Supplementary Information for

GABA transmission from mAL interneurons regulates aggression in *Drosophila* males

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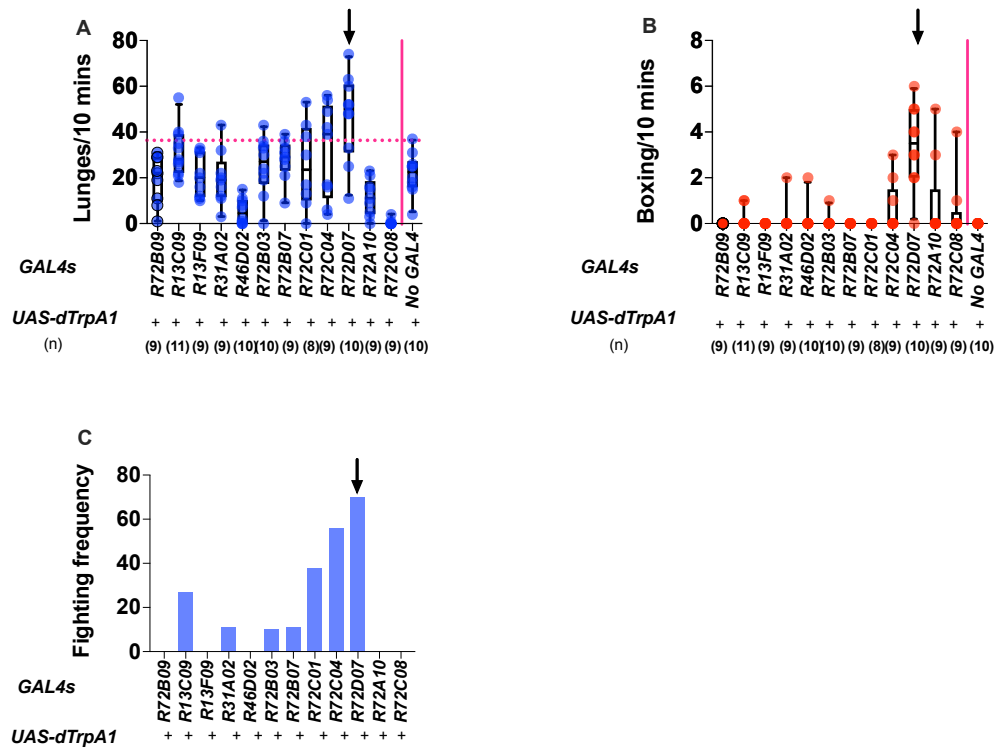
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> Figures S1 to S9
> Legends for Movies S1 to S5
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**Other supplementary materials for this manuscript include the following:**

> Movies S1 to S5
> Dataset S1 and Dataset S2

Fig.S1. Preliminary screening of 12 GAL4s to identify aggression promoting neurons: (*A*) Lunges and (*B*) boxing during thermogenetic activation of 12 GAL4-targeted neurons at 30°C. In *A* and *B*, data is plotted as box plot with whiskers extending to the 10th and the 90th percentile with a line at median. Each of the circles in *A* and *B* represents lunges per 10 mins and boxing per 10 mins, respectively at 30°C. GAL4 lines used, and number of fights performed for each GAL4 are indicated below respective figures. A pink solid vertical line separates the *No-GAL4/UAS-dTrpA1* control from the 12 *GAL4/UAS-dTrpA1* combinations in both *A* and *B*. A cut-off for lunges (pink dotted line) was set at the 90th percentile of the *No-GAL4/UAS-dTrpA1* control in *A*. *R72D07-GAL4/UAS-dTrpA1* and *R72C04-GAL4/UAS-dTrpA1* had medians for lunges above the cut-off in *A*. Since the median for *R72D07-GAL4/UAS-dTrpA1* was higher than that for *R72C04-GAL4/UASdTrpA1*, it was selected for further analysis. *R72D07-GAL4/UAS-dTrpA1* also had the highest median for boxing in *B*. (*C*) *R72D07-GAL4/UAS-dTrpA1* has the highest fighting frequency. Fighting frequency is defined as the percentage of fights with lunges above the cut-off. Black arrows in *A-C* point at *R72D07-GAL4/UAS-dTrpA1*.
