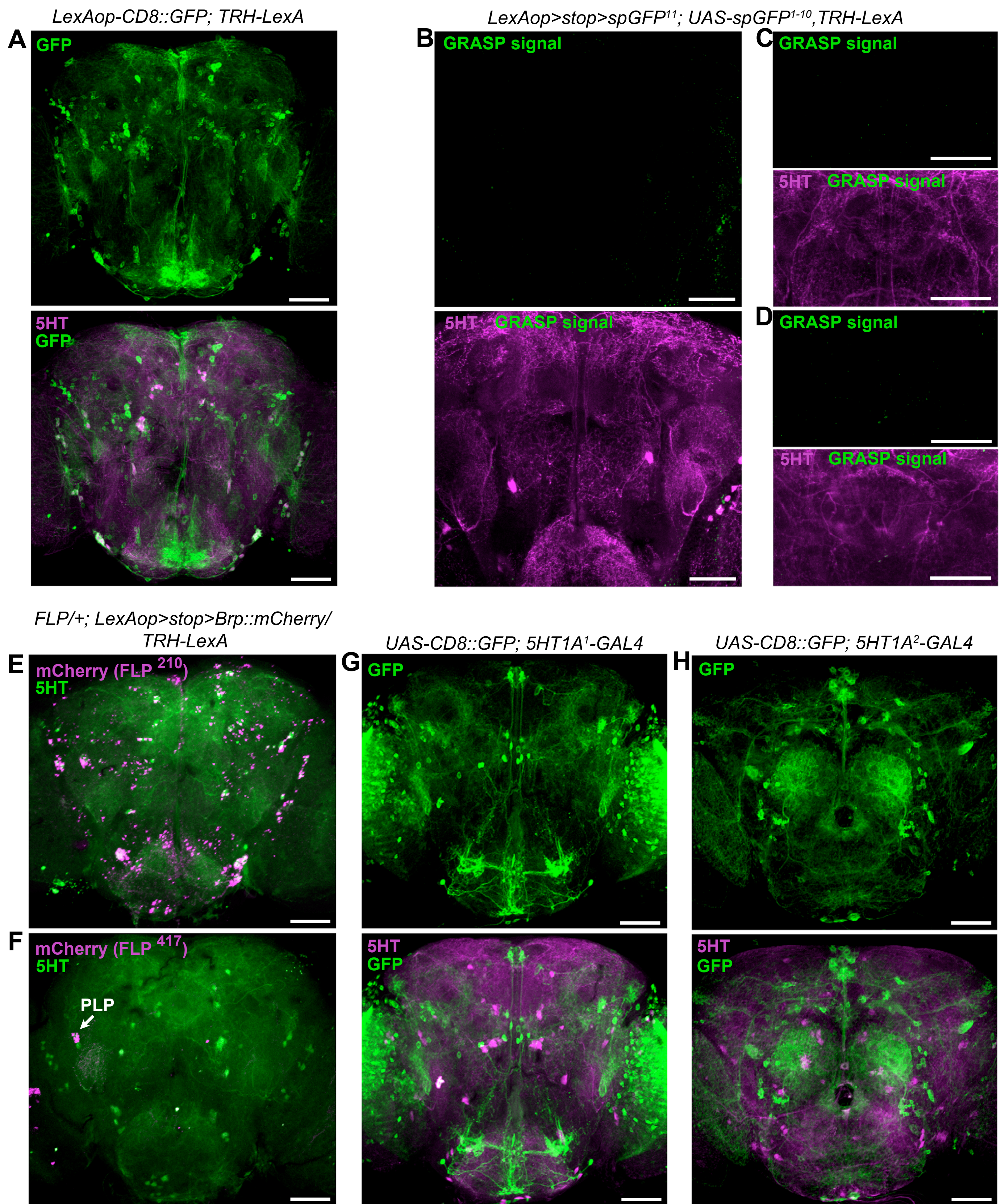

Figure S2, related to main Figure 4.
Positive and negative controls for GRASP combined with the intersectional strategy.

A. The *TRH-LexA* driven mCD8:GFP signal alone (green, upper panel) or counterstained with an anti-5HT antibody (magenta, lower panel).

