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Supplemental Information

Serotonin Signals Overcome Loser Mentality in *Drosophila*

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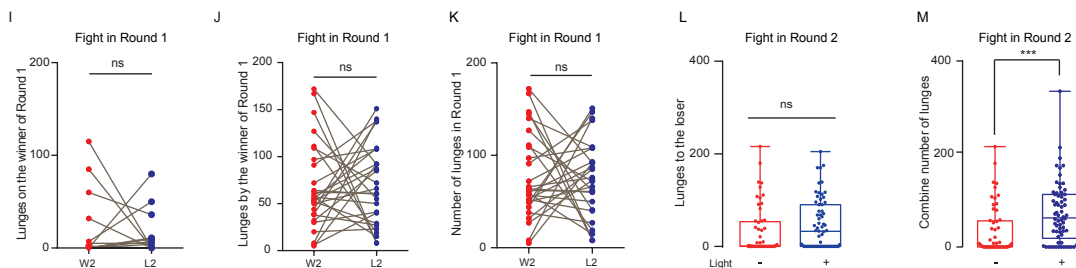
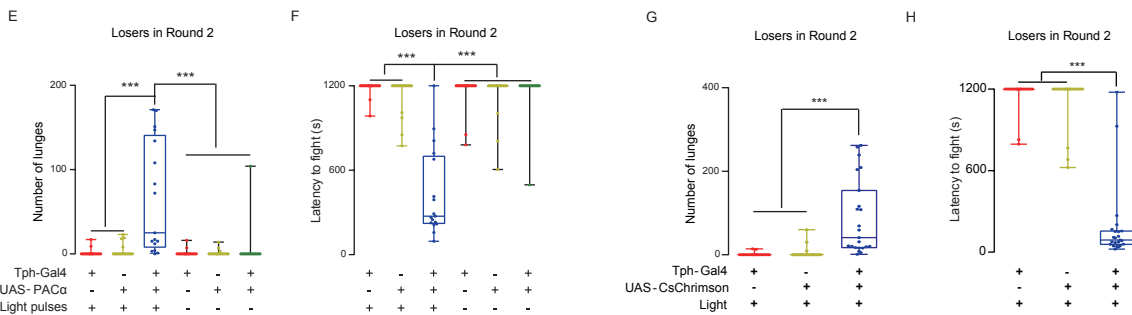
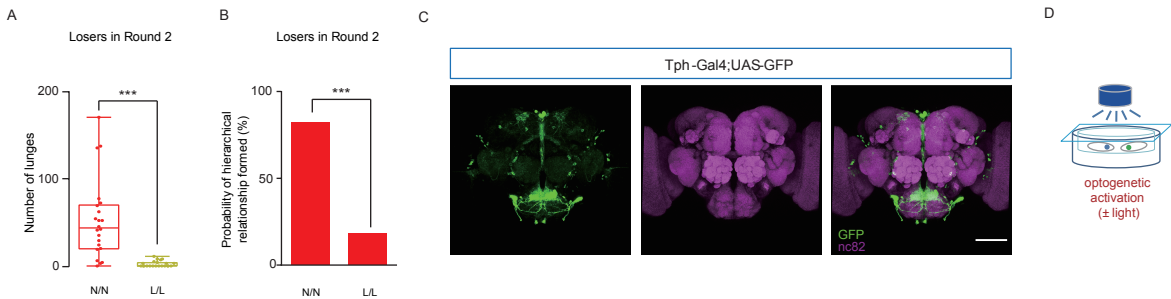


Figure S1. Loser Effect in *Drosophila*, Related to Figures 1 and 2. (A)

Fighting of loser–loser pairs (L/L) in Round 2 exhibited significantly fewer attacks than fighting of naïve–naïve pairs (N/N) in Round 1 (n = 22). (B) Fewer loser pairs established clear winner–loser relationships in Round 2 than did naïve pairs in Round 1 (n = 22). (C) Expression patterns of Tph-Gal4/UAS-mCD8::GFP. (D) Schema of optogenetic stimulation of the losers prior to a fight. (E, F) Optogenetic activation of 5-HT neurons via light pulses (10 Hz) for 80 s increased the attack intensity (E) and decreased the latency to fight (F) in Tph>PAC α losers (n = 17–20). (G, H) Optogenetic activation of 5-HT neurons via CsChrimson increased the attack intensity (G) and decreased the latency to fight (H) in losers (n = 20–23). (I–K) In light-activated Tph>PAC α flies, the new winner (W2) and loser (L2) in Round 2 were not determined by the numbers of attacks on their opponents (I), by the numbers of attacks from their opponents (J), or by the combined fighting intensity in Round 1 (K) (n = 27). (L, M) Optogenetic activation of 5-HT neurons in losers did not alter the attack actions from the winners (L) or the combined attacks of both winners and losers (M) (n = 57 and 66). All genotypes and experimental conditions are indicated with the plots. In the box-and-whisker plot, the whiskers mark the minimum and maximum, the box includes the 25th to 75th percentiles, and the line within the box indicates the median of the data set. The Mann–Whitney test was performed for (A) and (L)–(M), the Kruskal–Wallis test was performed for (E)–(H), Fisher’s exact test was performed for (B), and the paired t-test was performed for (I)–(K). ns, not significant (P > 0.05); ***P < 0.001. Scale bar, 100 μ m.