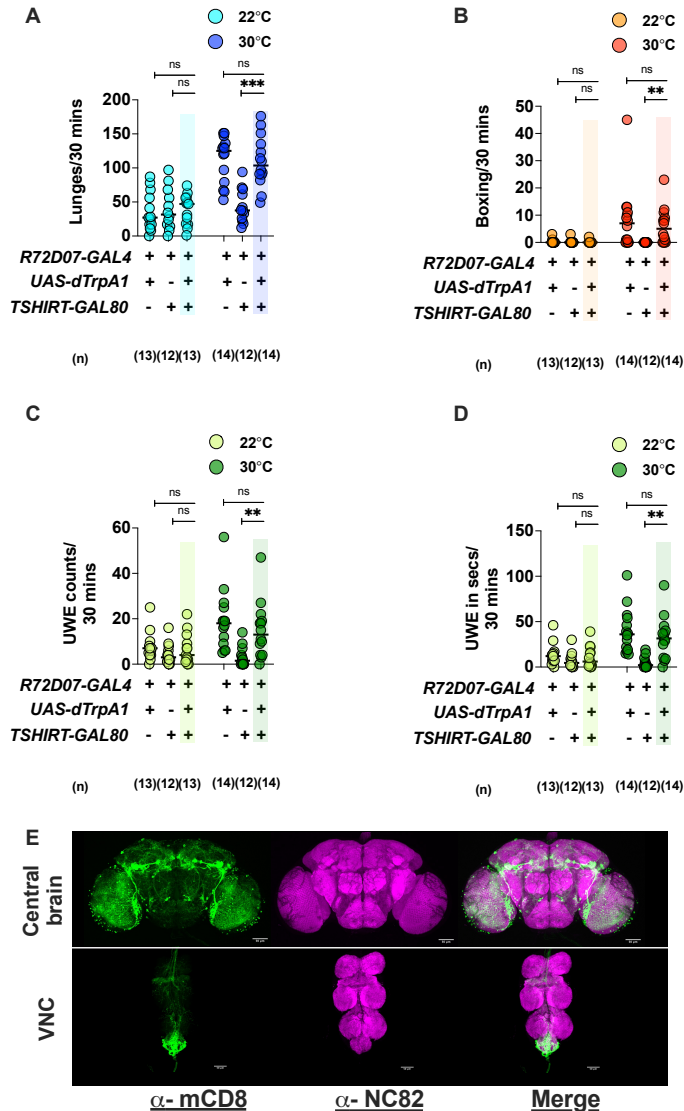
Fig. S2. Thermogenetic activation of *R72D07-GAL4* central brain neurons is sufficient for increasing both aggression and UWEs: *Tshirt-Gal80* was used to limit GAL4 activity in the central brain of *R72D07-GAL4*. Similar to the *R72D07-GAL4/UAS-dTrpA1* fights (**Fig.1** *A-D*), *Tshirt-Gal80*; *R72D07-GAL4/UAS-dTrpA1* fights display increased (*A*) lunges, (*B*) boxing, (*C*) UWE counts, and (*D*) UWE time, upon thermogenetic activation at 30°C. For *A-D*, each circle represents lunges per 30 mins, boxing per 30 mins, UWE counts per 30 mins, and UWE time in seconds per 30 mins, respectively. For *A-D*, Kruskal Wallis test followed by post-hoc Dunn's was performed for both the temperatures (**P < 0.01, ***P < 0.001, ns = not significant at *P* > 0.05). Genotypes and number of pairs used are shown below each plot (*E*) *Tshirt-Gal80* expression reduces GAL4-mediated *mCD8:GFP* expression in the VNC of *R72D07-GAL4*: Representative images of central brain (*upper panel*) and VNC (*lower panel*) of *Tshirt-Gal80*; *R72D07-GAL4/UAS-mCD8:GFP* male. α-mCD8 (green) marks the neurons targeted by *R72D07-GAL4* in presence of *Tshirt-Gal80*. Neuropil marker α-NC82 visualizes the brain and VNC. Images are maximum projection through all optical slices. (Scale bar, 50 *µ*m.)
