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Report

Single Serotonergic Neurons that Modulate Aggression in *Drosophila*

Olga V. Alekseyenko,[1,*] Yick-Bun Chan,[1] Maria de la Paz Fernandez,[2] Torsten Bülow,[3] Michael J. Pankratz,[3] and Edward A. Kravitz[1]

[1]Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA

[2]Instituto de Investigación en Biomedicina de Buenos Aires - CONICET - Partner Institute of the Max Planck Society, Buenos Aires C1425FQD, Argentina

[3]Molecular Brain Physiology and Behavior, LIMES Institute, University of Bonn, Bonn 53115, Germany

Summary

Monoamine serotonin (5HT) has been linked to aggression for many years across species [1–3]. However, elaboration of the neurochemical pathways that govern aggression has proven difficult because monoaminergic neurons also regulate other behaviors [4, 5]. There are approximately 100 serotonergic neurons in the *Drosophila* nervous system, and they influence sleep [6], circadian rhythms [7], memory [8, 9], and courtship [10]. In the *Drosophila* model of aggression [11], the acute shut down of the entire serotonergic system yields flies that fight less, whereas induced activation of 5HT neurons promotes aggression [12]. Using intersectional genetics, we restricted the population of 5HT neurons that can be reproducibly manipulated to identify those that modulate aggression. Although similar approaches were used recently to find aggression-modulating dopaminergic [13] and Fru^{M}-positive peptidergic [14] neurons, the downstream anatomical targets of the neurons that make up aggression-controlling circuits remain poorly understood. Here, we identified a symmetrical pair of serotonergic PLP neurons that are necessary for the proper escalation of aggression. Silencing these neurons reduced aggression in male flies, and activating them increased aggression in male flies. GFP reconstitution across synaptic partners (GRASP) [15] analyses suggest that 5HT-PLP neurons form contacts with 5HT1A receptor-expressing neurons in two distinct anatomical regions of the brain. Activation of these 5HT1A receptor-expressing neurons, in turn, caused reductions in aggression. Our studies, therefore, suggest that aggression may be held in check, at least in part, by inhibitory input from 5HT1A receptor-bearing neurons, which can be released by activation of the 5HT-PLP neurons.

Results and Discussion

Isolation and Anatomical Characterization of Individual Serotonergic Neurons

As in other species, serotonergic neurons in the fly nervous system display arbors of processes that ramify widely in multiple neuropil areas, through which they affect virtually all aspects of behavior. Therefore, we have used an intersectional genetics approach to isolate restricted sets of serotonergic neurons and manipulate their function in order to ask whether they are involved in the regulation of specific behaviors like aggression, or whether they exert multiple modulatory actions on many behaviors. We screened 65 enhancer-trap FLP recombinase transgenic lines (et-FLP) [13] with a serotonin-specific *TRH-Gal4* driver [12] and a *UAS>stop>mCD8::GFP* reporter, seeking to find combinations that resulted in reproducible GFP expression in small subsets of 5HT neurons. Although several broadly expressed FLP lines displayed a major overlap between the GFP-positive neurons and the total populations of 5HT neurons (for example, line FLP^{383}; see Figure 1A), only three FLP lines reproducibly targeted very restricted sets of 5HT neurons. We further characterized each type of the isolated 5HT neurons by identifying the areas of their arborization within known neuropil regions throughout the brains. The line FLP^{417} (Figure 1B) targeted 1–2 5HT-positive neurons from the posterior lateral protocerebrum (PLP) cluster on each side of the brain. The PLP cell bodies are located on the posterior surface of the brain, but their arbors form a dense neuropil throughout the ventrolateral protocerebrum and also ramify toward the central complex structures (Figure 1B). A second line, FLP^{550}, in combination with the *TRH-Gal4* driver, consistently labeled two large serotonergic neurons from the SE1 cluster (Figure 1C). These neurons arborize in the dorsal region of the subesophageal ganglion and send thick descending projections to the ventral nerve cord. The last of the selected 5HT-specific lines, FLP^{342}, targeted neurons from the posterior medial protocerebrum (PMP) cluster (Figure 1D) that send projections to the superior medial protocerebrum.

Serotonergic PLP Neurons Enhance Aggression

Our previous findings [12] demonstrated that acute disruption of serotonergic neurotransmission yielded male flies that could fight but displayed a dramatic reduction in the number of higher-intensity aggressive interactions. Here, we asked whether silencing of any of the genetically isolated 5HT neurons using the tetanus neurotoxin light chain (TNT) [16] had effects on aggression. TNT cleaves the synaptic-vesicle-associated protein, synaptobrevin, thereby chronically blocking transmitter release [17]. For these experiments, we paired socially naive males in multiwell plate aggression chambers [18]. The most important pattern in a *Drosophila* male aggressive attack is the lunge, a high-intensity behavioral pattern required for the establishment of dominance relationships. To demonstrate the dynamics of fights, we measured how long it takes to initiate higher-intensity attacks (the latency to the first lunge), the intensity levels displayed by the pair of flies (the number of lunges), and whether and when a dominance relationship was established as an outcome of a fight (the latency to establish dominance).

Early on we noticed that chronic silencing of large populations of 5HT neurons produced unhealthy flies that had difficulty landing on the food cup in the fight chamber and had profound locomotion deficits. As a consequence, they did not fight. This phenotype was observed with several broadly expressed lines, which targeted many 5HT neurons (FLP^{303}, FLP^{402}, FLP^{383}; Figure S1A available online). Thus, any

