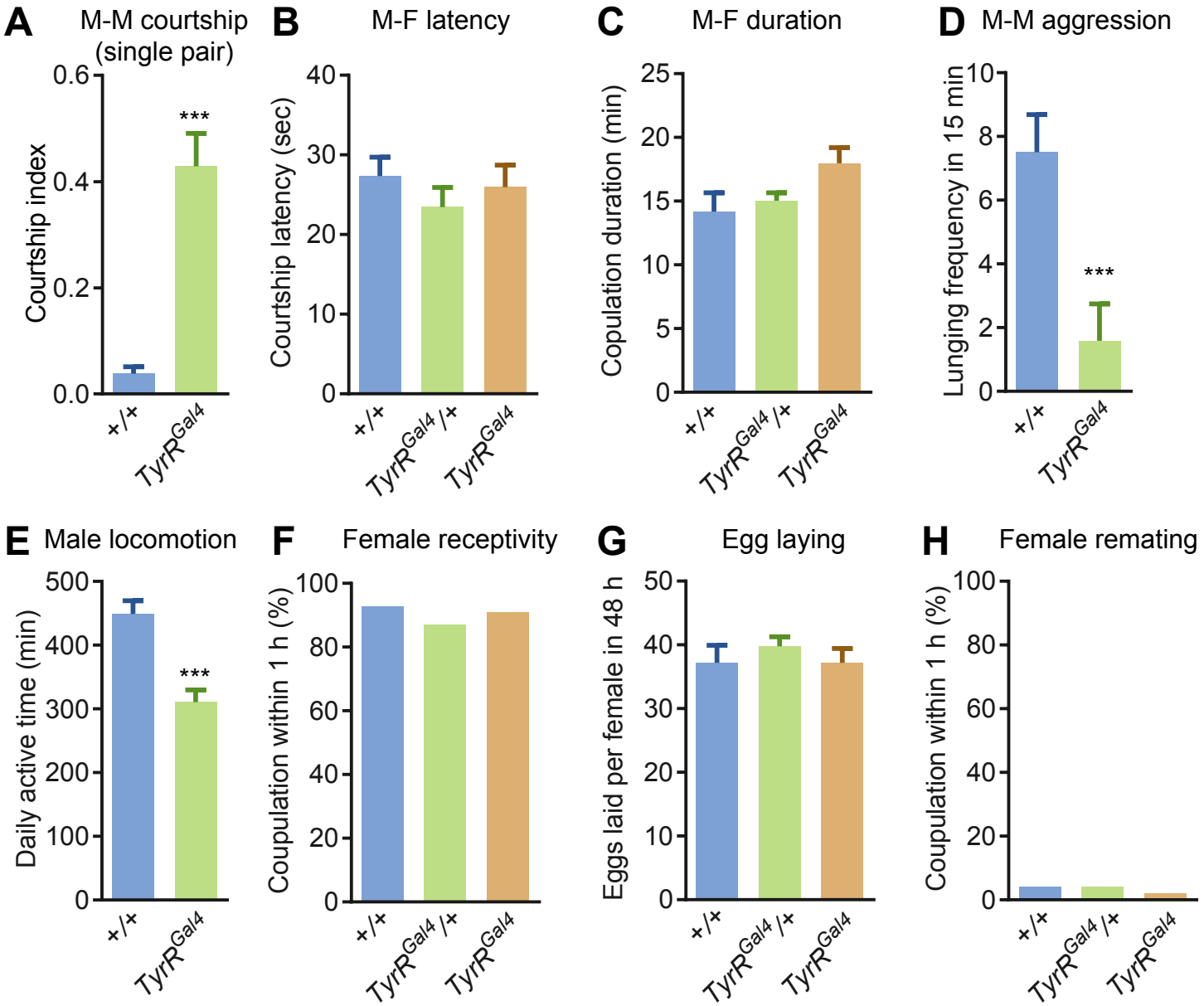