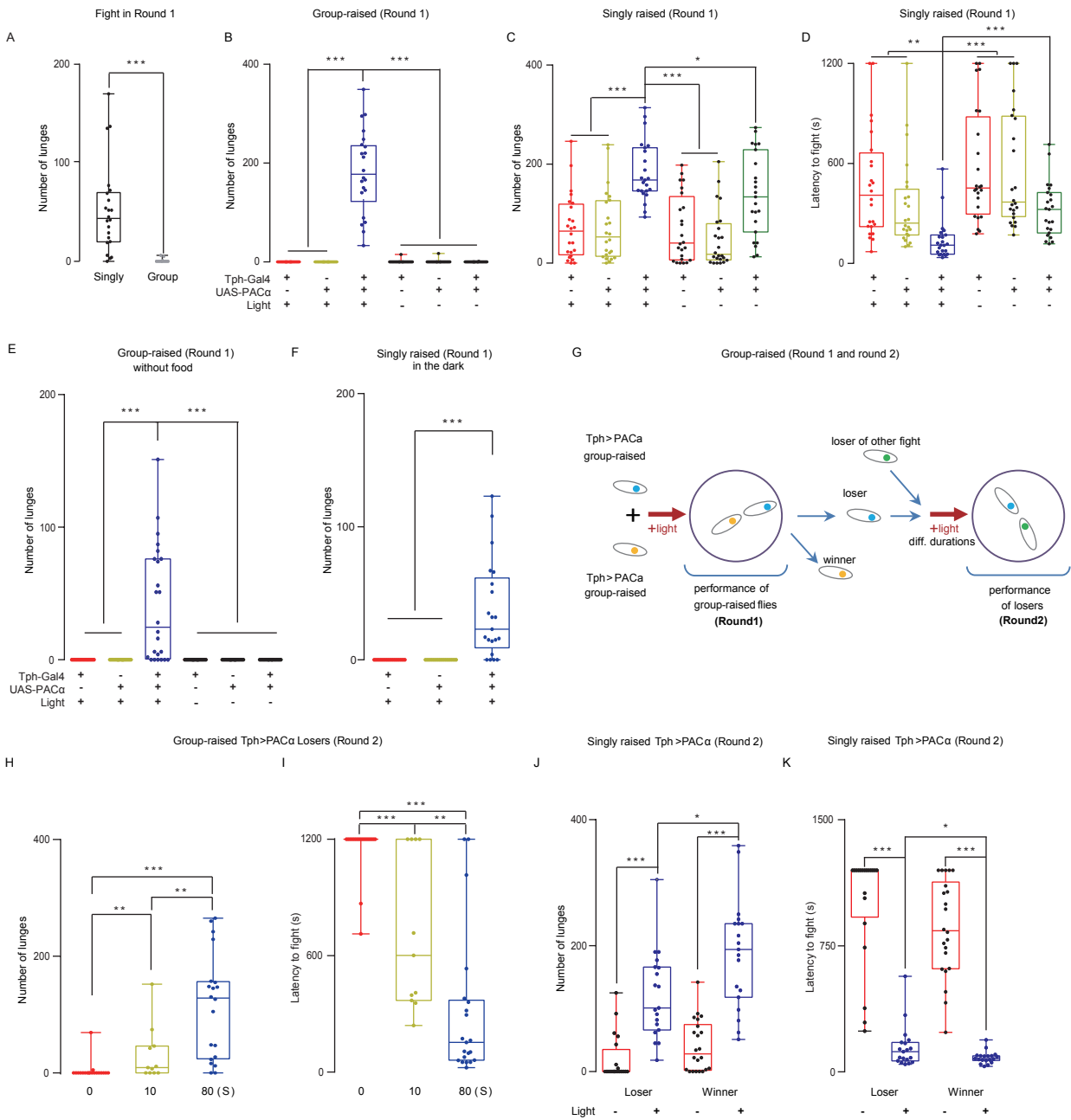


Figure S2. Activation of 5-HT Neurons Generally Increases

Aggression, Related to Figure 2. (A) Performance of wild-type flies in Round 1. The singly raised males displayed aggressive behavior in contrast to the group-raised males (n = 20–22). (B) Optogenetic activation of Tph-Gal4 neurons induced aggression in the group-raised males (n = 22–24). (C, D) Optogenetic activation of Tph-Gal4 neurons further increased the attack intensity (C) and reduced the latency to fight (D) of socially isolated males (n = 23–24). (E) Optogenetic activation of Tph-Gal4 neurons induced aggression without the presence of food (n = 21–24). (F) Optogenetic activation of Tph-Gal4 neurons induced aggressive actions in the dark (n = 21). (G) Schema of the experimental procedure for testing the loser effect in group-raised Tph>PAC α males for (H) and (I). Optogenetic activation of Tph-Gal4 neurons increased attack intensity (H) and decreased the latency to fight (I) in group-raised losers (n = 11–20). (J, K) Optogenetic activation of Tph-Gal4 neurons increased the attack intensity (J) and decreased the latency to fight (K) in winners after 45 minutes of rest (n = 19–22). All genotypes and experimental conditions are indicated with the plots. In the box-and-whisker plot, the whiskers mark the minimum and maximum, the box includes the 25th to 75th percentiles, and the line within the box indicates the median of the data set. The Mann–Whitney test was performed for (A); the Kruskal–Wallis test was performed for (B)–(F) and (H)–(K); *P < 0.05; **P < 0.01; ***P < 0.001.

Figure S3. Activation of PLP Neurons and Tk-Gal4 Neurons Is Not Sufficient for Overcoming the Loser Effect, Related to Figure 2. (A, B)

Thermal activation of serotonergic PLP neurons increased the attack intensity (A) and decreased the latency to fight (B) of socially isolated males in Round 1 (n = 22–23). (C, D) Thermal activation of serotonergic PLP neurons did not affect the attack intensity (C) or change the latency to fight (D) of losers in Round 2 (n = 15). (E, F) Photoactivation of Tk-Gal4 neurons increased the attack intensity (E) but did not affect the latency to fight (F) of socially isolated males in Round 1 (n = 24–27). (G, H) Activation of Tk-Gal4 neurons did not affect the attack intensity (G) or change the latency to fight (H) of losers in Round 2 (n = 21–25). All genotypes and experimental conditions are indicated with the plots. In the box-and-whisker plot, the whiskers mark the minimum and maximum, the box includes the 25th to 75th percentiles, and the line within the box indicates median of the data set. The Mann–Whitney test was performed for (A)-(D), the Kruskal–Wallis test was performed for (G)-(H), and one-way ANOVA was performed for (E) and (F). ns, not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$.

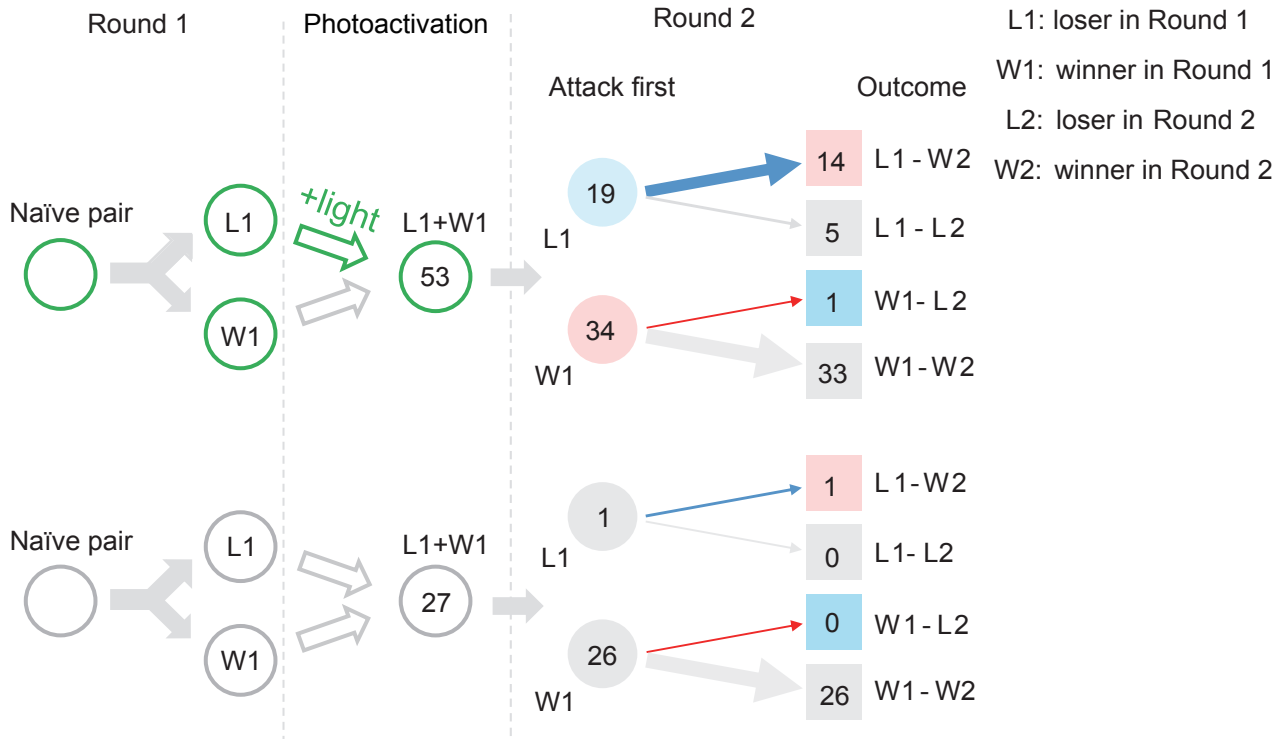
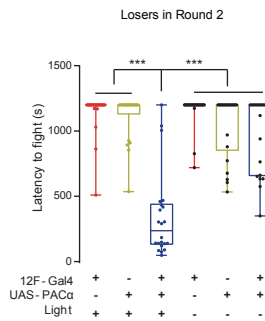