B-D. GRASP negative controls. Flies carrying the two spGFP components of GRASP without either the Gal4 driver or the FLP-recombinase showed no detectable GFP signal in the ventrolateral protocerebrum **(B)**, the ellipsoid body focal plane **(C)** or the fan-shaped body focal plane **(D).** The GRASP signal was visualized using a mouse anti-GFP-20 (Sigma) antibody (shown in green), while the anti-5HT immunostaining is shown in magenta.

E. Combination of the FLP^{210} line with the *TRH-LexA* line, which was used in GRASP experiments, targets a large population of 5HT neurons. The anti-5HT immunostaining pattern is shown in green, the mCherry signal driven by a combination of FLP^{210}, *TRH-LexA* and *LexAop>stop>BRP::mCherry* is shown in magenta.

F. Combination of the FLP^{417} line with the *TRH-LexA* line, which was used in GRASP experiments, targets the individual 5HT-PLP neurons. The anti-5HT immunostaining pattern is shown in green, the mCherry signal driven by a combination of FLP^{210}, *TRH-LexA* and *LexAop>stop>Brp::mCherry* is shown in magenta. The arrow points to a single targeted PLP neuron cell body.

G. *5HT1A^{1}-Gal4* driven mCD8::GFP signal alone (green, upper panel) or counterstained with anti-5HT antibody (magenta, lower panel).

H. *5HT1A^{2}-Gal4* driven mCD8::GFP signal alone (green, upper panel) or counterstained with anti-5HT antibody (magenta, lower panel).

A-H. Scale bar represents 50 μm.