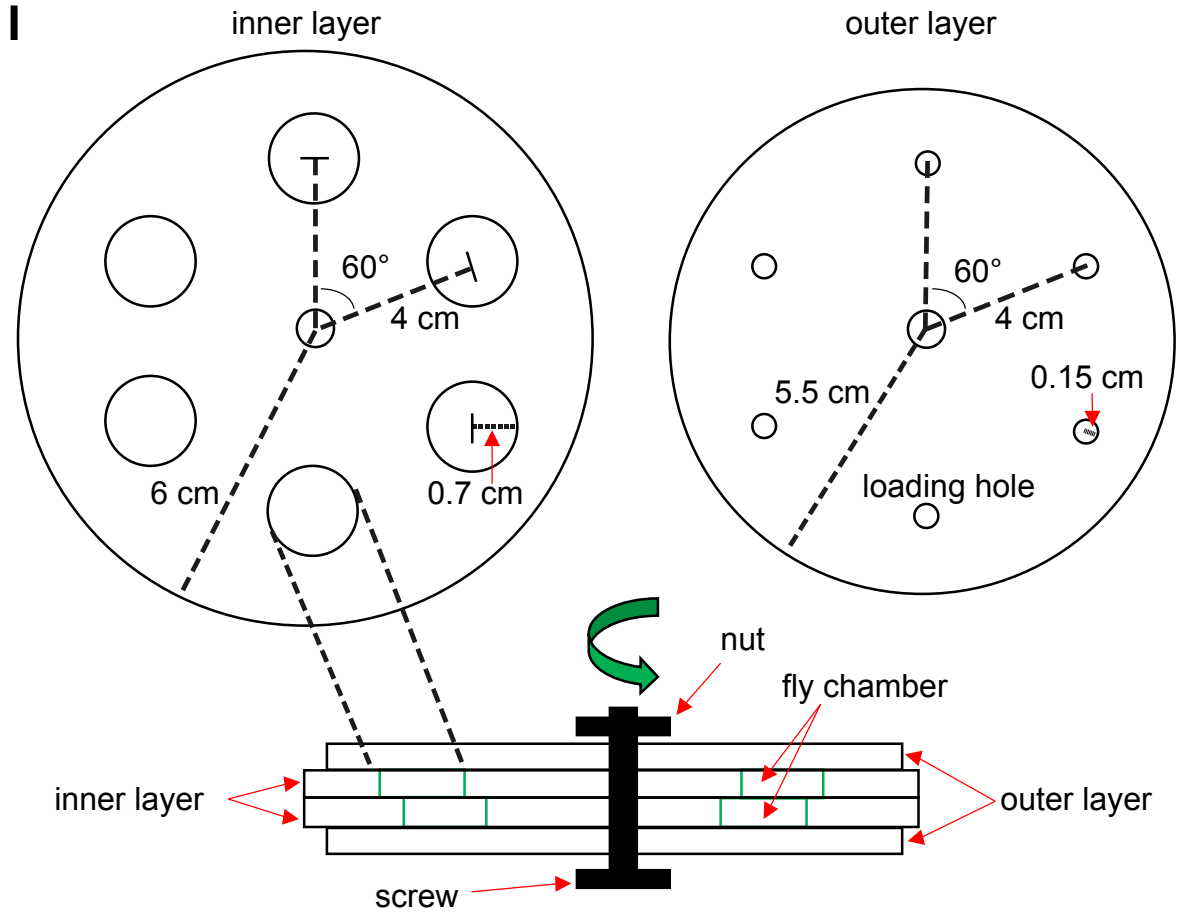
Figure S1 related to Figure 2