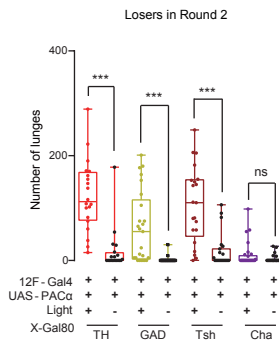
Figure S4. Initiator Effect Accompanies Reversal of Loser Effect,

Related to Figure 2. Familiar loser–winner pairs (Tph>PAC α) from Round 1 were put together for Round 2 after the losers had been light-stimulated. The status of initiators (L1 or W1 attacked first) were recorded and correlated to the fighting results. The flies in the control group (bottom) were not treated with light. L1: losers from Round 1; W1: winners from Round 1; L2: losers in Round 2; W2: winners in Round 2. The numbers inside the circles and boxes are the numbers of indicated flies. The expected events (L1 became L2 and W1 became W2) are shown in gray, while the unexpected events (L1 became W2 and W1 became L2) are shown in red (winning) or blue (losing). The thickness of the arrow lines indicates the relative frequency of the indicated events.

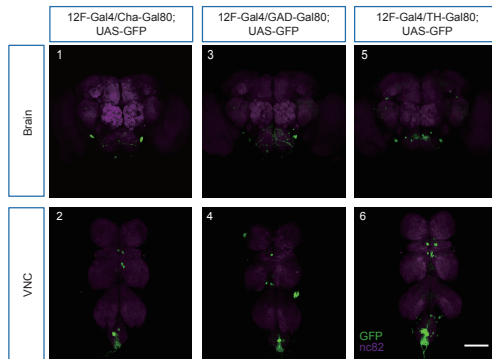
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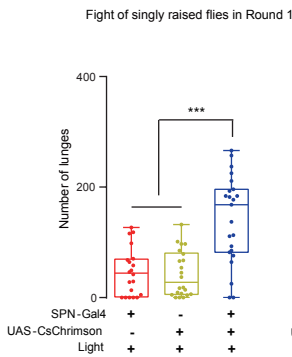
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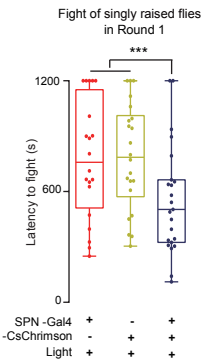
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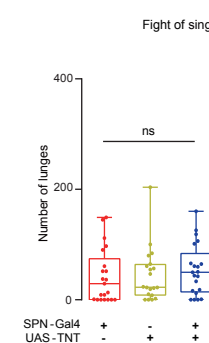
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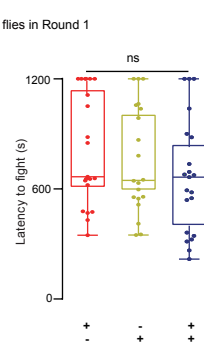
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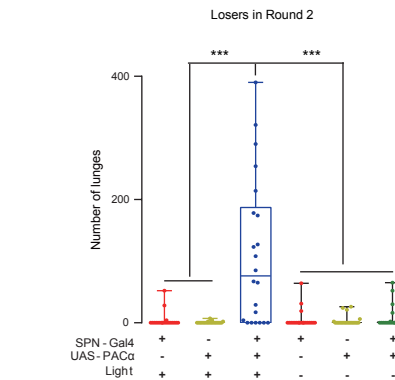
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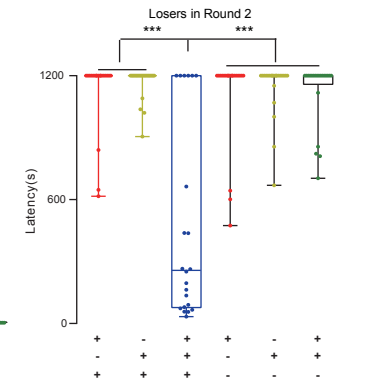
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I



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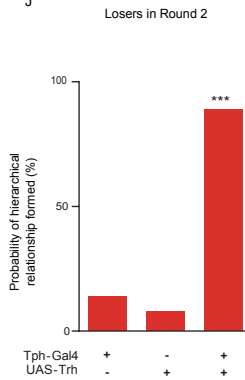


Figure S5. Enhancing Serotonergic Signals Effectively Extinguishes

the Loser Effect, Related to Figure 3. (A) Photoactivation of 12F-Gal4-labeled 5-HT neurons decreased the latency to fight in losers ($n = 22\text{--}24$). (B) Attack intensities of flies with 12F-Gal4 driving UAS-PAC α in the presence of different Gal80s ($n = 20\text{--}30$). (C) Expression patterns of 12F-Gal4 driving UAS-GFP in the presence of Cha-Gal80 (C1 and C2), Gad-Gal80 (C3 and C4), and Th-Gal80 (C5 and C6). (D, E) Optogenetic activation of SPN-split neurons increased the attack intensity (D) and decreased the latency to fight (E) in singly raised males ($n = 21\text{--}23$). (F, G) Silencing SPN-split neurons did not affect the attack intensity (F) or the latency to fight (G) in singly raised males ($n = 20\text{--}21$). (H, I) Optogenetic activation of SPN-split neurons via PAC α increased the attack intensity (H) and decreased the latency to fight (I) of losers ($n = 21\text{--}23$). (J) More loser–loser pairs reached a clear winner–loser status than controls when elevating the 5-HT levels in 5-HT neurons ($n = 21\text{--}28$). All genotypes and experimental conditions are indicated with the plots. In the box-and-whisker plot, the whiskers mark the minimum and maximum, the box includes the 25th to 75th percentiles, and the line within the box indicates median of the data set. The Kruskal–Wallis test was performed for (A), (B), (H), and (I); one-way ANOVA was performed for (D)–(G); and the chi-square test was performed for (J) (two-tailed $\chi^2 = 174.8$, $df = 2$). ns, not significant ($P > 0.05$); **** $P < 0.001$. Scale bar, 100 μm .