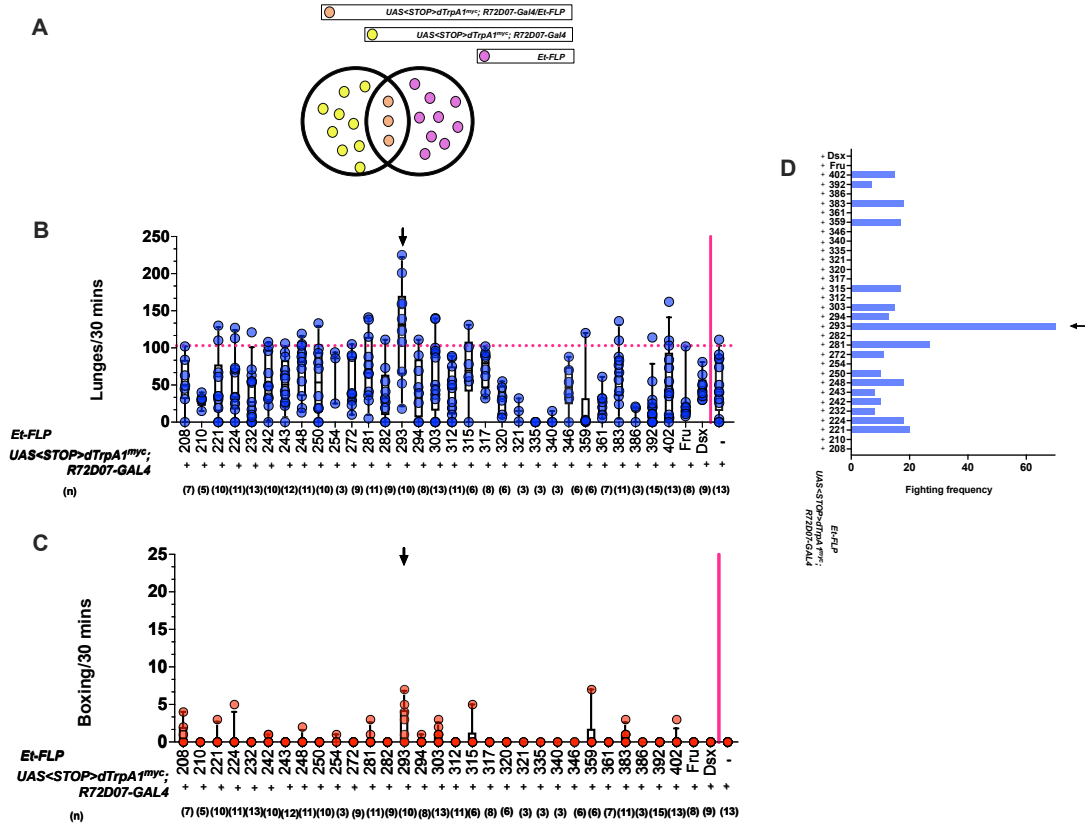
Fig. S3. Intersectional genetics strategy for reducing neurons: (*A*) Venn diagram showing the intersectional genetics strategy: Yellow circles represent neurons targeted in *UAS<STOP>dTrpA1^myc^; R72D07-Gal4*. Pink circles represent neurons targeted in different *Et-FLP* lines. Peach circles represent neurons targeted in *UAS<STOP>dTrpA1^myc^; R72D07-Gal4/Et-FLP* (or *R72D07∩Et-FLP*). (*B*) Lunges and (*C*) boxing during thermogenetic activation of 32 *R72D07∩Et-FLP* combinations at 30°C. In *B* and *C*, data is plotted as box plot with whiskers extending to the 10th and the 90th percentile with a line at median. Each of circles in *B* and *C* represents lunges per 30 mins and boxing per 30 mins respectively. Each of the 32 *Et-FLP* lines is denoted by a specific number and indicated below the figures. Number of pairs tested for each intersectional combination is also indicated below the figures. A pink solid vertical line separates *R72D07∩No-FLP* control from the 32 *R72D07∩Et-FLP* combinations in both *B* and *C*. A cut-off for lunges (pink dotted line) was set at the 90th percentile of the *R72D07∩No-FLP* control in *B*. *R72D07∩293-FLP* is the only combination to have a median for lunges above the cut-off. (*D*) *R72D07∩293-FLP* has the highest fighting frequency. Fighting frequency is defined as percentage of fights with lunges above the cut-off. Black arrows in *B-D* point at *R72D07∩293-FLP*.