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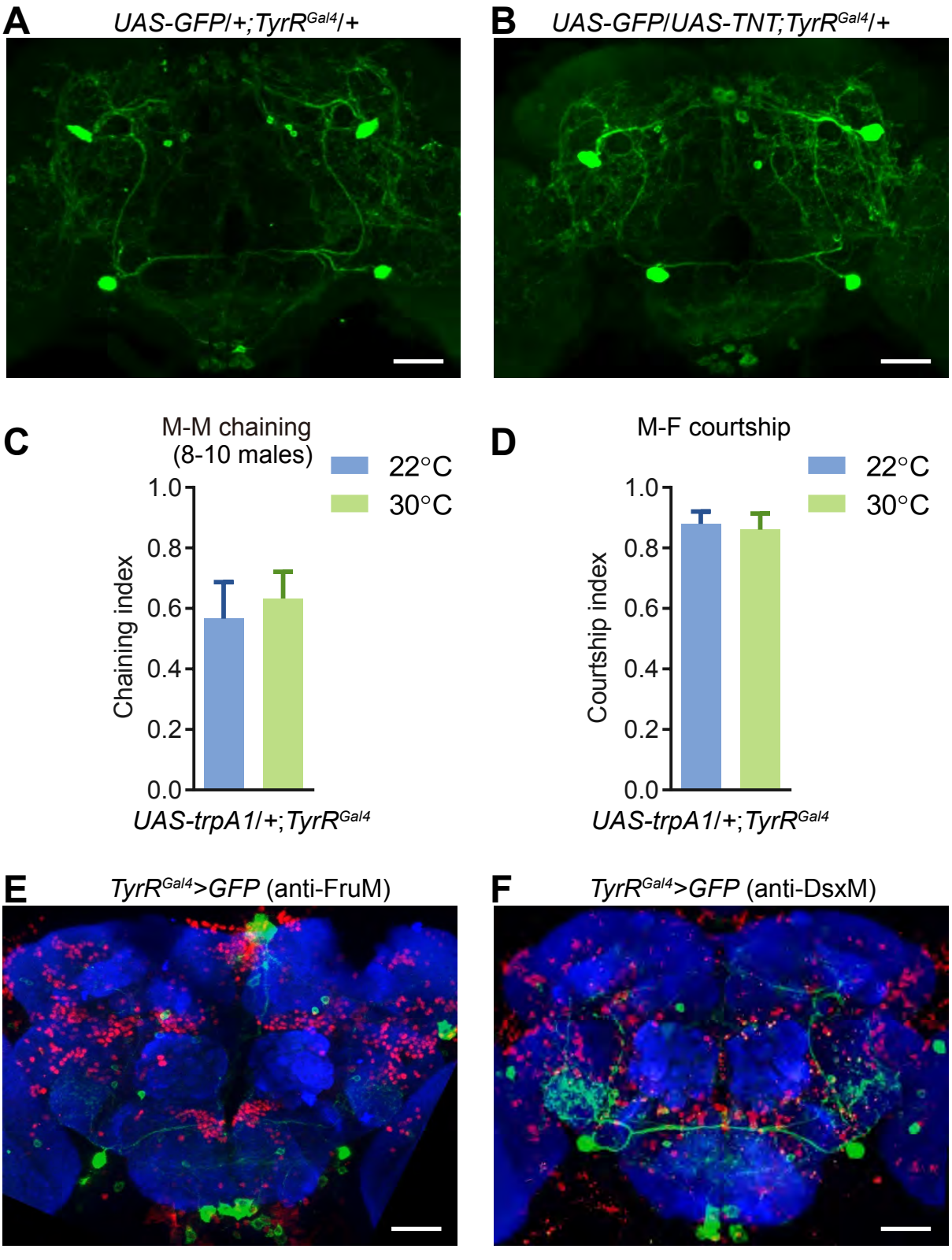
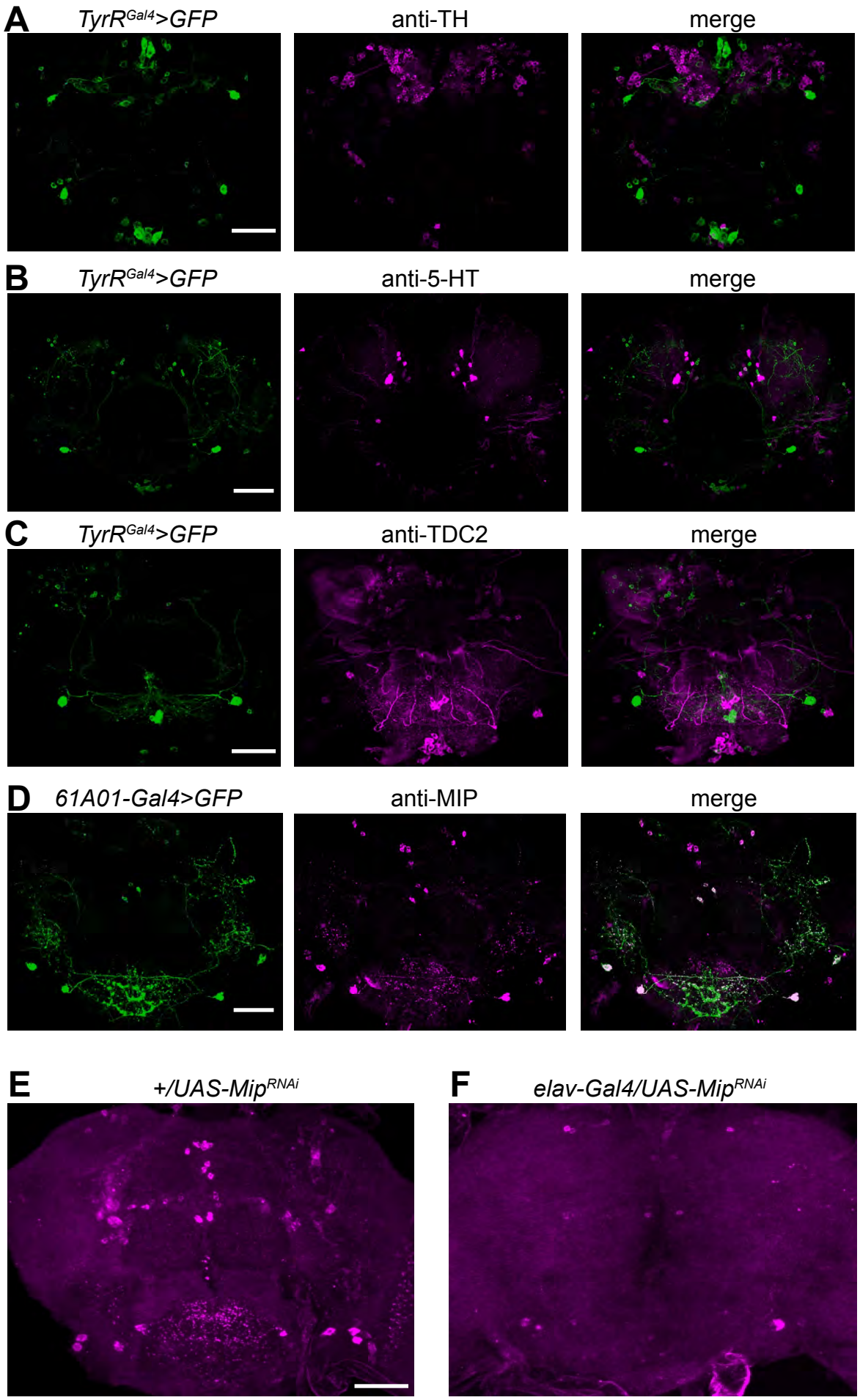