*Correspondence: olga_alekseenko@hms.harvard.edu

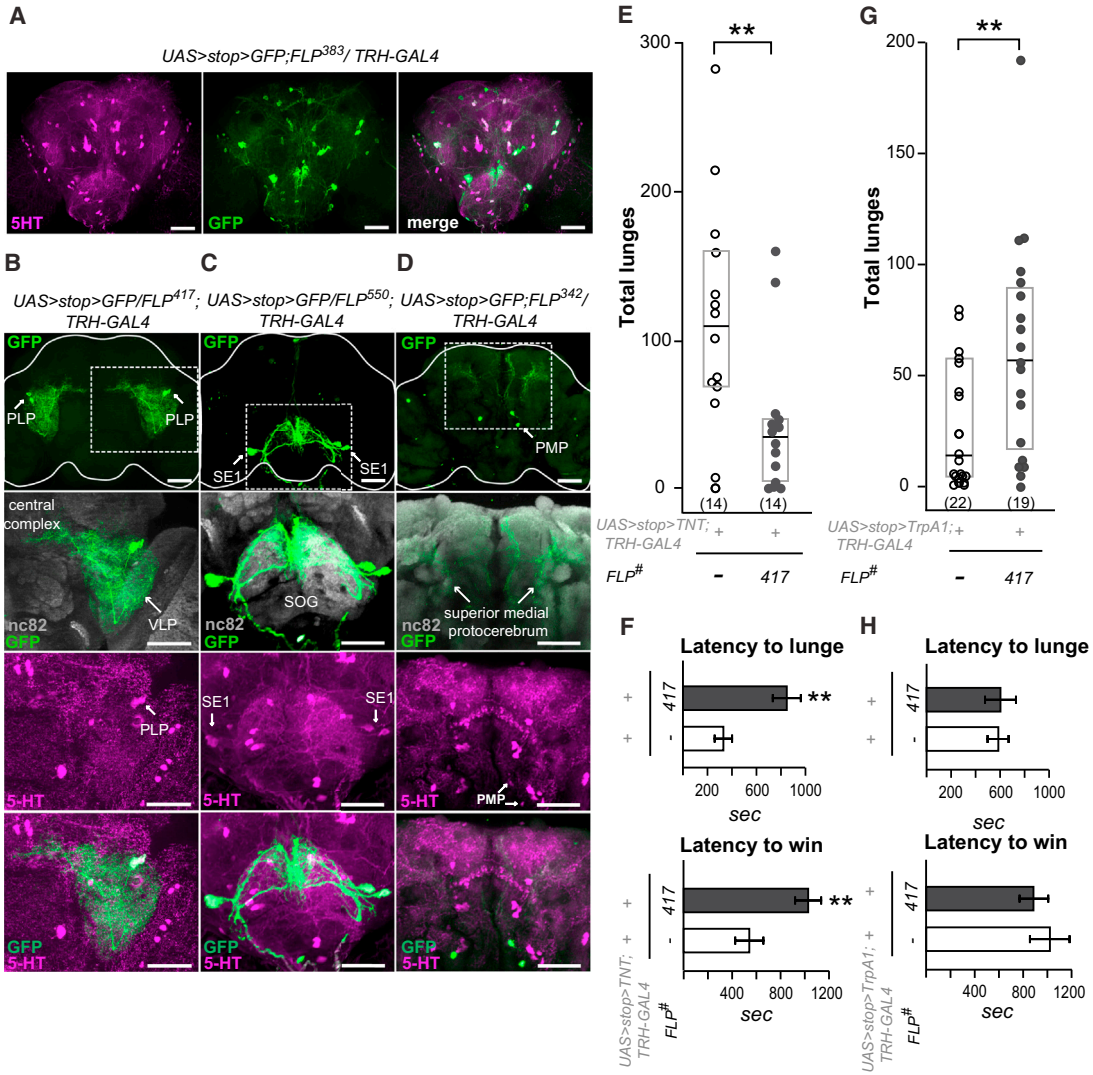

Figure 1. A Single Pair of Serotonergic PLP Neurons Enhances Aggression

(A–D) Serotonergic neurons identified by the enhancer trap (et)-FLP screen.

(A) Example of a broadly expressed FLP line that targets most of the 5HT neurons in the fly brain. The anti-5HT immunostaining pattern is shown in magenta; the membrane-tethered GFP signal driven by a combination of FLP^{383}, *TRH-Gal4*, and *UAS>stop>mCD8::GFP* is shown in green. The full z stack frontal projection is shown. Scale bars represent 50 μm.

(B–D) Individual 5HT neurons targeted by the use of different et-FLP lines. The mCD8::GFP signal amplified by anti-CD8 antibody staining is shown in green; the neuropil areas stained by an nc82 (anti-Bruchpilot) antibody are shown in gray; anti-5HT immunostaining is shown in magenta. Dotted boxes outline the magnified fields shown in the lower panels. The upper panels show full frontal projections. Scale bars represent 50 μm. Different frontal z stacks through either the anterior or the posterior areas of the same triple-stained brains were created when required to view the processes or cell bodies shown in the lower panels.

(B) The FLP^{417} line restricts GFP expression to 1–2 bilateral neurons from the PLP cluster (green). These neurons arborize within the ventrolateral protocerebrum (VLP) and send a midline-directed process toward the central complex (see Figure 3A for more details).

(C) The FLP^{550} line restricts GFP expression to 1–2 bilateral neurons from the SE1 cluster (green). These arborize within the subesophageal ganglion (SOG) and send descending projections to the ventral nerve cord.

(D) The FLP^{342} line restricts GFP expression to 1–2 bilateral neurons from the PMP cluster (green) that arborize in the superior medial protocerebrum.

For et-FLP line reproducibility and cell-count data, see Table S1.

(E–H) Manipulation of individual 5HT neurons from the PLP cluster targeted by FLP^{417} changes aggression.

(E) Total number of lunges performed by pairs of males with TNT-inactivated 5HT-PLP neurons.

(F) Latency to the first lunge and to the establishment of dominance in flies with TNT-inactivated 5HT-PLP neurons.

In (E) and (F), both genetic control and experimental flies were reared and fought at constant +25°C conditions. Note that the reduction in lunge numbers was not due to the increased latency to lunge because the number of lunges counted for 30 min after the first lunge rather than from the time of landing on the food surface was also reduced (FLP^{417}: 74.2 ± 17.5; controls: 134.9 ± 22.0; Mann-Whitney U = 54, p = 0.043).

(G) Total number of lunges performed by pairs of males with dTrpA1-activated 5HT-PLP neurons.

(H) Latency to the first lunge and to the establishment of dominance in flies with dTrpA1-activated 5HT-PLP neurons.

In (G) and (H), 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. Each dot in (E) and (G) represents the lunge count for an individual pair of flies. Data are presented as boxplots with a median line. The bottom and top of the box show the 25th and 75th percentile. Latencies in (F) and (H) are presented as means ± SEM. **p < 0.01 versus controls (white bar or white dots), analyzed by nonparametric two-independent-sample Mann-Whitney U test.

See Figure S1C for FLP parental control aggression data.

possible effects of individual 5HT neurons on aggression were probably masked by a major locomotion deficiency in these cases. Then, we checked the general activity of the flies with inactivated individual 5HT neurons and found that one of the restricted lines (FLP^{550}) also produced unhealthy flies with noticeable locomotion deficit (Figure S1A). These flies did not fight either. In contrast, inactivation of neurons from the PMP cluster targeted by FLP^{342} had no effects on either locomotion or aggression (data not shown). Only the PLP neurons targeted by FLP^{417} yielded an aggression phenotype that was not accompanied by substantial deficits in other behaviors. Therefore, for the rest of the study, we focused on the serotonergic PLP neurons. Inactivation of these neurons produced flies that not only lunged less often than controls (Figure 1E) but also took longer to start lunging and to establish dominance relationships (Figure 1F). To confirm the specificity of the observed aggression phenotype of the PLP neurons, we acutely activated them using the *UAS>stop>dTrpA1*^{Myc} transgene [19]. dTrpA1 is a temperature-sensitive cation channel that, when expressed in neurons, allows activation of the cells by small temperature increases [20]. We verified that the *dTrpA1*^{Myc} transgene was actually expressed in the neurons of interest by dissecting the experimental fly brains and processing them for anti-Myc staining after completion of the aggression assays. In contrast to the TNT silencing results, activation of the PLP neurons produced significant increases in the number of lunges (Figure 1G). This phenotype was similar to our previously demonstrated effects of induced activation of the entire population of 5HT neurons on the number of lunges [12]. However, activation of the PLP neurons did not reduce the latency to lunge, unlike what was observed with activation of the entire 5HT system. This suggests that other, yet-unidentified, 5HT neurons might also be involved in the modulation of aggression. Activation of the 5HT neurons targeted by either FLP^{550} or FLP^{342} had no effects on aggression (data not shown).