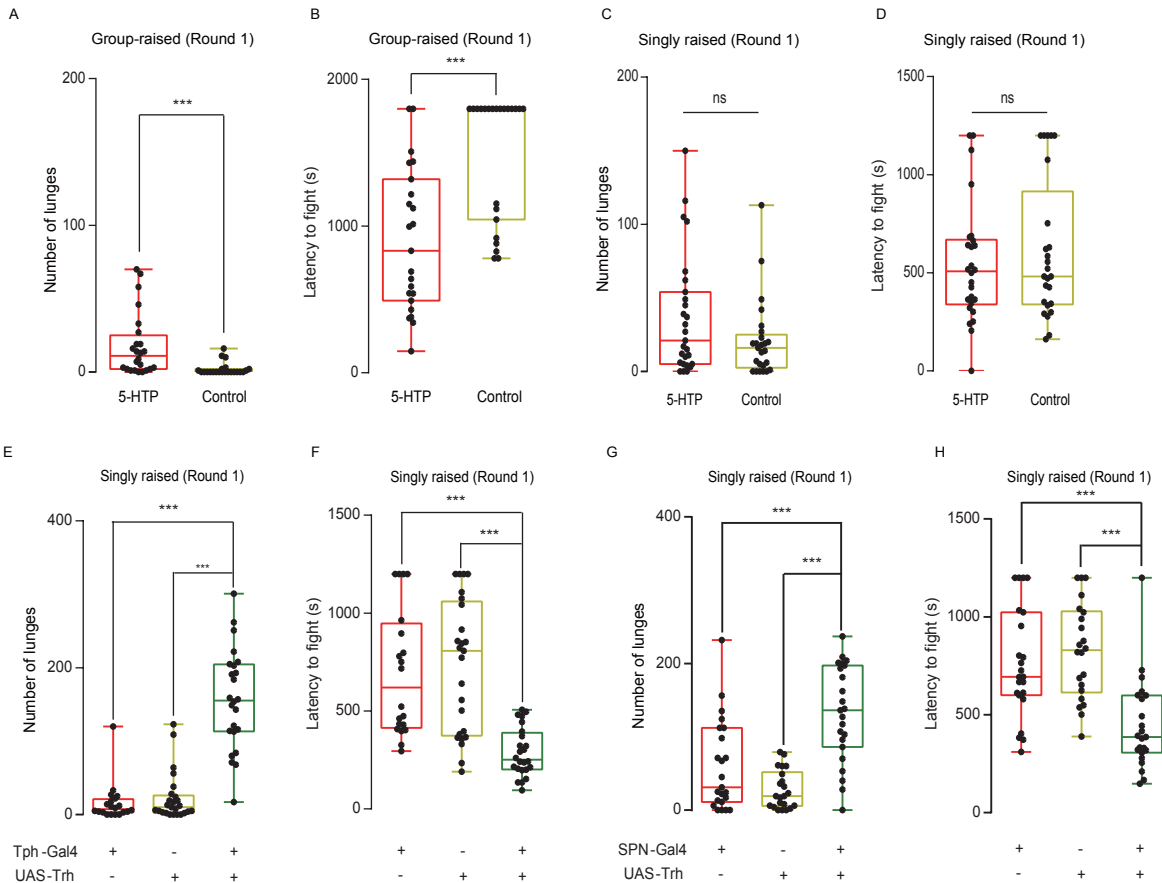
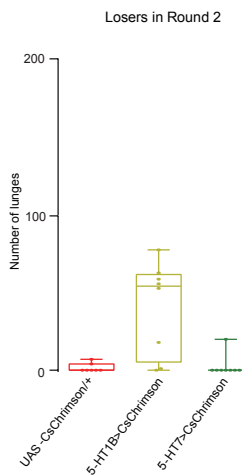


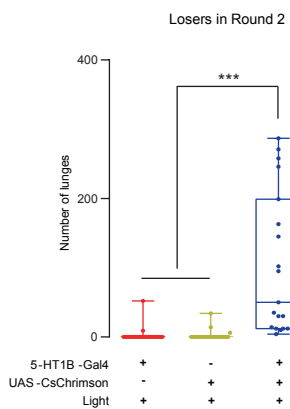
Figure S6. Enhanced 5-HT Level Increased Initial Levels of

Aggression, Related to Figure 3. (A, B) Pharmacologically increasing the 5-HT level promoted lunges (A) and decreased latency to fight (B) in group-raised males (n = 23–24). (C, D) Pharmacologically increasing the 5-HT level did not affect the lunge frequency (C) or latency to fight (D) in singly raised males (n = 25–26). (E) Overexpression of 5-HT in Tph neurons increased the lunge frequency in singly raised males (n = 20–25). (F) Overexpression of 5-HT in Tph neurons decreased the latency to fight of singly raised males (n = 20–25). (G) Overexpression of 5-HT in SPNs increased the lunge frequency in singly raised males (n = 22–23). (H) Overexpression of 5-HT in SPNs decreased the latency to fight in singly raised males (n = 22–23). All genotypes and experimental conditions are indicated with the plots. In the box-and-whisker plot, the whiskers mark the minimum and maximum, the box includes the 25th to 75th percentiles, and the line within the box indicates median of the data set. The Mann–Whitney test was performed for (A) and (B), the t-test was performed for (C) and (D), and the Kruskal–Wallis test was performed for (E)–(H). ns, not significant ($P > 0.05$); *** $P < 0.001$.

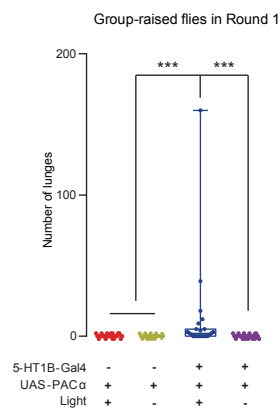
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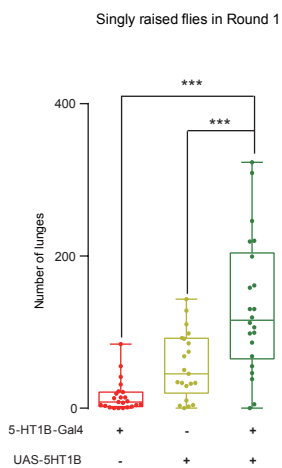
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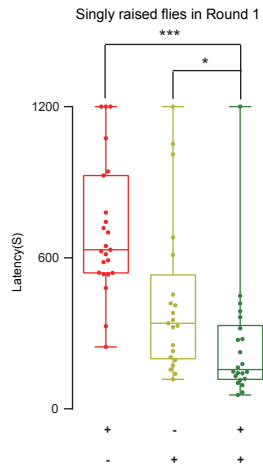
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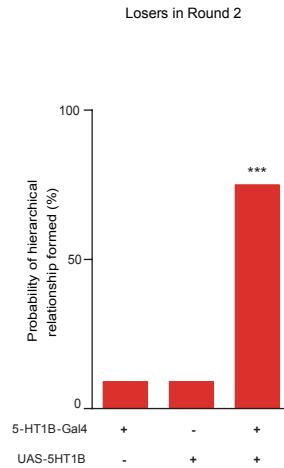