Figure S3 related to Figure 5



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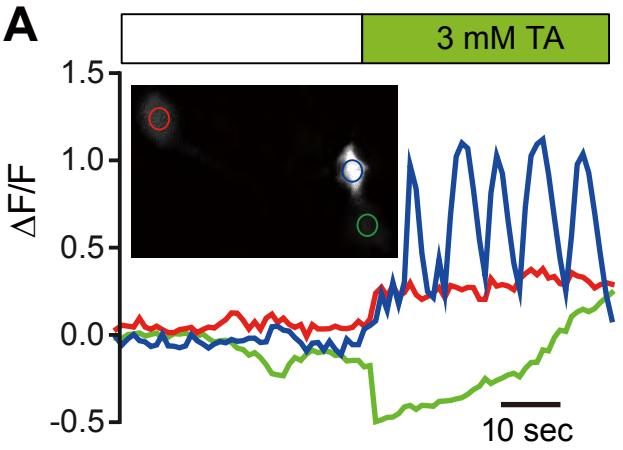

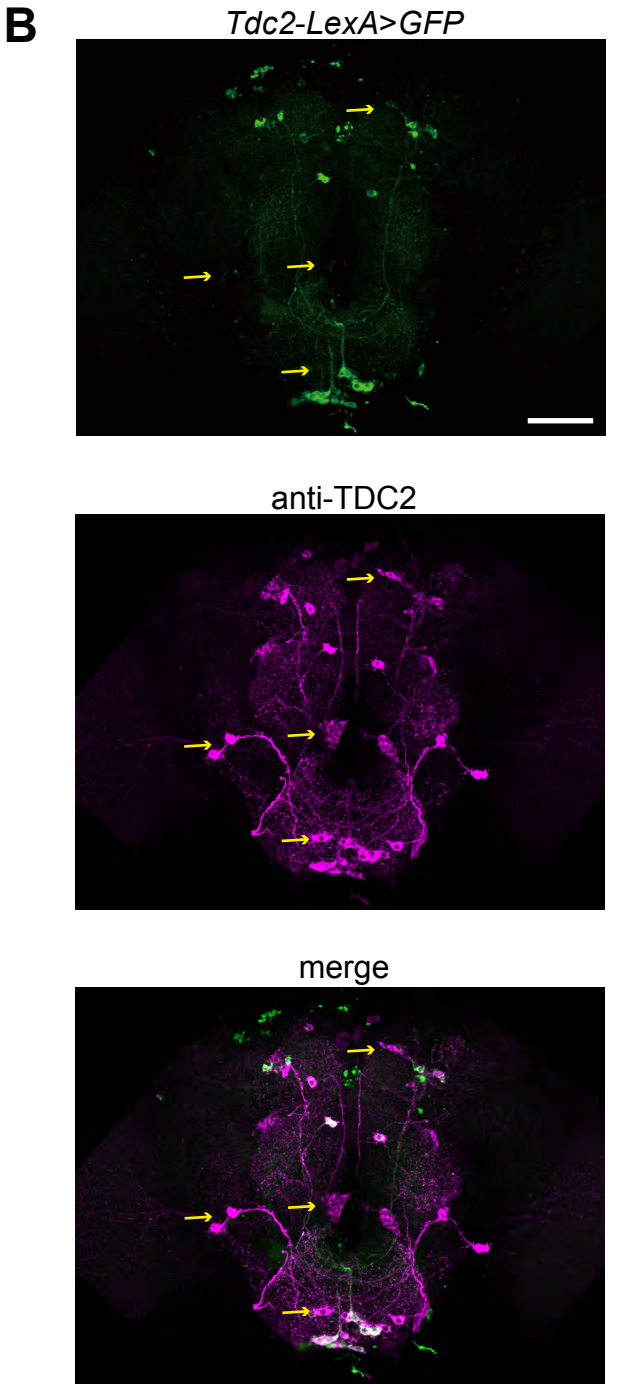

Legends to Supplemental Figures

Figure S1. Social behaviors of *TyrR^{Gal4}* males and post-mating behaviors of *TyrR^{Gal4}* females

(A) Courtship indices using one *fru^{M}* null (*fru^{LexA}*/*fru$^{4-40}$*) male as the target and one tester male of the indicated genotypes. p<0.001 compared to the control. n=13-14 trials/genotype. The "wild type" +/+ flies used here and in other panels are *w^{+}* outcrossed for five generations with *w^{1118}* flies.

(B) Courtship latency is the elapsed time between the introduction of the indicated male, and initiation of courtship with the female. n=24

(C) Copulation duration between +/+, heterozygous *TyrR^{Gal4}*/+ or homozygous *TyrR^{Gal4}* males. n=25.

(D) Male-male aggression assessed by measuring the lunging frequency between control and mutant males. n=12

(E) The "daily active time" is the total time per 24 h when flies are awake. n=23

(F) Receptivity of virgin wild-type (+/+), heterozygous or homozygous *TyrR^{Gal4}* females scored as the percentage of females that copulated with Canton-S males within 1 h. n=24-29 trials/genotype.