Figure S3

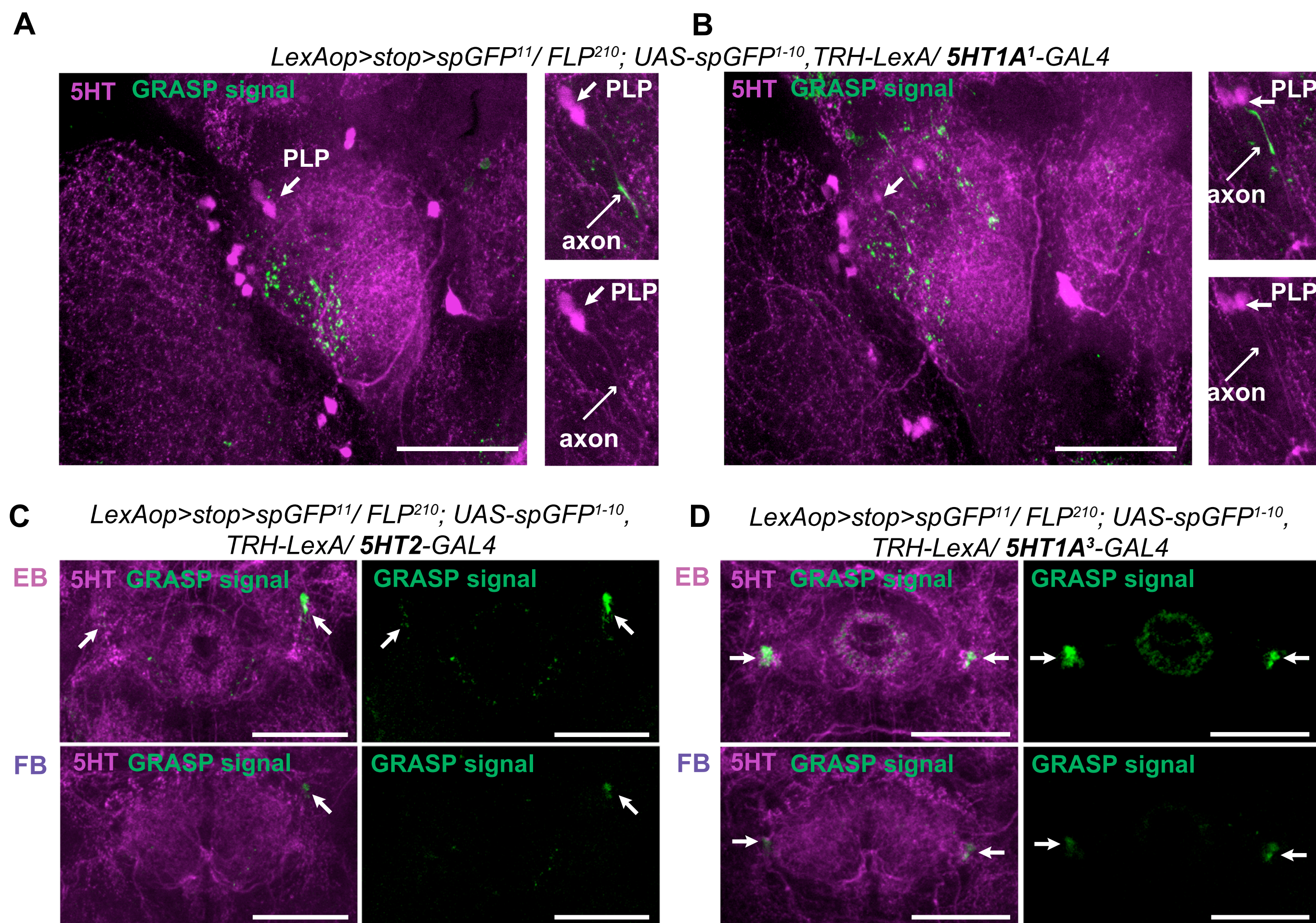

Figure S3, related to the main Figure 4.
Additional GRASP data.

A-B. Two examples of the reconstituted GFP (GRASP signal, green) between most of the serotonergic neurons and the $5HT1A^1$-expressing neurons, visualized using the mouse anti-GFP-3E6 antibody. The large panels show full frontal projections and the small panels show projections of the posterior regions of the same brain for better view of the PLP cell bodies and their axons. Note that a GRASP signal is detected over the axons of the serotonergic PLP neurons. Scale bar represents 50 μm.

C-D. Patterns of reconstituted GFP (GRASP signal, green) between most of serotonergic neurons and candidate 5HT receptor neurons in the areas of interests, which are visualized by anti-5HT immunostaining (magenta). Different frontal z-projections of the image stack were created to view the corresponding neuropils of the same brain. White arrows point to areas in which GRASP signal is observed. Scale bar – 50 μm. Both *5HT2-GAL4* **(C)** and *$5HT1A^3$-GAL4* **(D)** derived GRASP signal was observed in ellipsoid body, the region not innervated by aggression-modulation 5HT-PLP neurons. Scale bar represents 50 μm.

Table S1, related to main Figure 1.

Reproducibility of different enhancer trap (et-FLP) lines.

Et-FLP #	Description	Cells of interest	Unilateral	Bilateral	Other CNS cells [a]	*Cells in VNC* [a]
417 *(n=38)*	2-4 5HT cells from PLP cluster	76%	37%	39%	1-3 cells (55%) from PMP, SE1	1-2 cells (24%)
342 *(n=23)*	2-4 5HT cells from PMP cluster	73%	30%	43%	1-3 cells (43%) from PMP, SE3	1 cell (11%)
550 *(n=25)*	2-4 5HT cells from SE1 cluster	76%	12%	64%	1-6 cells (76%) from PI	6-8 cells (100%)

The percentage of brains that demonstrate GFP signal in the cells of interest across different preparations is shown. Isolated 5HT neurons are visualized by GFP expression (*et-FLP x UAS>stop>CD8::GFP; TRH-Gal4*).
FLP lines # 417, 342 and 550 target isolated 5-HT cells visualized by GFP expression. All lines used for the behavioral experiments had > 70% reproducibility across different brains.
[a] Other CNS and VNC cells targeted by these lines were not labeled consistently. Instead they showed varying numbers of neurons in the indicated range in different brain preparations. The percentage of brains with at least one extra cell labeled is shown in parentheses.

Table S2, related to main Figure 4.

Candidate serotonin receptor GAL4 lines used in GRASP experiments.