Figure S7. 5-HT1B-Positive Neurons Control Aggressive Arousal of

Losers, Related to Figure 4. (A) Survey of serotonin receptors for overcoming the loser effect. Neurons of 5-HT receptors in losers were activated optogenetically, and the numbers of attacks of loser pairs in Round 2 were quantified (n = 8). (B) Optogenetic activation of 5-HT1B receptor neurons via CsChrimson promoted aggression in losers (n = 19–23). (C) Activation of 5-HT1B neurons induced aggression in the group-raised males in Round 1 (n = 23–24). (D) Increased 5-HT1B level in 5-HT1B neurons elevated aggression in the singly raised males (n = 21–23). (E) Increased 5-HT1B level in 5-HT1B neurons reduced the latency to fight of singly raised males (n = 21–23). (F) More loser–loser pairs reached a clear winner–loser status when 5-HT1B levels were elevated in 5-HT1B neurons (n = 22–24). All genotypes and experimental conditions are indicated with the plots. In the box-and-whisker plot, the whiskers mark the minimum and maximum, the box includes the 25th to 75th percentiles, and the line within the box indicates median of the data set. The Kruskal–Wallis test was used for (B)–(E), and the chi-square test was used for (F) (two-tailed $\chi^2 = 135.8$, $df = 2$). *P < 0.05; ***P < 0.001.