To ask whether the 5HT-PLP neurons played roles in behaviors other than aggression, we expressed the TNT transgene in them and examined the following: (1) locomotion and sleep as indicators of general activity and (2) courtship as an example of a different social behavior. Flies with inactivated PLP neurons showed a small but significant deficit in locomotion (Figure 2A). This raises the question of whether the aggression-attenuating phenotype described above might be a consequence of the fact that these flies are simply "slower" than controls. However, a similar locomotion deficit was observed after dTrpA1-induced activation of the PLP neurons (Figure 2B), along with an increase of aggression (Figure 1G). These results indicate that the opposing aggression phenotypes caused by inhibition and activation of the 5HT-PLP neurons are not an indirect effect of a change in locomotor activity. An examination of 24 hr sleep patterns (Figures 2C and 2D) revealed that these flies slept on average less than controls during both day and night. They did, however, show normal diurnal sleep profiles and also the expected circadian anticipation of light and dark phases of the cycles. Importantly, their amount of sleep was not different from controls during the first hour of morning activity peak, when aggression assays were performed (Figure 2D, bracket). Next, we examined male courtship, as a different social behavior. We measured courtship vigor index, latency to court, and copulation success, and found no deficits in flies with inactivated PLP neurons (FLP^{417}; Figures 2E–2G); they performed courtship rituals as efficiently as controls. Thus, manipulation of 5HT-PLP neurons produced mild effects on activity-related behaviors that did not correlate with the observed effects on aggression or interfered with courtship behavior.

To rule out the possibility that FLP transgene insertion sites alone may contribute to the observed phenotypes, we examined flies carrying et-FLP transgenes without the Gal4 driver or UAS effector. None of the progeny of these flies crossed to wild-type Canton-S replicated the locomotion (Figure S1B), aggression (Figure S1C), or sleep (Figures S1D and S1E) phenotypes seen with the full complement of corresponding transgenes.

Targets of the Aggression-Modulating 5HT-PLP Neurons

The detailed morphological analysis showed that the densest arborizations of the 5HT-PLP neurons are in the ventrolateral protocerebrum (Figure 3A, top left), a region previously characterized as an integrative center for auditory [21], visual [22], and olfactory processing [23]. The 5HT-PLP neurons also ramify around the peduncles of the mushroom body (Figure 3A, white dotted line) and near the fan-shaped body of the central complex (Figure 3A, bottom left), whereas their cell bodies and axons are located close to the posterior surface of the brain. However, our attempts to determine putative target areas of the 5HT-PLP neurons through their dendritic and axonal morphology failed because both the presynaptic *nsyb::GFP* and the dendritic *DsCam::GFP* markers [19] labeled much of the dense arborization fields of those neurons (Figure 3B). Thus, in contrast to the clearly identifiable axonal and dendritic fields of dopaminergic neurons concerned with aggression [13], the input to and output from neuropil regions of the serotonergic PLP neurons remained unspecified.

The actions of serotonin are mediated via distinct types of receptors expressed on the surface of target neurons, where they commonly modulate the firing properties of neurons and/or change the effects of excitatory and inhibitory signals to and from the cells [24]. There are four known types of *Drosophila* serotonin receptors, 5HT1A [6, 25], 5HT1B [7], 5HT2 [26], and 5HT7 [10], any of which could be expressed by neurons downstream of the 5HT-PLP cells. Although a variety of Gal4 driver lines that presumably target 5HT receptor-bearing neurons exist, none has been fully evaluated because defined antibodies to subtypes of *Drosophila* serotonin receptors are unavailable. Therefore, we used the GFP reconstitution across synaptic partners (GRASP) technique [15] to identify downstream targets of the PLP neurons by visualizing the anatomical connections with their putative synaptic partners. The method is based on expressing two parts of the GFP molecule by two different neurons. If both parts of the GFP are in very close proximity, as in synaptic regions, GFP is reconstituted, and fluorescence is detected. We picked ten candidate 5HT receptor-Gal4 lines that targeted anatomical regions where arborization fields of the PLP neurons were observed (Table S2). We searched for possible connectivity or physical proximity between processes of those candidate receptor neurons and serotonergic neurons by the generation of a GRASP signal between them. To that end, we drove expression of one part of the GFP molecule ($spGFP^{1-10}$) by using candidate receptor-Gal4 lines, and we generated a *TRH-LexA* line to express the other portion of the GFP molecule ($spGFP^{11}$) in 5HT neurons by using the LexA/LexAop system. We found one *5HT1A*^{1}*-Gal4* line that produced reconstituted GFP signal in the ventrolateral protocerebrum, a second *5HT1A*^{2}*-Gal4* line that generated reconstituted GFP around the mushroom body peduncles, and three other lines (*5HT7-Gal4*, *5HT2-Gal4*, and