Gal4 line:	**Reference**	**Detected GRASP signal**	**Detected Intersectional GRASP**
5HT1B-Gal4	[S1]	MB	Did not proceed
5HT7-Gal4	[S2]	EB, AL, SOG	+, see Figure 4B
$5HT1A^{1}$-Gal4	#49397, BDSC	VLP, SOG	+, see Figure 4D
$5HT1A^{2}$-Gal4	[S3]	AL, SOG, peduncles of MB	+, see Figure 4C
$5HT1A^{3}$-Gal4	#50443, BDSC	EB	+, see Figure S3D
5HT1A-Gal4	#49583, BDSC	-	Did not proceed
5HT1A-Gal4	#38873, BDSC	SOG, MB, scattered	-
5HT2-Gal4	#50352, BDSC	EB, SOG	+, see Figure S3C
5HT2-Gal4	#49574, BDSC	-	Did not proceed
5HT2-Gal4	#38693, BDSC	scattered	-
5HT2-Gal4	#38744, BDSC	scattered	-

BDSC – Bloomington Drosophila Stock Center, MB – mushroom bodies, EB - ellipsoid body, AL – antennal lobes, SOG - suboesophageal ganglion, VLP - ventrolateral protocerebrum

Supplemental Experimental Procedures

Fly Stocks and crosses. The following fly lines were used in this study: w^{1118}, *Canton-S, 13XLexAop2-CD8::GFP* and various 5HT receptor-Gal4 lines (See Table S2) from the Bloomington Stock Center (Bloomington, IN), *5HT1A-Gal4* and *5HT7-Gal4* from Charles Nichols (LSU Health Sciences Center, New Orleans, USA), *UAS-spGFP*$^{1-10}$ and *LexAop-spGFP*^{11} from Kristin Scott (University of California, Berkeley, USA), *TRH-Gal4* was generated as previously described [S4]. *UAS>stop>CD8::GFP*, *UAS>stop>TNT, UAS>stop>dTrpA1*^{Myc}, *UAS>stop>nsyb::GFP* and *UAS>stop>DSCam::GFP* were obtained from Barry Dickson (The Research Institute of Molecular Pathology (IMP), Vienna, Austria). The line *13xLexAop2>stop>spGFP*^{11}*::CD4::HA-T2ABrp::mCherry* [S5] used to visualize neurons targeted by a combination of *TRH-LexA* and et-FLPs was a gift from Chi-Hon Lee (NICHD, Bethesda, USA). An enhancer trap (et)-FLP library was generated as described earlier [S6]. To obtain flies for behavioral experiments, females carrying *TRH-Gal4* in combination with corresponding *UAS>stop>*effector were crossed to the males of one of the et-FLP lines. For genetic controls, the same genotype females carrying *TRH-Gal4* in combination with corresponding *UAS>stop>*effector were crossed to w^{1118} males. In a second set of control experiments *Canton-S* females were crossed to males of different et-FLP lines (Figure S1B-E).

Generation of the *LexAop>stop>spGFP*^{11} line. To generate the *LexAop-FRT-stop-FRT-spGFP*^{11} line, we used the pLOT plasmid described in [S7] to obtain the spGFP^{11} fragment by PCR. This fragment was then cloned downstream of the LexAop2 sequence in plasmid pJFRC19 (#26224, Addgene) using the Not1 and Xba1 sites. We next used the pJFRC177 plasmid (#32149, Addgene) to amplify the STOP cassette and inserted it between the LexAop2 sequence and the spGFP11 fragment, using the BglII and xho1 sites. The resulting

sequence was verified by sequencing (see Supplemental Experimental Procedures). Transgenic flies were generated using PhiC31 mediated, site-specific insertion into an attP40 site (Genetic Services, Inc, Cambridge, MA).

Generation of the *TRH-LexA* line. The 1.7kb Trh promoter was amplified from pMB3-Trh [S4] by PCR (aaaggtaccTAGCTACTCGTTTTCGATTT-CCGC and aaactcgagATAAAAGTAAATATCTGGTACGACATTTG) and ligated into pENTR4 using the KpnI and XhoI sites. The promoter fragment was excised from pENTR4-Trh using EcoRV and KpnI, followed by ligation into pBPnlsLexA::p65 (Addgene), which previously was linearized with EcoRI, blunted and cut with KpnI to remove the Drosophila synthetic core promoter and the Gateway cassette. Transgenic flies were generated using PhiC31 mediated, site-specific insertion into an attP2 site (BestGene Inc, Chino Hills, CA).