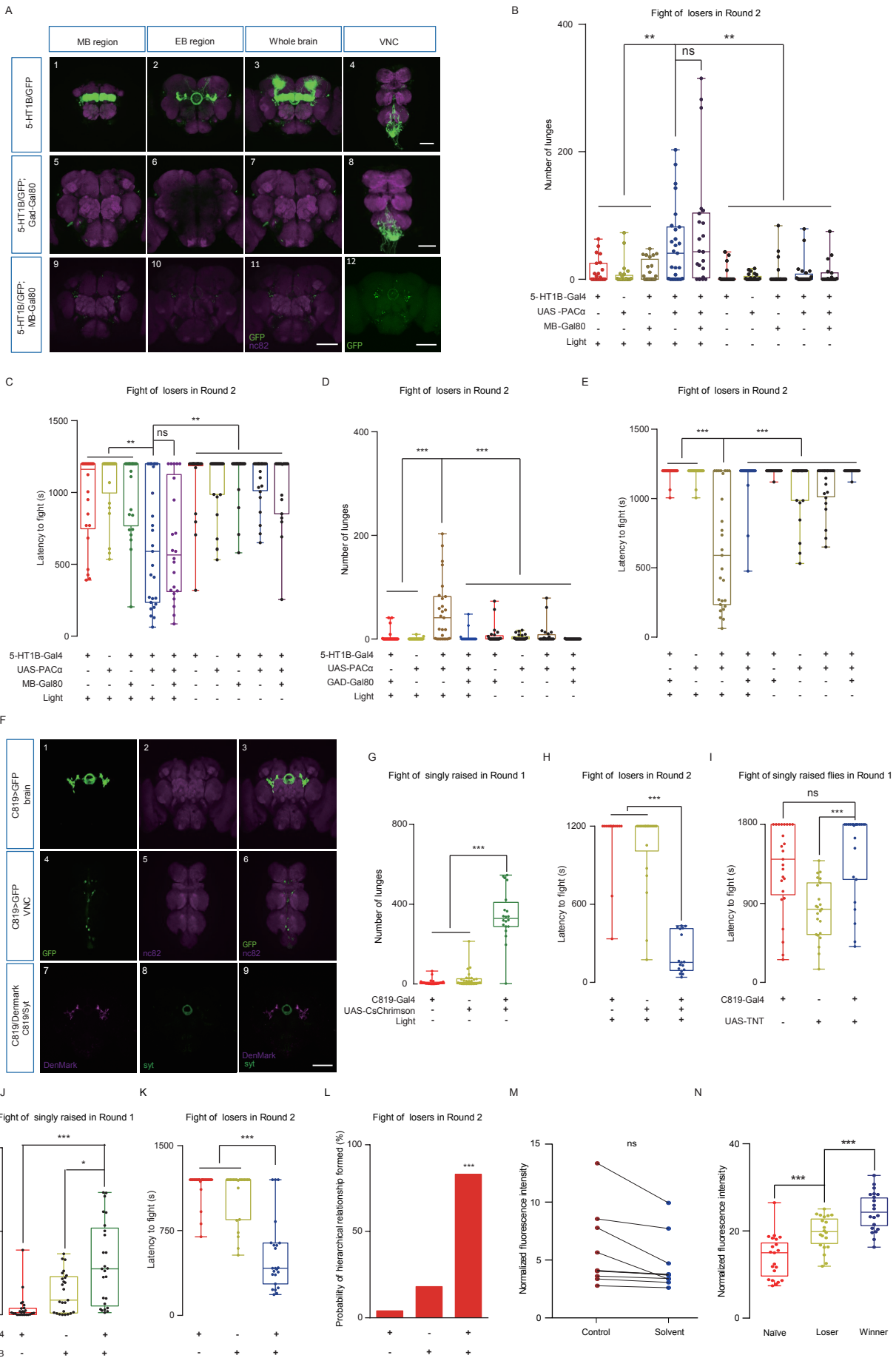


Figure S8. 5-HT1B-Positive C819 Neurons in EB Control Aggressive

Arousal of Losers, Related to Figure 5. (A) Expression patterns in males of 5-HT1B-Gal4/UAS-mCD8::GFP (A1-A4), 5-HT1B-GAL4/ UAS-mCD8::GFP; Gad-Gal80 (A5-A8), and 5-HT1B-GAL4/ UAS-mCD8::GFP; MB-Gal80 (A9-A11) in the MB, ellipsoid body, and whole brain. GFP signals in A12 were enhanced specifically from A11 to show the remaining neurons. (B, C) Attack intensity (B) and latency to fight (C) remained unchanged in loser pairs of 5-HT1B-Gal4 driving UAS-PAC α with or without MB-Gal80, which prevents optogenetic activation of MB neurons (n = 19–27). (D, E) The attack intensity was decreased (D) and the latency to fight was increased (E) in loser pairs of 5-HT1B-Gal4 driving UAS-PAC α with Gad-Gal80 (n = 23–27). (F) Expression patterns of C819-Gal4/UAS-mCD8::GFP in the brain (F1-F3) and VNC (F4-F6). F7 shows the postsynaptic patterns of C819 neurons as indicated by C819>DenMark, whereas F8 shows the pre-synaptic patterns of C819 neurons as indicated by C819>Synaptotagmin-GFP (syt). (G) Activation of C819 neurons increased the lunge frequency of singly raised males (n = 20–30). (H) Optogenetic activation of C819 neurons decreased the latency to fight in losers (n = 14–22). (I) Inhibition of synaptic transmission in C819 neurons did not change the latency to fight in socially isolated males (n = 23–25). (J) Elevating expression levels of the 5-HT1B receptor in C819 neurons increased lunge frequency of singly raised males (n = 25). (K) Elevating expression levels of the 5-HT1B receptor in C819 neurons decreased the latency to fight in losers (n = 22–24). (L) More loser–loser pairs formed stable winner–loser relationships when the 5-HT1B level was elevated in C819 neurons (n = 22–24). (M) Application of solvent alone, instead of 5-HT, did not increase the activity of C819 neurons (n = 10). (N) The activity of R2/R4m neurons in both winners and losers was higher than that in naïve flies (n = 20–21). All genotypes and experimental conditions are indicated with the plots. In the box-and-whisker plot, the whiskers mark the minimum and maximum, the box includes the 25th to 75th percentiles, and the line in the box indicates the median of the data set. The Kruskal–Wallis test was performed for (B)-(E), (G)-(K). The chi-square test was performed for (L) (two-tailed $\chi^2 = 156.2$, $df = 2$), the paired t-test was performed for (M), and one-way ANOVA was performed for (N). ns, not significant ($P > 0.05$); * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bar, 100 μm .

Supplementary Table S1. *Drosophila* strains, reagents, software, and source data essential to reproduce results presented in the manuscript, Related to Figures 1–8 and Transparent Methods.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-GFP (rabbit)	Thermo Fisher	CAT# A-6455 RRID AB_221570
fasciclin II (mouse)	DSHB	CAT# ab141801 RRID AB_28235
nc82 (mouse)	DSHB	CAT# ZF-0313 RRID AB_2314866
Goat anti-Rabbit IgG (H+L) Alexa Fluor 488	ZSGB-Bio	CAT# ZF-0511
Goat Anti-Mouse IgG (H+L) Alexa Fluor 635	Thermo Fisher	CAT# A31574 RRID AB_2536185
Chemicals, Peptides, and Recombinant Proteins		
5-Hydroxy-L-tryptophan	Sigma-Aldrich	CAT# H9772-1G
Serotonin hydrochloride	Alfa	CAT# B21263
Adenosine 5'-triphosphate disodium salt hydrate	Sigma	CAT# A2383-5G
Experimental Models: Organisms/Strains		
<i>Drosophila</i> : Wild type Canton S	Li Liu	N/A
<i>Drosophila</i> : <i>Tph-Gal4</i>	Yi Rao	Park et al. 2006
<i>Drosophila</i> : <i>UAS-PACα</i>	B. Kottler and M. Schwarzel.	N/A
<i>Drosophila</i> : <i>UAS-NaChBac (TM6B)</i>	Bloomington Drosophila Stock Center	RRID: BDSC_9468
<i>Drosophila</i> : <i>UAS>stop>TrpA1;TRH-Gal4</i>	Olga V. Alekseyenko, Edward A. Kravitz	N/A
<i>Drosophila</i> : <i>FLP # 417</i>	Olga V. Alekseyenko, Edward A. Kravitz	N/A
<i>Drosophila</i> : <i>Tk-GAL4</i>	Bloomington Drosophila Stock Center	RRID: BDSC_51975
<i>Drosophila</i> : <i>12F-Gal4</i>	Olga V. Alekseyenko, Edward A. Kravitz	N/A
<i>Drosophila</i> : <i>UAS-mCD8::GFP</i>	Y. Li and A. Guo	N/A

<i>Drosophila: Trh-Gal4</i> (second chromosome)	Olga V. Alekseyenko, Edward A. Kravitz	N/A
<i>Drosophila: Trh-Gal4</i> (third chromosome)	Olga V. Alekseyenko, Edward A. Kravitz	N/A
<i>Drosophila: UAS-Trh</i>	Bloomington Drosophila Stock Center	RRID: BDSC_27638
<i>Drosophila: UAS-Trh-RNAi</i>	Julie H. Simpson	Albin et al., 2015
<i>Drosophila: Th-Gal80</i>	Y. Li and A. Guo	N/A
<i>Drosophila: Cha-Gal80</i>	Y. Li and A. Guo	N/A
<i>Drosophila: Gad-Gal80</i>	Y. Li and A. Guo	N/A
<i>Drosophila: MB-Gal80</i>	Y. Li and A. Guo	N/A
<i>Drosophila: SL3 (5-HT1B-Gal4)</i>	Yi Rao	Quan et al. 2005
<i>Drosophila: HMS6 (5-HT1B-Gal4)</i>	Yi Rao	Quan et al. 2005
<i>Drosophila: HMS1 (UAS-5-HT1B)</i>	Yi Rao	Quan et al. 2005
<i>Drosophila: UAS-TNT</i>	Y. Li and A. Guo	N/A
<i>Drosophila: 20XUAS-IVS-CsChrimson.mVenus (attP40)</i>	Bloomington Drosophila Stock Center	RRID: BDSC_5515
<i>Drosophila: 13XLexAop2-IVS CsChrimson.mVenus (attP2)</i>	Bloomington Drosophila Stock Center	RRID: BDSC_5519
<i>Drosophila: 5-HT2A-Gal4</i>	Yi Rao	N/A
<i>Drosophila: 5-HT2B-Gal4</i>	Yi Rao	N/A
<i>Drosophila: 5-HT7-Gal4</i>	Yi Rao	N/A
<i>Drosophila: tsh-Gal80</i>	L. Vosshall	N/A
<i>Drosophila: C819-Gal4</i>	Li Liu	N/A
<i>Drosophila: UAS-Denmark</i>	Y. Li and A. Guo	N/A
<i>Drosophila: UAS-Synaptotagmin-GFP</i>	Y. Li and A. Guo	N/A
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<i>Drosophila: GMR15A01-Gal4 (attp2)</i>	Bloomington Drosophila Stock Center	RRID: BDSC_48670
<i>Drosophila: 20XUAS-IVS-GCaMP6m (attP40)</i>	Bloomington Drosophila Stock Center	RRID: BDSC_42748
<i>Drosophila: LexAop-P2X₂;UAS-GCamp6m</i>	Yufeng Pan	N/A