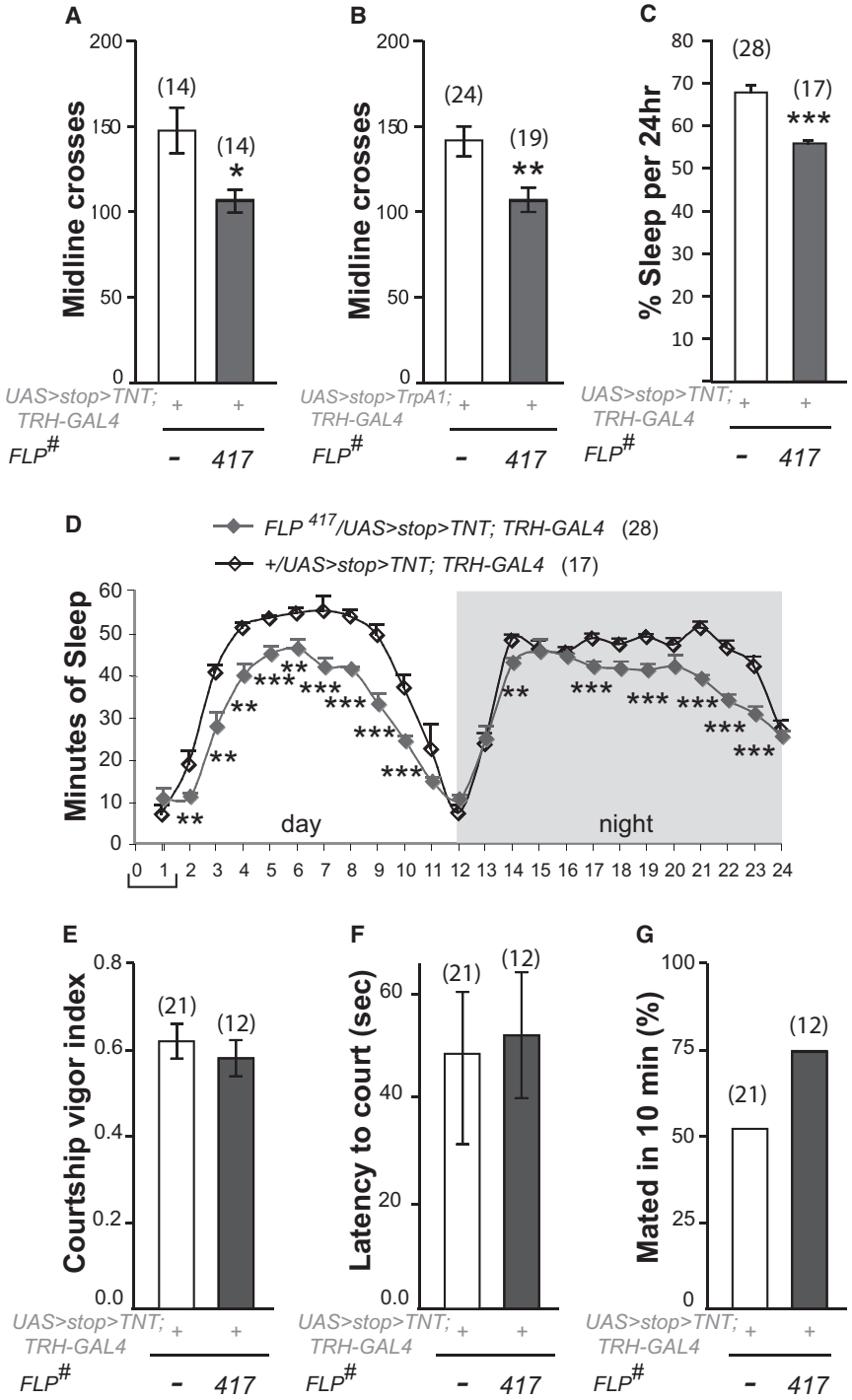

Figure 2. Manipulation of 5HT-PLP Neurons Has Selective Effects on Behavior

(A) Inactivation of 5HT-PLP neurons produced a mild locomotion deficit.

(B) Induced activation of 5HT-PLP neurons produced a mild locomotion deficit.

(C) The inactivation of 5HT-PLP neurons produced sleep deficit, measured by average percentage of sleep per 24 hr.

The data in (A)–(C) are presented as means ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus controls (white bar), analyzed using a nonparametric two-independent-sample Mann-Whitney U test.

(D) Distribution of sleep during averaged 24 hr periods in flies with inactivated 5HT-PLP neurons. Gray line indicates experimental flies; black line indicates controls. Data are presented as means ± SEM; **$p < 0.01$, ***$p < 0.001$ versus the corresponding hour data point of the control group, analyzed by an unpaired t test. The bracket shows the time of day when aggression assays were performed.

(E–G) Courtship behavior is unaffected by TNT inactivation of the aggression-modulating PLP (FLP^{417}) neurons. The data for the courtship vigor index (E) and for the latency to court (F) are presented as means ± SEM. Courtship success is calculated as the percentage of males that mated within 10 min of the assay (G).

See also Figures S1B–S1E for FLP parental control data.

5HT1A^3-Gal4) that resulted in reconstituted GFP signals in central complex structures (Table S2).

The novel *TRH-LexA* line that we used to express the spGFP[11] component of GRASP in serotonergic neurons has a broad expression pattern involving most of the 5HT neurons in the central brain area, but it also targets some nonserotonergic cells (Figure S2A). Therefore, we sought to combine the GRASP method with the intersectional strategy to express spGFP[11] in restricted sets of 5HT neurons in order to identify their potential synaptic partners (see drawing in Figure 4A). We generated a *LexAop>stop>spGFP[11]* line and used it first in combination with *TRH-LexA* and one of the broadly expressed lines, FLP^{210}, which targets most of serotonergic neurons, including the 5HT-PLP neurons (Figure S2E). This was done to reconfirm the reconstituted GFP patterns described above when using the regular GRASP method. As expected, the combination of *TRH-LexA* and FLP^{210} resulted in a similar but better-defined reconstituted GFP signal between 5HT neurons and their close candidate downstream synaptic partners (Figures 4B–4D). Detailed image analysis revealed that only three of the five candidate serotonin receptor GAL4 lines showed a GFP signal overlapping with or near areas of interest as potential targets of the 5HT-PLP neurons. These were the *5HT7-Gal4* line that showed putative synaptic contacts near the fan-shaped body of the central complex (Figure 4B), the *5HT1A^2-Gal4* line around the peduncles of the mushroom bodies (Figure 4C), and the *5HT1A^1-Gal4* line in the ventrolateral protocerebrum (Figure 4D). In some brain samples, a 5HT1A^1-derived GRASP signal was detected over the axons of the 5HT-PLP cells (Figures S3A and S3B), suggesting that close contacts might exist between 5HT1A^1-bearing neurons and the axons of serotonergic PLP neurons. With the 5HT7 and 5HT1A^2-derived GRASP signals, it was difficult to say whether the GFP puncta were derived from synaptic contacts with PLP neurons or with other serotonergic neurons branching in the same areas. Our further attempts to restrict the GRASP analysis to a