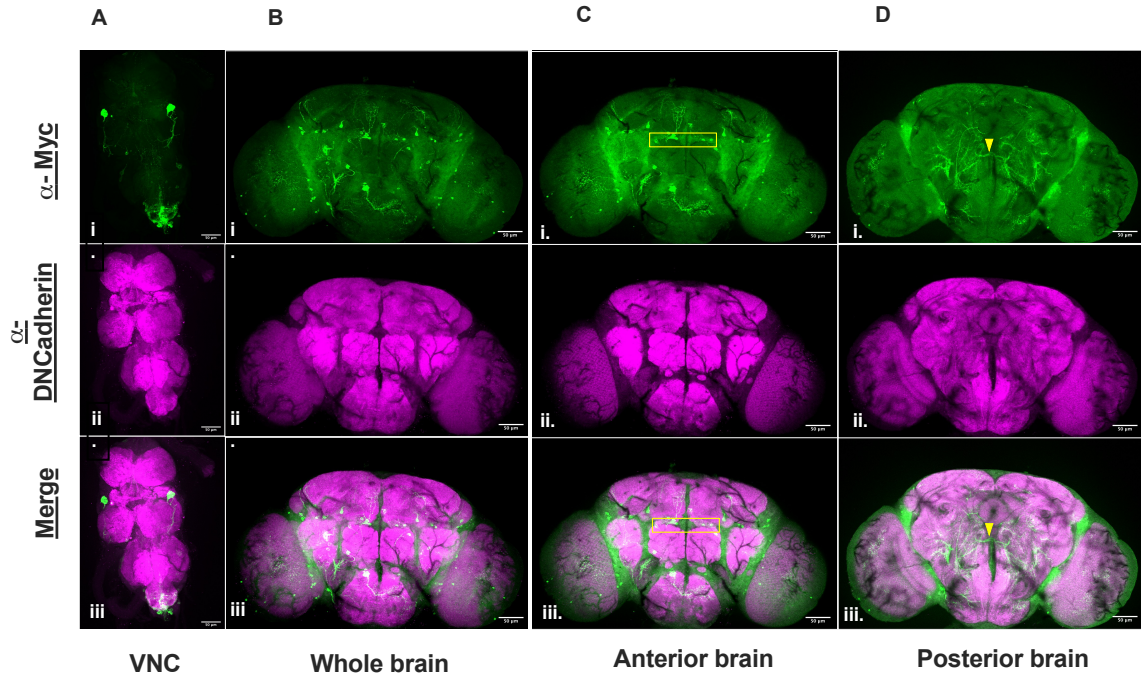
Fig. S4. *R72D07∩293-FLP* **brain includes a small group of mAL resembling neurons:** Compared to *R72D07-GAL4* (**Fig.2 *A* and *B***), the intersectional combination of *R72D07∩293-FLP* has reduced number of neurons in both (*A*) VNC and (*B*) central brain. α-Myc (green) reveals the neurons targeted by *R72D07∩293-FLP*. The neuropil marker α-DNCadherin (magenta) visualizes the brain and VNC. *R72D07∩293-FLP* also targets a small group of mAL resembling neurons. (*C*) Cell bodies of this group are located above ALs (yellow rectangle), like mAL. (*D*) Processes of this group exhibit midline crossing (yellow arrow), and descend to the SOG, like mAL. *A* and *B* are maximum projection through all the optical slices of the confocal stack. *C* and *D* are maximum projection of optical slices containing the cell bodies and processes of the mAL-resembling group, respectively. (Scale bar, 50 *μ*m)
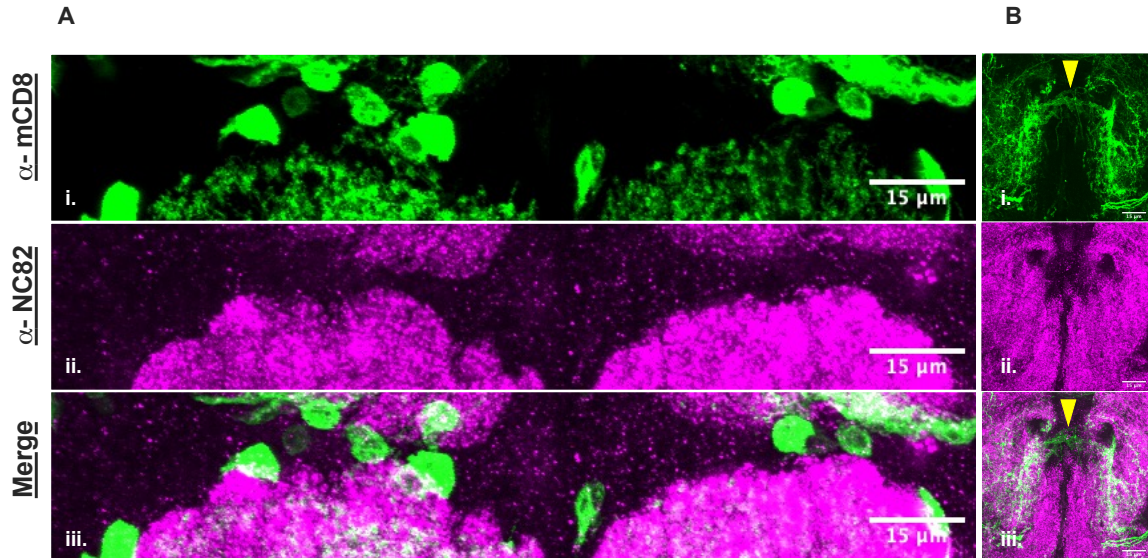