<i>Drosophila:</i> <i>UAS-myrGFP.QUAS-mtdTomato-3xHA</i> <i>(attP8); trans-Tango (attP40)</i>	Bloomington Drosophila Stock Center	RRID: BDSC_77124
<i>Drosophila: LexAop-spGFP11;</i> <i>UAS-spGFP1-10</i>	Mark Wu	N/A
<i>Drosophila: SPN split-Gal4</i>	Thomas Preat	N/A
Software and Algorithms		
Prism6	GraphPad Software	URL: http://www.graphpad.com/ ; RRID:SCR_002798
MATLAB R2012a	MathWorks	URL: http://www.mathworks.com/products/matlab/ ; RRID:SCR_001622

1 **Transparent Methods**

2 **Fly stocks and genetics.** Flies were usually reared at 25°C and 60% humidity in a
3 12:12-h light:dark regimen (light on at 08:00) unless otherwise indicated. The
4 standard fly media included water (1000 mL), cornmeal (77.7 g), yeast (32.1 g), agar
5 (8 g), calcium chloride (0.726 g), sucrose (31.62 g), glucose (63.2 g), potassium
6 sorbate (2 g), and methyl p-hydroxybenzoate (1.5 g). The flies used for the
7 optogenetic experiments were reared in the dark. Flies for CsChrimson experiments
8 were raised without all-trans-retinal.

9 The details of the fly stocks are listed in Supplementary Table S1. Brief descriptions
10 of three lines (CS, 12F-Gal4, and SPNsplit-Gal4) are provided below. The wild-type
11 strain used was Canton S (CS; stock number: 105666; Kyoto Stock Center). Flies
12 with 12F-Gal4 were obtained from O. Alekseyenko and E. Kravitz. The transgene
13 for 12F-Gal4 was first generated by fusing the short regulatory sequence of the
14 neural-specific Trh gene (CG9122) with Gal4; it was then inserted into the second
15 chromosome (Alekseyenko et al., 2010). The SPN split-Gal4 flies were obtained
16 from T. Preat and included two transgenes: an activation domain fused with a
17 regulatory sequence from VT026326 (inserted into the atp40 site) and a
18 DNA-binding domain fused with a regulatory sequence from VT057280 (inserted
19 into the atp2 site) (Scheunemann et al., 2018). Behavioral assays were carried out at
20 25°C and 60% humidity between 15:00 and 19:00.

21

22 **Aggression assay.** The protocol for measuring aggression was adapted from previous
23 work with some modifications (Zhou et al., 2008). The circular fighting chamber had
24 a radius of 7 mm and contained a central food patch (radius of 4 mm). The inside
25 height of the chamber was 3.5 mm. Among the socially isolated flies, newly emerged
26 flies were collected after eclosion and reared individually in a 2-mL Eppendorf tube
27 containing 0.5 mL of food. Flies aged 6 to 8 days were used for behavioral tests. To
28 distinguish the flies from one another, they were lightly anesthetized with carbon
29 dioxide and marked on the thorax with acrylic paint of different colors. Painted flies
30 were allowed to recover for at least 48 h before aggression assays were carried out.

31

32 Antagonistic interactions between a pair were quantified by the number of lunges and
33 the latency to fight. The number of lunges was measured by the number of combined
34 lunges performed by both flies within the first 20 min of observation. The latency to
35 fight was measured as the duration of time from the placement of the flies into the
36 chamber to the first aggressive action. To simplify quantification, when no aggressive
37 actions had occurred throughout the 20 min period, the latency to fight was assigned a

38 1200 s. In a few experiments, the observation period was 30 min. The first fly to lunge
39 was defined as the initiator. The criterion of assigning winners and losers was based
40 on a five-lunge/five-retreat rule (Yurkovic et al., 2006).

41

42 **Social defeat assay.** The protocol for the social defeat assay was adapted from
43 previous work with some modifications (Yurkovic et al., 2006). For the Round 1
44 fight, two singly raised males were placed in a standard fighting chamber for 30 min.
45 After 30 min of fighting, most naïve–naïve pairs formed a stable winner–loser
46 relationship. The loser flies were then returned to their home vials for 30 min and
47 paired for 20 min with a loser or winner for Round 2. The assignment of winners and
48 losers was based on a five-lunge/five-retreat criterion. We quantified the fighting
49 intensity with the combined number of lunges and latency of fighting (the elapsed
50 time until the first lunge was performed).

51

52 **State persistence assay.** After naïve–naïve pairs of Tph>PAC α males formed stable
53 winner–loser relationships, we removed the winners, illuminated the losers inside the
54 fighting chambers for 80 s, and returned the losers to their home tubes for 15, 30, or
55 60 min. We then tested the aggression of these losers.

56

57 **Territory assay.** The protocol for the territory assay was adapted from previous work
58 with some modifications (Zhou et al., 2008). A Tph>PAC α male and Tph>NaChBac
59 male were paired in a fighting chamber. When a Tph>PAC α male became a loser, we
60 illuminated the flies with blue light for 80 s to activate the 5-HT neurons of the
61 Tph>PAC α loser. The duration of time that each fly stayed on the food patch of the
62 fighting chamber within the first 5 min was quantified.

63

64 **Threat display assay.** A fly (the threatener) displays threat-like actions prior to
65 engaging in physical contact for a fight (Duistermars et al., 2018). We quantified the
66 threat displays of a threatener by analyze the number of wing elevation and charges
67 toward the opponent in the first 5 min (0-5 min) after placing them in a fighting
68 chamber.

69

70 **Courtship motivation assay.** The analysis for courtship motivation was adapted
71 from previous work with some modifications (Teseo et al., 2016). We used
72 decapitated females, which usually stood and exhibited basic activities for several
73 hours but did not respond to courtship attempts from males, thus allowing us to
74 quantify the males' motivation to mate objectively. In contrast, intact females avoid,
75 reject, or mate with males, influencing the males' behavior and thus increasing the
76 complexity of data analysis.

77

78 After 30 min of fighting, the winners and losers were separated and returned to their
79 home vials for 30 min. We then individually tested the courtship attempts of winners,
80 losers, and singly raised naïve flies toward decapitated virgin females for 20 min
81 each.

82

83 **Optogenetic stimulation.** For continuous optogenetic stimulation, a 460-nm blue
84 light source (Denjoy DY400-4) was placed 5 mm above the fighting chambers to
85 illuminate the flies inside for 80 s before starting video recording. The light intensity
86 (122 mW/cm^2) was measured with a spectrometer (CCS200/M, Thorlabs) at the site
87 of illumination. The fighting chambers were topped with a transparent glass sheet for
88 illumination and observation.

89 The intensity and duration of light illumination were determined by pilot
90 experiments. Half of the minimal intensity causing paralysis in *elav>PAC α* flies was
91 chosen for optogenetic manipulation. At this intensity, the exposure time (80 s) was
92 found to induce the highest number of lunges in group-raised *Tph>PAC α* flies.
93 Under this condition, the behavioral effects induced by *PAC α* and *CsChrimson* were
94 similar with most drivers.