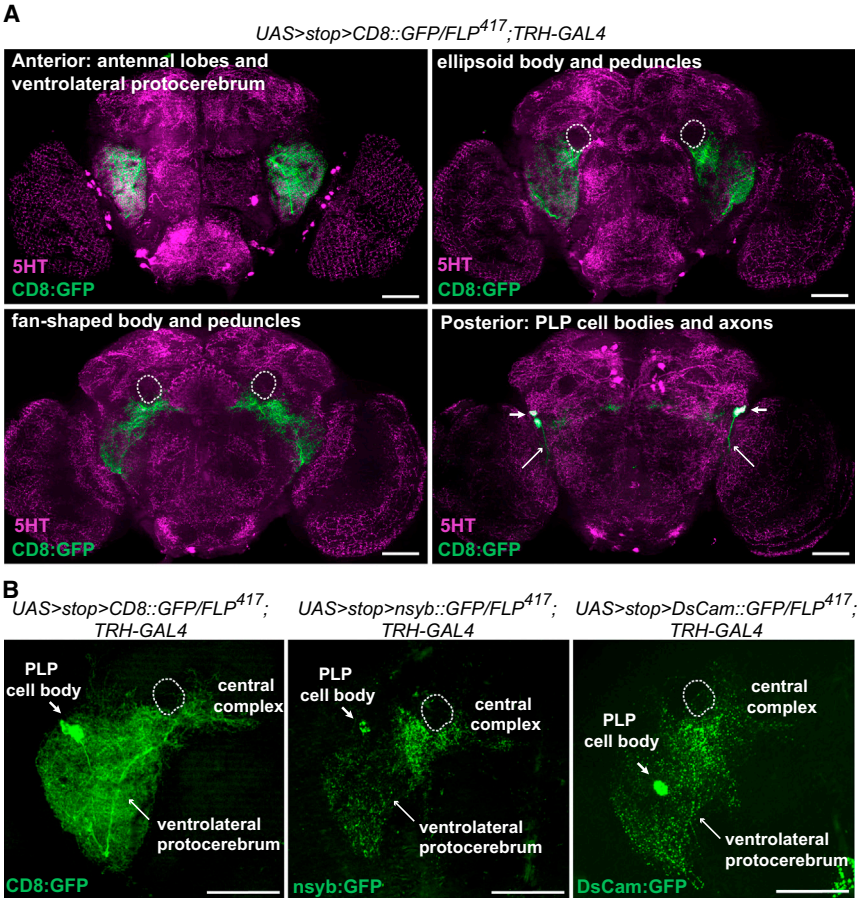

Figure 3. Anatomical Characterization of the Aggression-Modulating 5HT-PLP Neurons

(A) Arborization patterns of the PLP neurons visualized by membrane-bound CD8::GFP (green) relative to anti-5HT-labeled neuropil regions of the brain (magenta). These are displayed in frontal z projections of an image stack through the VLP and antennal lobes (top left), the ellipsoid body of the central complex and the peduncles of the mushroom bodies (top right), the fan-shaped body of the central complex (bottom left), and a posterior view of the brain, where the PLP cell bodies and their axons are located (bottom right). Scale bars represent 50 μm. Short arrows point to cell bodies; long arrows point to axons of the PLP neurons. A dotted line outlines the peduncles of the mushroom bodies that are not stained by anti-5HT antibodies.

(B) Polarity of the serotonergic PLP neurons. Left: the total arborization field of the PLP neurons visualized using membrane-bound CD8::GFP. Center: the putative presynaptic terminals of the PLP neurons revealed using the presynaptic marker nsyb::GFP. Right: the putative dendritic arbors of the PLP neurons, visualized by expression of the postsynaptic marker DsCam:GFP. Full z stack frontal projections are shown. Scale bars represent 50 μm.

single-cell level by utilizing a combination that should target only the 5HT-PLP neurons (e.g., using *TRH-LexA* and FLP^{417}; Figure S2F) yielded no GFP signal. It is possible that a successful reconstitution of GFP between individual neurons depends on the density and shape of their synaptic contacts, setting a limitation on the use of this approach at the present time.

Thus, the GRASP data showed that the processes of 5HT-PLP neurons are closely apposed to those of 5HT1A receptor-bearing neurons in the ventrolateral protocerebrum and near the peduncles of the mushroom body and also to those of 5HT7 receptor-bearing neurons near the fan-shaped body.

Induced Activation of 5HT1A Receptor-Bearing Neurons Reduces Aggression

We next asked whether neurons that express 5HT1A receptors might serve as downstream targets in pathways involved with the serotonergic modulation of aggression. In mammalian systems [27] and in *Drosophila* [28], activation of 5HT1A receptors inhibits cAMP production, hyperpolarizes neurons, and reduces neuronal excitability [27]. Activation of neurons bearing 5HT1A receptors in behaving animals, however, should yield opposite effects and might offer possible clues as to the normal behavioral roles served by some of these neurons. To test this hypothesis, we expressed the dTrpA1 channel in both populations of 5HT1A-bearing neurons and examined the aggressive behavior of flies shortly after thermal activation of the channel. We found that the number of lunges in flies with activated 5HT1A-bearing neurons using both the *5HT1A^{1}-Gal4* (ventrolateral protocerebrum target area) and *5HT1A^{2}-Gal4* (peduncle target area) drivers was significantly lower than in controls (Figure 4E). The magnitude of the effect on the number of lunges (~2-fold decrease) was similar to that observed with TNT inactivation of the 5HT-PLP neurons. This suggests that much of the PLP neuronal influence on aggression is mediated via 5HT1A receptor-bearing neurons. This, however, does not eliminate the possibility that other aggression-related neurons or circuits might also receive modulating influences from the 5HT-PLP neurons. Activation of the neurons expressing 5HT7 receptors had no effects on aggression (Figure 4E). These data, combined with the fact that activation of the 5HT-PLP neurons results in an enhancement of aggression (see above), raise the possibility that 5HT released from activated PLP neurons might inhibit 5HT1A-bearing neurons, which are key components of a descending aggression-suppressing pathway. Inhibition of an inhibitory pathway could subsequently lead to the display of higher levels of aggression. This suggestion compares favorably with a model proposed in a vertebrate system in which activation of 5HT1A postsynaptic receptors, located on GABAergic interneurons, triggers hyperpolarizing responses to released 5HT. These hyperpolarizing responses reduce the postsynaptic neuronal excitability and firing rates, thereby relieving the inhibition on the system [29]. Mammalian 5HT1A receptors show differential brain-region-specific transcriptional regulation [30] and are implicated in the regulation of mood, emotions, and stress responses. In addition, they are candidate targets in the management of various neuropsychiatric disorders [31]. Moreover, similar inhibitory control mechanisms have been reported in *Drosophila* feeding circuits [32] and in a hierarchical inhibition switch observed in appetitive memory performance [33, 34].

Displays of appropriate levels of aggression rely on the ability of an animal to analyze many factors, including the following: the correct identification and evaluation of