(G) Number of eggs laid per +/+, heterozygous or homozygous *TyrR^{Gal4}* females during the 48 h post mating. n=22-25.

(H) Re-mating frequency of +/+, heterozygous or homozygous *TyrR^{Gal4}* females 48 h after the initial mating. The males were Canton S. n=22-24.

(I) Custom-built plate for assaying courtship latency. The apparatus consisted of two inner layers and two outer layers. We inserted the test fly and target fly into separate small chambers of the inner layers through the loading holes in the outer layers. The two flies were combined after we rotated the inner layers to make the chambers connect. The error bars indicate means ±SEMs. We used Fisher's exact test for (F, H) and the Mann-Whitney test for other panels. ***p<0.001.

Figure S2. *TyrR* neurons do not express Fruitless or Doublesex

(A-B) *TyrR* reporter expression (*TyrR^{Gal4}>UAS-mCD8-GFP*) in the absence (A) and presence (B) of *UAS-TNT*. Anti-GFP, green. n=5-7.

(C) Chaining among groups of males in which we thermally hyperactivated *TyrR* neurons with *UAS-trpA1* in the mutant background. n=5

(D) Male-female courtship indices in males with thermally hyperactivated *TyrR* neurons in the mutant background. n=11

(E) *TyrR*-expressing neurons did not express FruM. Anti-GFP (Green, *TyrR^{Gal4}>UAS-mCD8-GFP*), anti-FruM (magenta), and anti-nc82 (blue).

(F) *TyrR*-expressing neurons did not express DsxM. Anti-GFP (Green, *TyrR^{Gal4}>UAS-mCD8-GFP*), anti-DsxM (magenta), and anti-nc82 (blue).

The scale bars represent 50 μm.

Figure S3. Relative brain expression of the *TyR* reporter with 5-HT, TH, Tdc2 and MIP, and the knockdown efficiency of the *Mip^{RNAi}*

(A-C) *TyrR* reporter expression (*TyrR^{Gal4}>UAS-mCD8-GFP*) detected by anti-GFP

(green).
(A) Anti-tyrosine hydroxylase (TH), magenta.
(B) Anti-serotonin (5-HT), magenta.
(C) Anti-tyrosine decarboxylase (Tdc2).
(D) Co-staining of anti-MIP with the *61A01>mCD8-GFP* reporter. Anti-GFP, green; anti-MIP, magenta.
(E and F) Anti-MIP staining (magenta) of brains from animals with the *UAS-Mip^{RNAi}* transgene alone (E) or *UAS-Mip^{RNAi}* expressed under the control of the elav-Gal4 (F). The scale bars represent 50 μm.

Figure S4. Ca^{2+} oscillations induced by 3 mM TA in a *TyrR* neuron, and relative expression of the *Tdc2-LexA* reporter and anti-TDC2.
(A) The blue, red and green traces correspond to three different neurons indicated in the inset. ~30% of the tested cells stimulated with 3 mM TA displayed oscillations.
(B) *Tdc2-LexA* recapitulated a portion of *Tdc2* expression. *Tdc2-LexA>UAS-mCD8-GFP.* Anti-GFP, green. Anti-TDC2, magenta. The yellow arrows indicate neurons labeled by anti-TDC2 but not the *Tdc2-LexA* reporter. The scale bar represents 50 μm.