For GRASP experiments *TRH-LexA* was recombined with *UAS-spGFP*$^{1-10}$. To obtain experimental flies, females carrying *LexAop>stop>spGFP*^{11} in combination with *TRH-LexA*, *UAS-spGFP*$^{1-10}$ were crossed to males carrying one of the *et-FLP* lines combined with one of the *5HT-receptor-Gal4* drivers.

Immunohistochemistry. Adult male brains were dissected, fixed, treated with primary and secondary antibodies, and prepared for confocal imaging as described previously [S8]. The following primary antibodies were used: mouse anti-GFP-3E6 anti-GFP (1:500) (Invitrogen, Carlsbad, CA), mouse anti-GFP-20 (1:100) (Sigma-Aldrich, St. Louis, Missouri), rat anti-mouse CD8a (1:100) (Caltag Laboratories, Invitrogen, Carlsbad, CA), rabbit anti-5HT (1:1000) (Sigma-Aldrich, St. Louis, Missouri), mouse nc82 (1:20) (Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-Myc (1:4000) (Abcam, Cambridge, MA). The secondary antibodies used included: Alexa Fluor 488-, Alexa Fluor 594- and Alexa Fluor 647-conjugated cross-adsorbed antibodies (Invitrogen, Carlsbad, CA). Confocal Z-stacks were

acquired using an Olympus Fluoview FV1000 confocal microscope with a UAPO 20x water-immersion or 40x oil-immersion objective, or using Nikon Eclipse 90i fluorescent microscope with an OptiGrid apparatus and NIS-Elements software. Images were processed with ImageJ imaging software.

GFP immunostaining for GRASP. We used two different antibodies for GRASP experiments - mouse anti-GFP-20 (Sigma) and mouse anti-GFP-3E6 (Invitrogen). Mouse anti-GFP-20 (Sigma) has been shown to label reconstituted GFP specifically, producing no signal with either part of spGFP alone [S7]. Our data demonstrated the same property of this antibody. Thus, mouse anti-GFP-20 (Sigma) was used for the majority of GRASP experiments (see Figure 4 for the GRASP signal and Figure S2, B-D for the negative control). Another antibody, mouse anti-GFP-3E6 (Invitrogen), produced a much stronger specific signal for reconstituted GFP, but also resulted in weak staining of Gal4-induced *UAS-spGFP*$^{1-10}$ expression alone. LexA-driven *LexAop-spGFP*^{11} expression was not detected by either antibody (data not shown), as was previously reported by others [S7, S9]. We used the mouse anti-GFP-3E6 (Invitrogen) antibody to visualize the 5HT1A-derived GRASP signal on the axons of the serotonergic PLP neurons (Figure S3A-B), which was too weak to detect using the mouse anti-GFP-20 (Sigma) antibody. In our system, *LexA-LexAop* components were used to drive the expression of spGFP^{11} in serotonergic neurons. Therefore the GRASP signal observed on the axons of the serotonergic PLP neurons could not originate from the background detection of Gal4-induced *UAS-spGFP*$^{1-10}$.

Cell counts: The reproducibility of different enhancer trap (et-FLP) lines was checked by dissecting multiple brains for each line and staining them with anti-GFP and anti-5HT antibody. Lines that targeted identifiable individual pairs of 5HT neurons in at least 70% of all brain preparations were considered reproducible (Table S1). Other cells targeted by these

lines (ranging from 1 to 6 cells per brain) were not labeled consistently. Instead they showed varying numbers of neurons in the indicated range in different brain preparations.