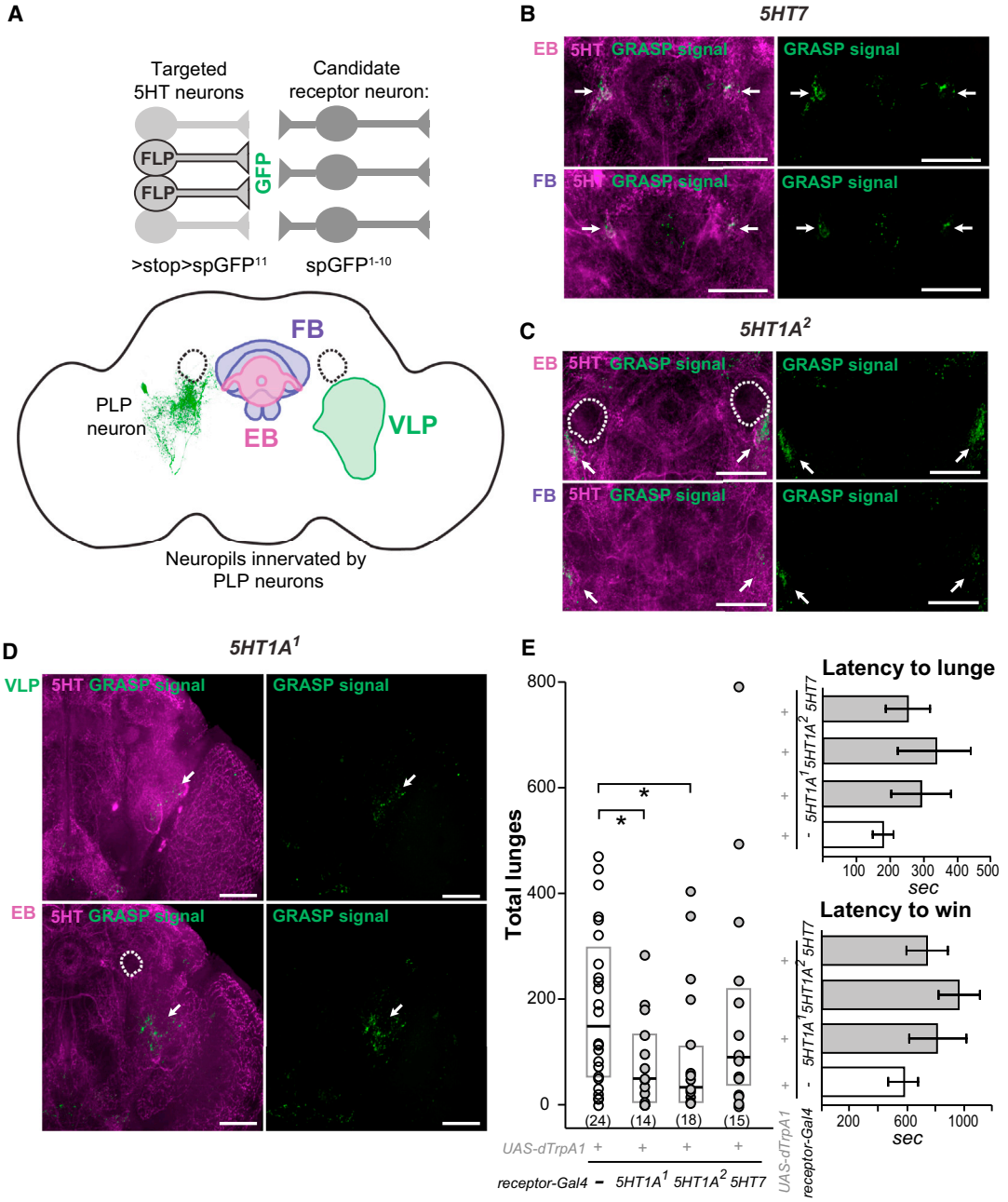

Figure 4. Putative Targets of 5HT-PLP Neurons Determined Using the Anatomical and Functional Analyses

(A) A schematic illustration of the combined use of the GRASP and FLP-recombinase techniques to find possible synaptic connections between serotonergic neurons and target neurons that express different subtypes of 5HT receptors. We used the FLP^{210} line that targets most of the serotonergic neurons in the brain to restrict the expression of the spGFP^{11} part of GFP driven by the *TRH-LexA*. The other part of GFP, spGFP$^{1-10}$, was expressed under control of different 5HT receptor Gal4 drivers. We found GRASP signals in three neuropil regions where arborization of 5HT-PLP neurons was observed: at the VLP, around peduncles of the mushroom bodies at the ellipsoid body (EB) focal plane, and near fan-shaped body (FB). The use of the FLP^{417} line that further restricted the expression of the spGFP^{11} to aggression-modulating 5HT-PLP neurons yielded no detectable GRASP signals.

(B–D) Patterns of reconstituted GFP (GRASP signal, green) between most of the serotonergic neurons and candidate 5HT receptor neurons in the areas of interest, visualized by anti-5HT immunostaining (magenta). The genotypes used for GRASP experiments with different 5HT receptor-Gal4 drivers were as follows: *w^{1118}; LexAop>stop>spGFP^{11}/FLP^{210}; UAS-spGFP$^{1-10}$, TRH-LexA/5HT receptor-Gal4*. Different frontal z projections of the image stack were created to view the corresponding neuropils of the same brain. The three neuropil regions (VLP, EB, and FB) were examined for each receptor type, but only regions that showed GRASP signal are shown. The dotted circles outline the peduncles of the mushroom bodies that were not stained by the anti-5HT antibody. White arrows point to areas in which GRASP signal is observed. Scale bars represent 50 μm. For positive and negative GRASP controls, see Figure S2; for additional GRASP data, see Figure S3.

(E) dTrpA1-induced activation of 5HT1A receptor neurons decreases the total number of lunges. Each dot represents the lunge count for an individual pair of flies. Data are presented as boxplots with a median line. The bottom and top of the box show the 25th and 75th percentile. *p < 0.05 versus corresponding control (white dots), analyzed by nonparametric two-independent-sample Mann-Whitney U test. Latency to the first lunge and to the establishment of dominance in flies with dTrpA1-activated 5HT receptor neurons was not changed. The latencies are presented as means ± SEM.

the abilities of potential competitors; the evaluation of the value of a territory and the likelihood of acquiring it; and the physiological state of the animal. Multiple sensory systems and circuits will be utilized in making such evaluations. The fixed number of neurons and neuronal circuits in nervous systems might limit the abilities of an animal to evaluate such a multiplicity of factors, but great flexibility is introduced into the system by the availability of neuromodulators. These have the capability of rapidly, efficiently, and reversibly reconfiguring the networks of neurons without changing the "hard-wiring." The studies reported here illustrate the modulation by 1–2 pairs of serotonergic neurons that enhance aggression. Other modulatory neurons and systems that influence aggression have been identified previously in *Drosophila*, including dopaminergic neurons [13], Fru^M-positive octopamine neurons that influence the behavioral choice between courtship and aggression [35], Fru^M-positive tachykinin [14] neurons that enhance aggression, and neuropeptide F circuits that decrease aggression [36]. The arbors of processes of the 5HT-PLP neurons examined here densely innervate several integrative centers in the fly brain, but thus far, they do not seem to overlap with the processes of the other reported aggression-influencing neuromodulatory neurons. The 5HT-PLP neurons do not coexpress Fru^M or Dsx (O.V.A., unpublished data). Thus, the modulatory control of the male-specific higher-level aggression appears to involve both sex-specific regulatory factors [14, 35] and other as-yet-unidentified control elements. Our studies further suggest that going to higher-intensity levels in fights may be held in check by inhibition, which can be released by activation of the 5HT-PLP neurons. Learning more about the neurons and neuronal circuits involved with a suggested downstream aggression-suppressing system and with the sensory systems that trigger aggression in the first place will be essential steps in further unraveling the complex circuitry that controls the release of aggression in *Drosophila*.