Supplemental Experimental Procedures

Fly stocks

We obtained the following strains from the Bloomington Drosophila Stock Center (stock numbers indicated): *61A01-Gal4* (39269), *61A01-lexA* (54909), *UAS-TyrR^{RNAi}* (25857), *Tdc2-Df* (3367), *TyrR-Df* (9207), *UAS-mCD8-GFP* (5137), *VGluT-Gal80* (55847), *LexAop2-mCherry* (52271), *UAS-ChAT^{RNAi}* (25856), *UAS-Mip^{RNAi}* (26246), *UAS-TNT* (28838), *UAS-TNTin* (28840) and *UAS-GCaMP6f* (42747). Other lines used were: *Tdc2^{RO54}* S1], *Tβh^{nM18}* [S2], *fru^{LexA}* [S3], *fru^{NP21}-Gal4* [S4], *fru^{FLP}* and *UAS>stop>mCD8-GFP* [S5], *UAS-trpA1* [S6], *UAS-NaChBac* [S7], *Tsh-Gal80* [S8], *UAS-kir2.1* [S9], *UAS-mCD4-spGFP$^{1-10}$* and *LexAop-mCD4-spGFP^{11}* [S10].

Behavioral assays

Post-mating behaviors: Single female and male virgins were paired in a chamber (a 24-well tissue culture plate with loading holes on the cover for transferring flies into each well) and videotaped for 1 h. We tabulated the percent of females that copulated within 1 h. The females that copulated were transferred individually to food vials for 48 h, and the numbers of eggs laid by each female were counted manually. We then retested the females for receptivity in the same manner as in the pairings with naive Canton-S males and females.

Courtship assays: We introduced a test male fly and a target fly into the same courtship chamber and recorded their behavior with a video camera. We recorded male-male courtship for 20 min. In cases in which we used a female target, we videotaped for up to 20 min, and then terminated the recordings once the females successfully copulated. The copulation duration was the time that elapsed between the initiation of copulation and when the male fly left the female. To determine the courtship latency, we

prepared a custom-made plate with four layers (Figure S1I). We placed the test fly and target fly into the small chambers of the inner layers through the loading holes on the outer layers, separately. The two flies met after we rotated the inner layers to enable the chambers to connect.

To quantify the courtship index, we tabulated the percentage of time during the 20 observation that a test male performed any courtship step towards a female target or another male. To determine the chaining index, we combined 8-10 males in a 60 mm tissue culture dish, and calculated the percentage of the observation time (10 min) in which ≥3 flies engaged in simultaneous courtship.

Sexual discrimination assays: We placed a decapitated w^{1118} virgin female and a decapitated w^{1118} male in a courtship chamber. We then introduced the test male into the chamber and counted the total time during which the test male directed courtship behavior toward either the decapitated female or the decapitated male.

Thermogenetic activation experiments: We collected male flies expressing *UAS-trpA1* shortly after eclosion, and aged the animals individually for 4-10 days at 18°C. To activate TRPA1, we raised the temperature during the behavior assays from 22°C to 30°C for 10 min.

Aggression assays: Pairs of male flies were introduced into the fighting chamber S11] by taking advantage of their positive phototaxis and negative geotaxis tendencies. After the flies entered the chamber, the entrance was covered up with 20 mm × 20 mm glass slides. The behaviors were recorded for 15 min using a digital video camera. We measured the aggression of the male flies by assaying the number of lunges over 15 min, since lunging is the most frequently observed aggressive behavior exhibited by male flies [S12]. Lunging is a stereotyped behavioral pattern in which one fly rears up on its hind legs and snaps down onto its opponent [S13].

Locomotion assays: We assayed locomotor activity using the *Drosophila* Activity Monitoring System (DAMS, Trikinetics). Individual male flies were lightly anesthetized and introduced into polycarbonate tubes. Midline crossing activity was sampled every minute and pooled into 30 min bins for analysis.

Generation of the $TyrR^{Gal4}$ mutant

We PCR amplified from isogenic w^{1118} flies a 3 kb genomic DNA extending from the 5' end of the start codon, and a 3 kb genomic DNA extending from the 3' side of the first exon of the *TyrR* gene, which is located at 90C2-90C3 on the 3rd chromosome Each homologous arm was subcloned into the pw35*Gal4* vector [S14]. The transgenic flies were generated by first obtaining random insertions of the transgenes (BestGene) and then by mobilizing the transgenes and screening for targeted insertions as described previously [14]. The following primer sequences were used to perform the screening for homologous recombination: GCCCCTCTGATTGATGACCC (F1); GAGCACTTGAGCTTTTTAAGTCG (R1); CGTCGGCAAGAGACATCCAC (F2); GTCATCTCTATTGCGAACCACTCCG (R2). The following primers were used to confirm the deletion: TAAGCGCCATTTATCCGTCG (F3); ACGAAGATGCCCACGAGAAG (R3).