Fig. S5. *R72D07-GAL4* **targets a small group of mAL-resembling neurons:** The *R72D07-GAL4* labels a small group of approximately 3 to 5 cells per brain hemisphere with anatomical resemblance to mAL. (*A*) Cell bodies and (*B*) processes of the mAL-resembling group are visualized by α-mCD8 signal (green) in a *R72D07-GAL4/UAS-mCD8* brain, visualized by the neuropil marker α-NC82 (magenta). Like the mAL neurons, cell bodies of this group are located above ALs, and the processes exhibit midline crossing (yellow arrow) and descend towards SOG. Both *A* and *B* are maximum projections through the optical slices containing the cell bodies and processes of the mAL-resembling group, respectively. (Scale bar, 15 *μ*m)
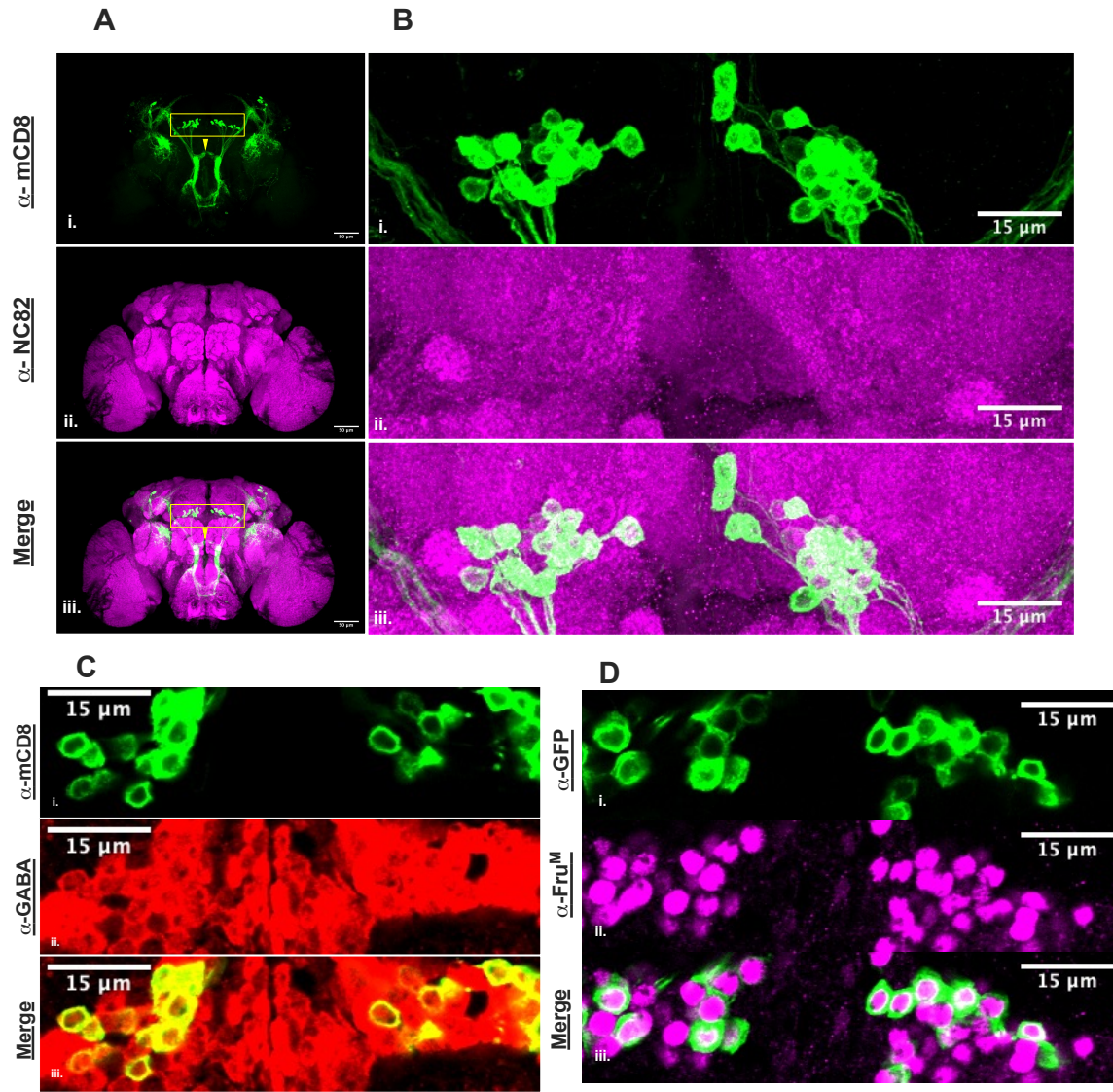
Fig. S6. *R43D01-GAL4* **primarily drives into the FruM⁺ GABA⁺ mAL cluster:** (*A*) Cell bodies of the mAL cluster are located above ALs (yellow rectangle). mAL processes show midline crossing (yellow arrow) before descending to the SOG (Scale bar, 50 μm). (*B*) Magnified image of mAL cell bodies on the ALs. In *A* and *B*, α-mCD8 (green) marks the neurons and α-NC82 (magenta) visualizes the brain. (*C*) mAL cells (green, α-mCD8) are GABA⁺ (red, α-GABA). (D) mAL cells (green, α-GFP) are FruM⁺ (magenta, α-FruM). *A* and *B* are maximum projection through all the optical slices of the confocal stack. In *C* and *D*, overlap of signals in a single optical slice is shown for accurate representation. (Scale bar, 15 μm in *B-D*).
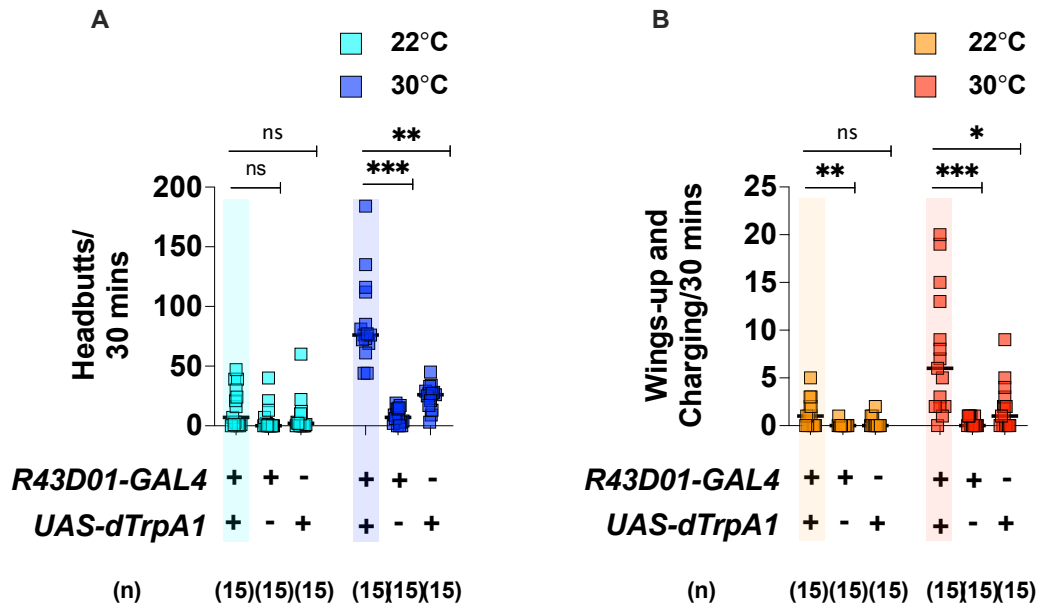
Fig. S7. Thermogenetic activation of mAL neurons increases aggression in interfemale fights: "Headbutts" and "wings-up and charging" are motor programs of aggression used by *Drosophila* females. Thermogenetic activation of mAL neurons at 30°C in females significantly increases (*A*) headbutts, and (*B*) Wings-up and charging, compared to the parental controls. For *A* and *B*, each square represents headbutts per 30 mins and Wings-up and charging per 30 mins respectively. For A and B, Kruskal Wallis test followed by post-hoc Dunn's was performed for the control and experimental temperatures of 22°C and 30°C respectively (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns = not significant at $P > 0.05$). Genotypes and number of pairs used are shown below each plot.