95

96 To generate light pulses for optogenetic activation, a 480-nm square LED of 20×20
97 mm (RJH100B160A1-1500T; Ruijiahong) was controlled by an Arduino board with
98 a custom script to deliver 10-Hz pulses (pulse width of 20 ms) to a fighting chamber
99 at a 45-degree angle. The illumination duration and light intensity for
100 photoactivation experiments were the same as described above.

101

102 **Thermogenetic activation.** Activating the 5HT-PLP neurons required thermogenetic
103 manipulation. The protocol was adapted from previous work with some
104 modifications (Alekseyenko et al., 2014). Newly emerged flies were collected after
105 eclosion and maintained at 18°C for 10 to 12 days before testing. The *dTrpA1*
106 activation in Round 1 was conducted at 32°C to increase the efficacy of *dTrpA1*
107 activation in the experimental groups, whereas the temperature-controlled groups
108 were tested at 22°C . Losers were generated by allowing a pair to fight at 22°C for 30
109 min. The losers were then tested at 32°C to evaluate their fighting behavior with the
110 losers fought at 22°C , serving as the temperature controls.

111

112 **Drug treatment.** The pharmacological protocol was adapted from previous work with
113 some modifications (Dierick and Greenspan, 2007). Newly emerged males were

114 collected after eclosion and reared individually in a 2-mL Eppendorf tube containing
115 0.5 mL of food for 3 days, then transferred into a new 2-mL Eppendorf tube
116 containing 0.5 mL of food mixed with 5-HTP (H9772; Sigma) at a final concentration
117 of 50 mM for 3 days.

118

119 **Immunohistochemistry.** The immunohistochemistry protocol was adapted from
120 previous work with some modifications (Zhan et al., 2016). Dissection of intact brains
121 and VNCs of adult male flies was performed in cold phosphate-buffered saline and
122 fixed in 4% fresh paraformaldehyde solution for 3 to 4 h on ice. The tissues were then
123 washed with 0.1% Triton X-100 in 1× phosphate-buffered saline (PBT) three times
124 (15 min each), blocked for 30 min with PBT containing 5% normal goat serum, and
125 incubated with a primary antibody in a blocking buffer for 24 h at 4°C. After washing
126 with PBT three times, the tissues were incubated with a secondary antibody in PBT
127 for 24 h at 4°C. The nc82 signals served as counterstaining unless otherwise indicated.
128 We used the same protocol as above for *trans*-Tango imaging. The flies for
129 *trans*-Tango analysis were raised at 18°C for 10 to 20 days before dissection.

130

131 **Functional fluorescence imaging.** Previously established methods for calcium
132 imaging were used with minor modifications (Chen et al., 2017). Adult
133 hemolymph-like saline (AHL) consisting of 108 mM NaCl, 5 mM KCl, 2 mM CaCl₂,
134 8.2 mM MgCl₂, 4 mM NaHCO₃, 1 mM NaH₂PO₄-H₂O, 5 mM trehalose, 10 mM
135 sucrose, and 5 mM HEPES (pH 7.5) was used to bathe the brains for dissection and
136 imaging. Flies were briefly anesthetized on ice, and the brains were quickly dissected
137 into AHL at room temperature. For imaging, an O-ring (inner diameter of 10 mm) was
138 glued to a glass slide to form a small reservoir to hold a brain in AHL.

139

140 To measure the levels of activity in C819 neurons after fights, two C819>GCaMP6;
141 myr::RFP males were placed in a fighting chamber for 30 to 60 min of fighting. The
142 brains of losers and winners were dissected and imaged *in vitro*. We used
143 C819>GCaMP6; myr::RFP males placed individually in the fighting chamber for 30
144 to 60 min as the socially isolated controls (socially isolated males treated similarly but
145 singly in a fighting chamber for the same duration).

146

147 To measure the changes in the activity of C819 neurons after 5-HT application,
148 whole-brain explants of C819>GCaMP6; myr::RFP were positioned on circular
149 coverslips (5-mm diameter) and placed in a recording chamber containing AHL.
150 Serotonin hydrochloride (1 mM, dissolved in AHL) was gently delivered by a pipette
151 into the chamber. The control group received only AHL.

152

153 For the ATP application experiments, whole-brain explants of
154 GMR15A01-LexA/LexAop-P2X2; C819/UAS-GCamp6m (or the control:
155 GMR15A01-lexA/+; C819/UAS-GCamp6m) were placed in a recording chamber
156 containing AHL. ATP dissolved in AHL was gently delivered into the chamber to
157 reach a final ATP concentration of 2.5 mM.

158

159 Calcium imaging was performed using an SP8 confocal microscope (Leica, Wetzlar,
160 Germany) with a 20× objective (Figures 5H and 5I) or a 40× water immersion
161 objective (Figures 5F, 5G and Figures 6C-6E). All settings were kept constant
162 between the experimental conditions. Images were taken in 2.0-μm steps and acquired
163 at 512 × 512 pixels. GCaMP fluorescence was measured with excitation at 488 nm.
164 Fluorescence signals were recorded at 0.7 Hz. Images were processed with
165 customized scripts of MATLAB (MathWorks).

166

167 RFP signals were used to analyze the data of the activity in C819 neurons (after fights
168 and 5-HT application). First, the raw data were loaded using the Bio-Formats library
169 (Linkert et al., 2010). Next, the three-dimensional region of interest (ROI) of the EB
170 was manually defined according to the RFP signal. Finally, the green fluorescent
171 protein intensities within the ROI were averaged to represent the quantified GCaMP
172 signal.

173

174 For the imaging experiments performed to evaluate the functional connectivity
175 between P1 and C819 neurons, the fly of GMR15A01-LexA/LexAop-P2X2;
176 C819/UAS-GCaMP6m did not have an RFP transgene, so the three-dimensional ROIs
177 were built manually with the help of a baseline GFP signal from the GCaMP. With the
178 same three-dimensional ROI for one time series, the GCaMP fluorescent values were
179 then obtained by averaging signals across all the pixels at each time point. The change
180 in fluorescent intensity was calculated as follows: $\Delta F/F_0 = (F_t - F_0) / F_0 \times 100$,
181 where F_t is the fluorescent value at time t , and F_0 is the averaged value of three-time
182 points near time zero (before ATP was applied). All imaging and analyses were
183 performed blinded to the experimental conditions.

184

185 **Statistical analysis.** Statistical analysis was performed using Prism 6 (GraphPad
186 Software, La Jolla, CA, USA). All experiments were performed in parallel with both
187 the experimental and control groups. All data points in a data set were plotted in a
188 box-and-whisker plot; the whiskers marked the minimum and maximum of the data
189 set, the box included data from the 25th to 75th percentiles, and the line within
190 indicated the median. When two groups of normally distributed data were compared,
191 we performed Student's t-test. The Kruskal-Wallis test was used to analyze

192 non-normally distributed data. Analysis of variance (ANOVA) was used to analyze
193 multiple comparisons among data with normal distributions. Following ANOVA, the
194 Bonferroni post hoc test was conducted to determine statistical significance.

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