Generation of *TyrR* transgenes

We generated the *UAS-TyrR* transgene by subcloning the *TyrR* cDNA into the pUAST vector [S15]. To generate flies harboring a transgene encoding the entire *TyrR* genomic region, we used ϕ31-mediated transgenesis (Bestgene) to transfer the DNA from the CH322-156H15 BAC plasmid to the attP40 site.

Immunohistochemistry

Fly tissues were dissected in ice-cold 0.3% PBST (0.3% Triton X-100 in phosphate-buffered saline) buffer and fixed in 4% paraformaldehyde at room temperature for ~20 min. Samples were then incubated with primary antibodies diluted in 5% goat serum, 0.3% PBST for 48 h at 4°C. Samples were washed 5 times with 0.3% PBST before application of secondary antibodies for 24 h at 4°C. After washing 5 times with 0.3% PBST, the samples were mounted with VectaShield (Vector Labs) on glass slides. To visualize native GRASP fluorescence, we dissected brains in ice-cold PBL (0.075 M lysine, 0.1 M sodium phosphate buffer), fixed for 30 min in 4% paraformaldehyde in PBL at 22°C, washed three times with 0.3% PBST (0.3% Triton X-100 in phosphate-buffered saline) and then blocked with 10% normal goat serum in PBST. We performed the imaging using a Zeiss LSM700 confocal microscope, and processed the images using ImageJ.

The primary antibodies used were chicken anti-GFP (1:1000, Invitrogen, A-10262), rabbit anti-GFP (1:1000, Invitrogen, A-11122), rabbit anti-DsRed (1:1000, Clonetech, 632496), mouse anti-nc82 (1:50, Developmental Studies Hybridoma Bank), rabbit anti-Tdc2 (1:500, Abcam, ab128225), rabbit anti-tyrosine hydroxylase (TH; 1:500, Millipore, AB152), rabbit anti-serotonin (1:500, Sigma, S5545), rabbit anti-MIP S16] rabbit anti-FruM (1:10000) [S17] and rat anti-DsxM (1:500) [S18]. Secondary fluorescent antibodies were AlexaFluor 488 goat anti-chicken (1:1000; Invitrogen, A-11039), AlexaFluor 488 goat anti-Mouse (1:1000; Invitrogen, A-11001), AlexaFluor 488 Goat anti-rabbit (1:1000; Invitrogen, A-11008), AlexaFluor 568 goat anti-rabbit (1:1000; Invitrogen, A-11011), AlexaFluor 568 goat anti-mouse (1:1000; Invitrogen, A-11004), AlexaFluor 568 goat anti-rat (1:1000; Invitrogen, A-11077), AlexaFluor 633 goat anti-rabbit (1:1000; Invitrogen, A-21070) and AlexaFluor 633 goat anti-mouse (1:1000; Invitrogen, A-21050).

Ca^{2+} imaging in brains

We performed the analysis using 1-7-day-old mutant (*$TyrR^{Gal4}$*) or control flies (*$TyrR^{Gal4}$*/+) expressing *UAS-GCaMP6f*. We anaesthetized the flies on ice, opened the head capsule under saline buffer, transferred the tissue to a custom imaging chamber (5.5 x 8.0 cm, Figure S5E), fixed the tissue in 1% low melting gel (Lonza, SeaPlaque Agarose) and viewed the Ca^{2+} dynamics using a Zeiss LSM700 confocal microscope. To record the video, we used the physiology module of the Zen2012 software at a rate of one frame/sec. We imaged the spontaneous baseline (F) GCaMP6f response for 30 sec (30 frames), and then added ~5 ml test chemicals to the bath using a transfer pipette. The base line (F) was used to calculate the ΔF. We calculated the average ΔF

for each time point from ≥5 samples.

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