The experimental males used in the original screen were raised under normal temperature conditions (+25°C). In the *UAS>stop>dTrpA1*^{Myc} experiments, however, the flies were raised at +19°C, and subsequently tested at +27°C for aggression. They underwent brain dissections and anti-Myc antibody staining afterwards to check the expression pattern of the *dTrpA1*^{Myc} transgene. Under these conditions, Myc expression was more broadly distributed. Myc staining was confirmed in the neurons of interest, however, the additional cells that occasionally expressed GFP in the original screen (Table S1) were found to express Myc in a consistent manner in all brain preparations. We suspected that growing flies at the lower temperature required for the dTrpA1 experiments likely changed the efficiency of the recombinase enzyme. To test this hypothesis we took flies of the same genotypes as in the original GFP screen, but grew them at +19°C similar to the *dTrpA1*^{Myc} experimental conditions. As expected, the resultant GFP expression patterns were similar to the anti-Myc staining in *dTrpA1*^{Myc} experiments. For each tested FLP line the GFP signal was now consistently present in the neurons that were occasionally targeted when the flies were reared at +25°C. Thus, growing flies at the low temperature leads to an increase of FLP efficiency. The underlying mechanisms of this phenomenon remain unknown.

Behavioral Assays. Flies were reared on a standard cornmeal medium at +25°C and 50% relative humidity on a 12:12hr light:dark cycle. Pupae were picked and placed in individual 16x100 mm glass vials containing 1.5 ml of standard food medium, where they emerged and were kept in isolation for 4-6 days before testing. One day before the aggression assays, flies were anesthetized with CO_2, a small dot of acrylic paint was placed on the thorax, and the flies were returned to their isolation vials to recover. All experiments were performed within

the first 1-1.5 hr after lights-on.

For *UAS>stop>dTrpA1*^{Myc} data, both genetic control and experimental flies were reared at +19°C and transferred to a +27°C experimental room 15 min before the aggression assay. At the completion of the assay experimental flies were re-captured and individual fly brains were collected and processed for anti-Myc staining to ensure that the *dTrpA1*^{Myc} transgene was expressed in the neurons of interest.

Aggression assay. Males of the same genotype and the same age were paired and allowed to interact in individual chambers of 12-well polystyrene plates as previously described [S10]. Each chamber contained a food cup (filled with fly food) with a headless female in the center to attract males to the food surface. All fights were videotaped and the following parameters were quantified: time to land on the food cup and to initiate a first low-intensity encounter, latency to the first attack/lunge (calculated as “time to the first lunge” minus “time to the first low-intensity encounter”), number of lunges performed by both flies in 30 min after the first encounter, and the latency to establish a dominance relationship. In some experiments where the latency to initiate lunging differed between the control and experimental flies, the lunges also were counted for the 30 min from the time of the first lunge. Dominance relationships were determined by observing the winning fly gaining control of the food cup territory by lunging and chasing the loser off repeatedly.

Courtship assay. A single experimental male and a virgin CS female were placed by aspiration into round chambers (10 mm in diameter, 5 mm in height) and all interactions were recorded for up to 60 min. The latency to court and copulate, and the time spent courting were determined from the videos. A Courtship Vigor Index was calculated as the fraction of time that a male spent courting the female (includes tapping, wing extension and vibration, and attempted copulation) during a 10 min period after the first response to the female or until

the onset of copulation.

Locomotion. Locomotion was measured by counting the numbers of midline crosses by both flies within the first 5 min after loading the flies into the fight chambers.

Activity and sleep: The activity and sleep of individual flies was recorded for 3 consecutive days using a TriKinetics Drosophila Activity Monitors (DAM) (TriKinetics Inc, Waltham, MA). Activity counts were summed across all wake bins, defined by at least one beam crossing in 5 min, and then averaged per minute. A sleep episode was defined as a 5-min bin of uninterrupted rest with the DAM system. Sleep and activity data were averaged across three days using an Excel-based "Sleep Counting Macro" [S11].

Statistical Analyses. All data were analyzed using the SPSS 16.0 for Mac statistical software package (SPSS, Chicago, IL). For pairwise comparisons the nonparametric two-independent-sample Mann-Whitney test was used. Two-tailed P values were determined with the significance level set at *- $P < 0.05$; ** -$p<0.01$; ***-$p<0.001$. In case where outlier data points were detected, the outliers were excluded and the data analysis was run again to confirm that observed significant differences were not due to the outliers.

13XLexAop2>stop>spGFP^{11} sequence:

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