In summary, using a *Drosophila* model system and an intersectional genetic strategy, we identified a pair of serotonergic neurons in the PLP cluster that modulate aggressive behavior. These neurons arborize through several neuropil regions in the central brain, where they influence the escalation of aggression, at least in part, via 5HT1A receptor-bearing neurons and also independently influence locomotion and sleep. The single-cell resolution in identification of neuronal connections and explorations of their functions in behaving animals provides an entry point into unraveling the circuitry associated with complex behaviors like aggression.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.09.051.

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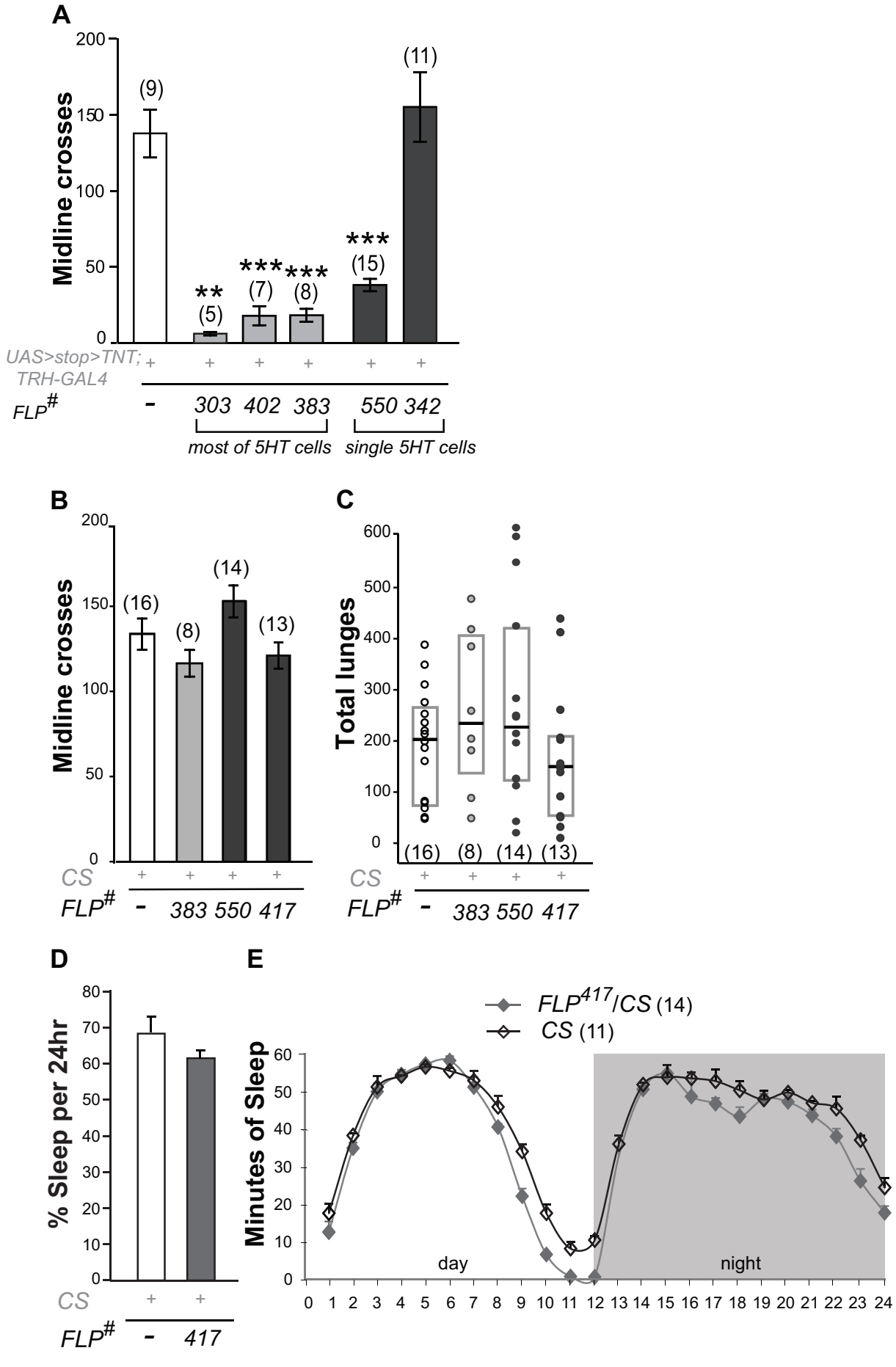
Current Biology, Volume *24*

Supplemental Information

Single Serotonergic Neurons that Modulate Aggression in *Drosophila*

Olga V. Alekseyenko, Yick-Bun Chan, Maria de la Paz Fernandez, Torsten Bülow, Michael Pankratz, and Edward A. Kravitz

Figure S1


A
Midline crosses
200
150
100
50
0
(9)
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(5)
***
(7)
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(8)
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(15)
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UAS>stop>TNT; TRH-GAL4
FLP#
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303 402 383
550 342
most of 5HT cells
single 5HT cells
B
Midline crosses
(16)
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383 550 417
C
Total lunges
600
500
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300
200
100
0
(16) (8) (14) (13)
CS
FLP#
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383 550 417
D
% Sleep per 24hr
80
70
60
50
40
30
20
10
0
CS
FLP#
-
417
E
FLP417/CS (14)
CS (11)
Minutes of Sleep
60
50
40
30
20
10
0
day
night
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