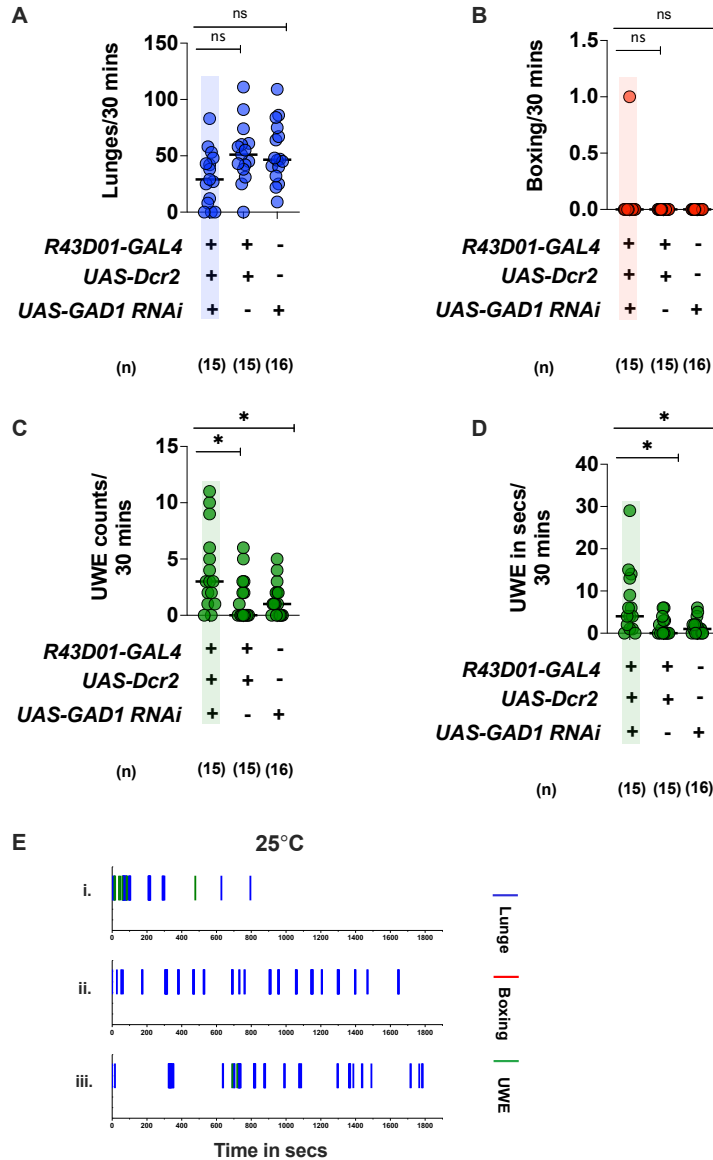
Fig. S8. GABA knockdown in mAL neurons reduces lunges and increases UWEs in intermale fights: mAL *GAD1 RNAi* fights have the lowest median for (*A*) lunges (also see text). mAL *GAD1 RNAi* fights have significantly increased (*C*) UWE counts, and (*D*) UWE time, compared to the parental controls. For *A-D*, each circle represents lunges per 30 mins, boxing per 30 mins, UWE counts per 30 mins, and UWE time in seconds per 30 mins, respectively. For *A-D*, Kruskal Wallis test followed by post-hoc Dunn's was performed (*$P< 0.05$, ns = not significant at $P > 0.05$). Genotypes and number of pairs used are shown below each plot. (E) *GAD1 RNAi* in mAL neurons decreases lunges and increases UWEs in intermale fights: The x-axis represents the observation period which is 1,800 s (or 30 min). Representative fights of (*i*) *UAS-Dcr2/UAS-GAD1RNAi; R43D01-GAL4* (*ii*) *UAS-Dcr2; R43D01-GAL4* only control (*iii*) *UAS-GAD1 RNAi* only control.