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

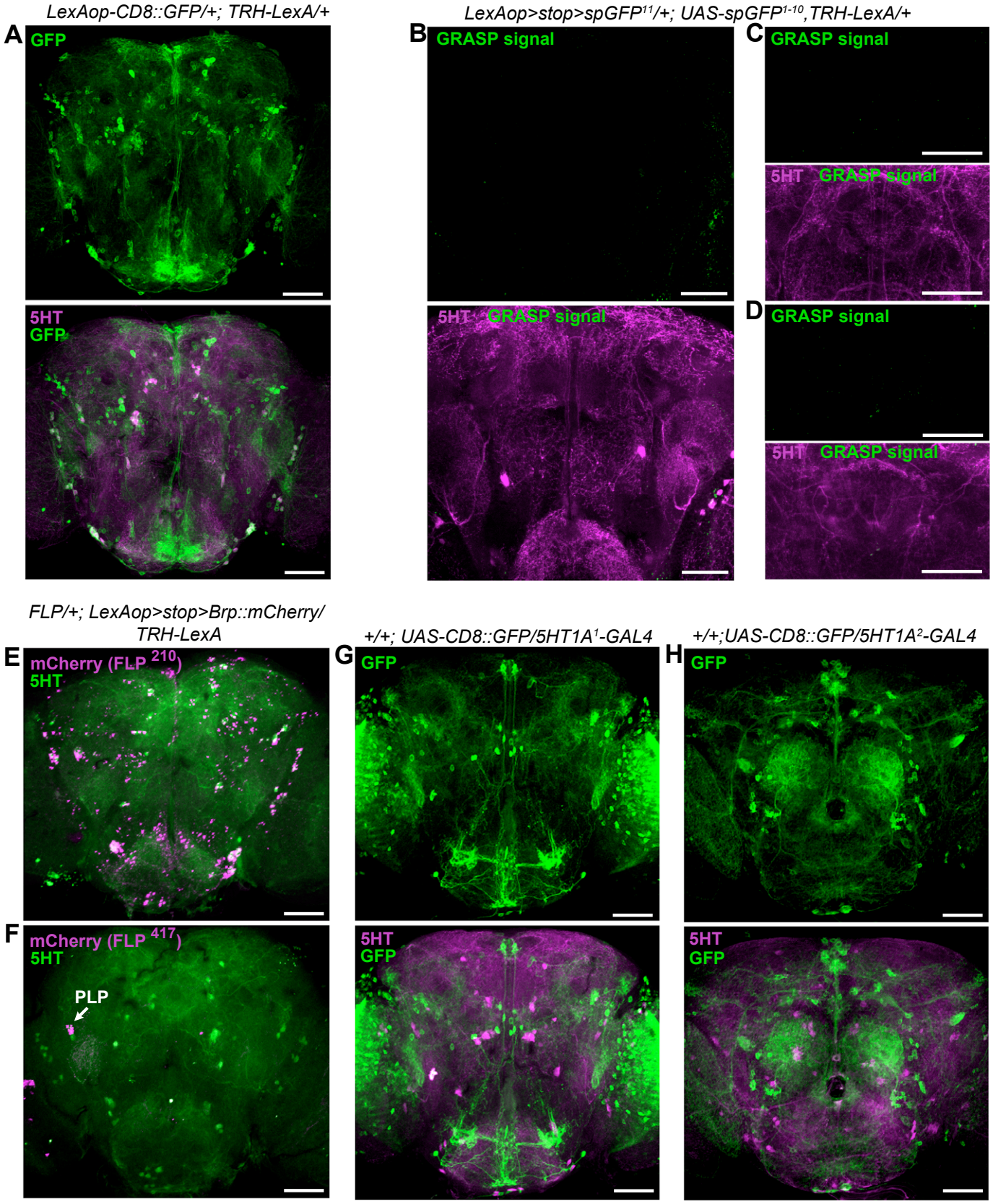

Figure S2, related to main Figure 4.
Positive and negative controls for GRASP combined with the intersectional strategy.

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^{417}, *TRH-LexA* and *LexAop>stop>Brp::mCherry* is shown in magenta. The arrow points to a targeted single 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

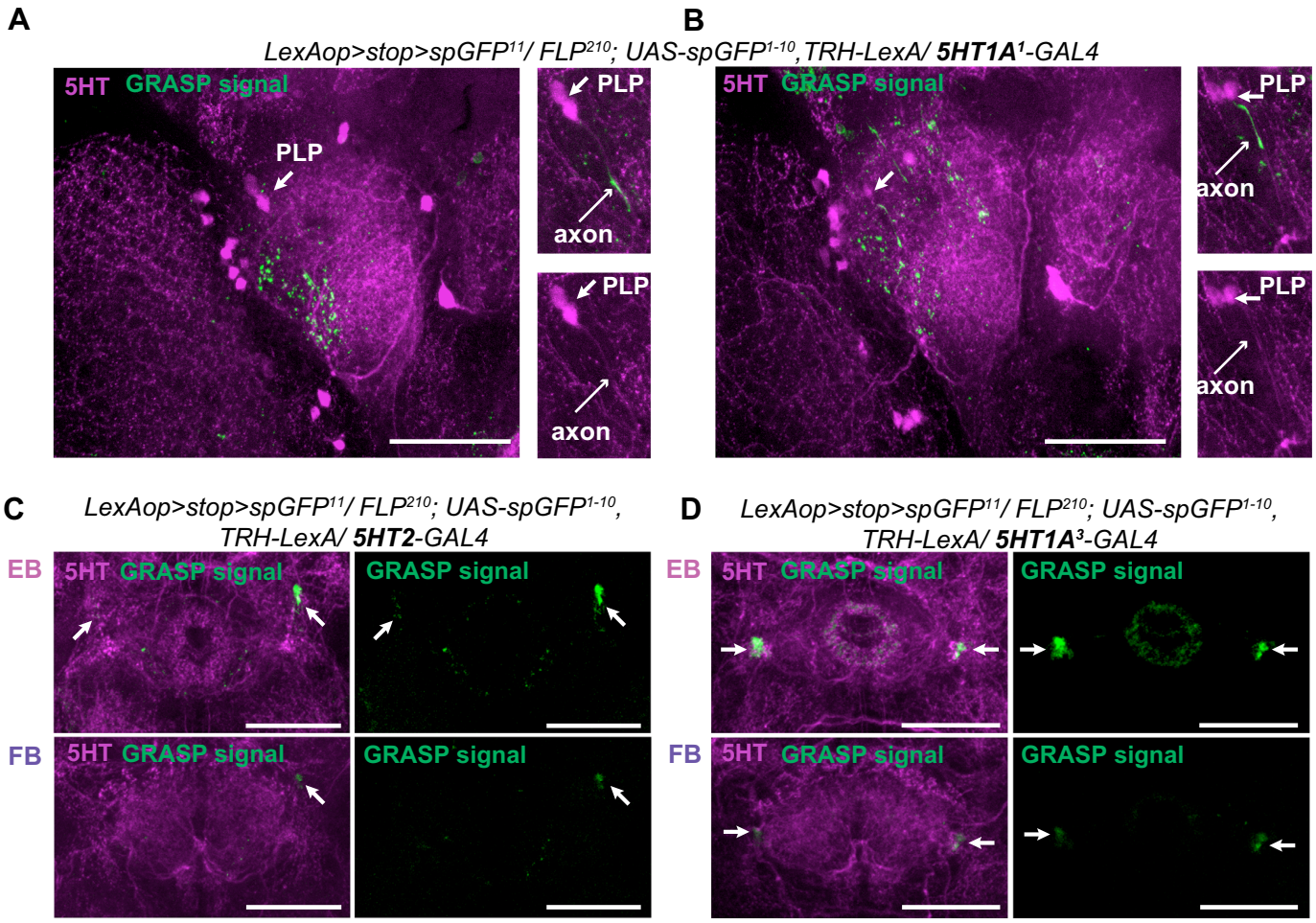

Figure S3, related to the main Figure 4.
Additional GRASP data.

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. 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:

GACTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGA
CAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGG
CATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATC
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