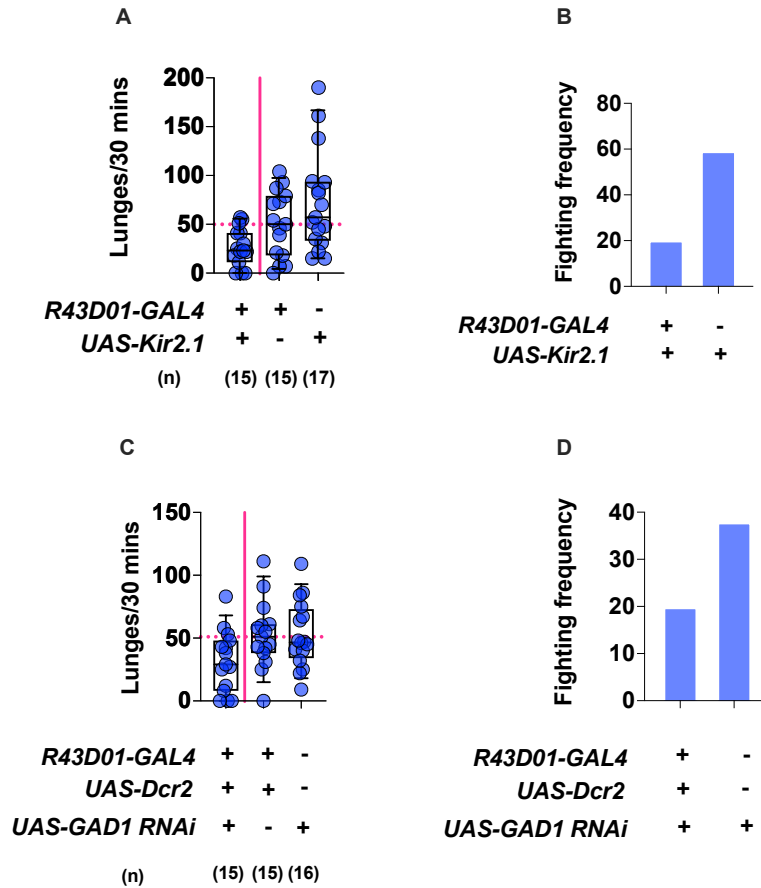
Fig. S9. Suboptimal mAL activation decreases lunges in intermale fights: (*A*) mAL inactivation by Kir2.1 has the lowest median for lunges. Data is plotted as box plot with whiskers extending to the 10th and the 90th percentile with a line at median in *A*. A vertical pink solid line separates the experimental line with mAL inactivation from the two parental control lines. For calculating fighting frequency, a cut-off (pink dotted line) is set at the median or the 50th percentile of the *R43D01-GAL4* only parental controls. Fighting frequency is described as the percentage of fights with lunge numbers above the cut-off. (*B*) Experimental fights with mAL inactivation have reduced fighting frequency. Only 20% of these fights have lunge numbers above the cut-off, whereas 59% of the *UAS-Kir2.1* only parental control fights have lunges above the cut-off. (*C*) mAL *GAD1* RNAi has the lowest median for lunges. Data is plotted as box plot with whiskers extending to the 10th and the 90th percentile with a line at median in *C*. A vertical pink solid line separates the experimental line with mAL *GAD1* RNAi from the two parental control lines. For calculating fighting frequency, a cut-off (pink dotted line) was set at the median or the 50th percentile of the *UAS-Dcr2; R43D01-GAL4* only parental controls. Fighting frequency is described as the percentage of fights with lunge numbers above the cut-off. (*D*) mAL *GAD1 RNAi* fights have reduced fighting frequency. Only 18% of these fights have lunges above the cut-off, whereas 38% of the *UAS-GAD1 RNAi* only parental fights have lunges above the cut-off. Each of the circles in *A* and *C* represents lunges per 30 mins. Genotypes are indicated below respective plots.

Legends for Supplementary movies 1-5

Movie S1. Thermogenetically activated *R72D07-GAL4/UAS-dTrpA1* intermale fight: dTrpA1-activated *R72D07-GAL4* intermale fight is characterized by increased lunging, boxing, and UWEs.

Movie S2. Thermogenetically activated *R72D07-GAL4/UAS-dTrpA1* intermale fight: Lateral view of a dTrpA1-activated *R72D07-GAL4* intermale fight: Behavior chamber was modified to facilitate recording. This fight was recorded only for the purpose of demonstration and not used in any calculation. dTrpA1-activated *R72D07-GAL4* intermale fight is characterized by increased lunging, boxing and UWEs.

Movie S3. Thermogenetically activated *R72D07∩293-FLP* intermale fight: dTrpA1-activated *R72D07∩293-FLP* intermale fight is characterized by increased lunging without any observable increase in boxing or UWEs.

Movie S4. Thermogenetic activation of mAL neurons increases intermale lunging: dTrpA1-mediated mAL activation increases intermale lunging without any observable change in boxing or UWEs.

Movie S5. Silencing of mAL neurons decreases intermale aggression but increases UWEs: Kir2.1-mediated mAL silencing decreases intermale lunging but increases intermale UWEs.

Dataset 1: List of full genotypes

Dataset 2: List of *P* values