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Supplemental information

GABAergic signaling shapes multiple aspects of *Drosophila* courtship motor behavior

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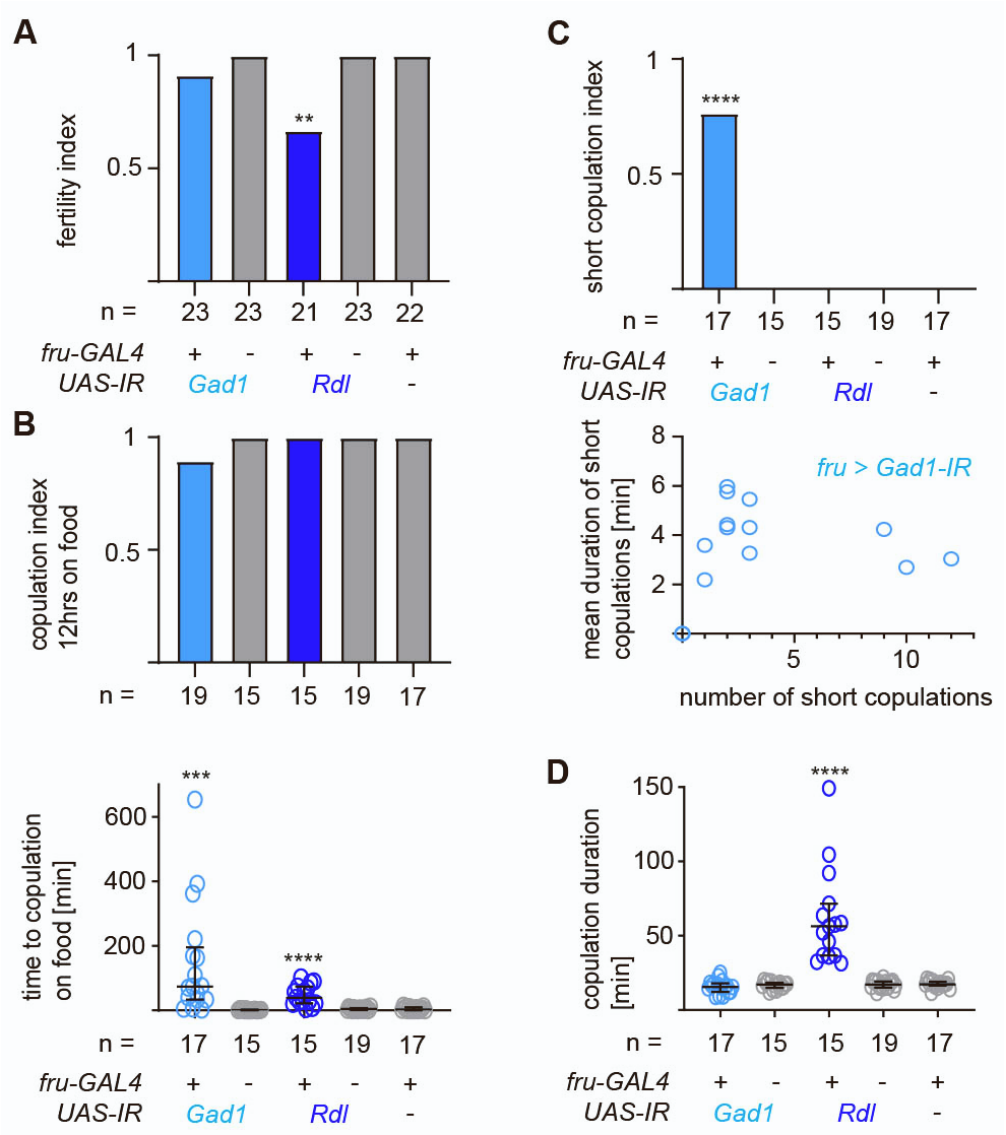

Figure S1. Copulation behavior upon knockdown of GABAergic signaling in *fruitless* neurons in a long-term assay, related to Figure 1.

A Fertility index (fraction of males producing offspring after being paired with 3 virgin flies on food for 3 days) of male flies upon RNAi-mediated knockdown of *Gad1* or *Rdl* in *fruitless* neurons and respective genetic controls. **p ≤ 0.0036, Fisher exact tests. **B** Copulation index in a long-term mating assay on food (fraction of males copulating for longer than 8 min when paired with 3 virgin flies on food for 12 hrs) and time to first copulation in the same assay. ***p ≤ 0.0002, ****p< 0.0001, Kruskal-Wallis test with Dunn's multiple comparison. **C** Short copulation index (fraction of males copulating for shorter than 8 min when paired with 3 virgin flies on food before they achieve their first copulation lasting longer than 8 min) for knockdown and control males. ****p< 0.0001, Fisher exact tests. Below: Number of short copulations and mean duration of short copulations for individual males depleted for Gad1 in *fru+* neurons (each datapoint represents one fly). **D** Copulation duration (only copulations lasting longer than 8 min included) for knockdown and control males, ****p< 0.0001, Kruskal-Wallis test with Dunn's multiple comparison. All experiments are performed with wild type virgin females. n indicates number of flies tested. In all scatterplots, error bars indicate median with interquartile range.

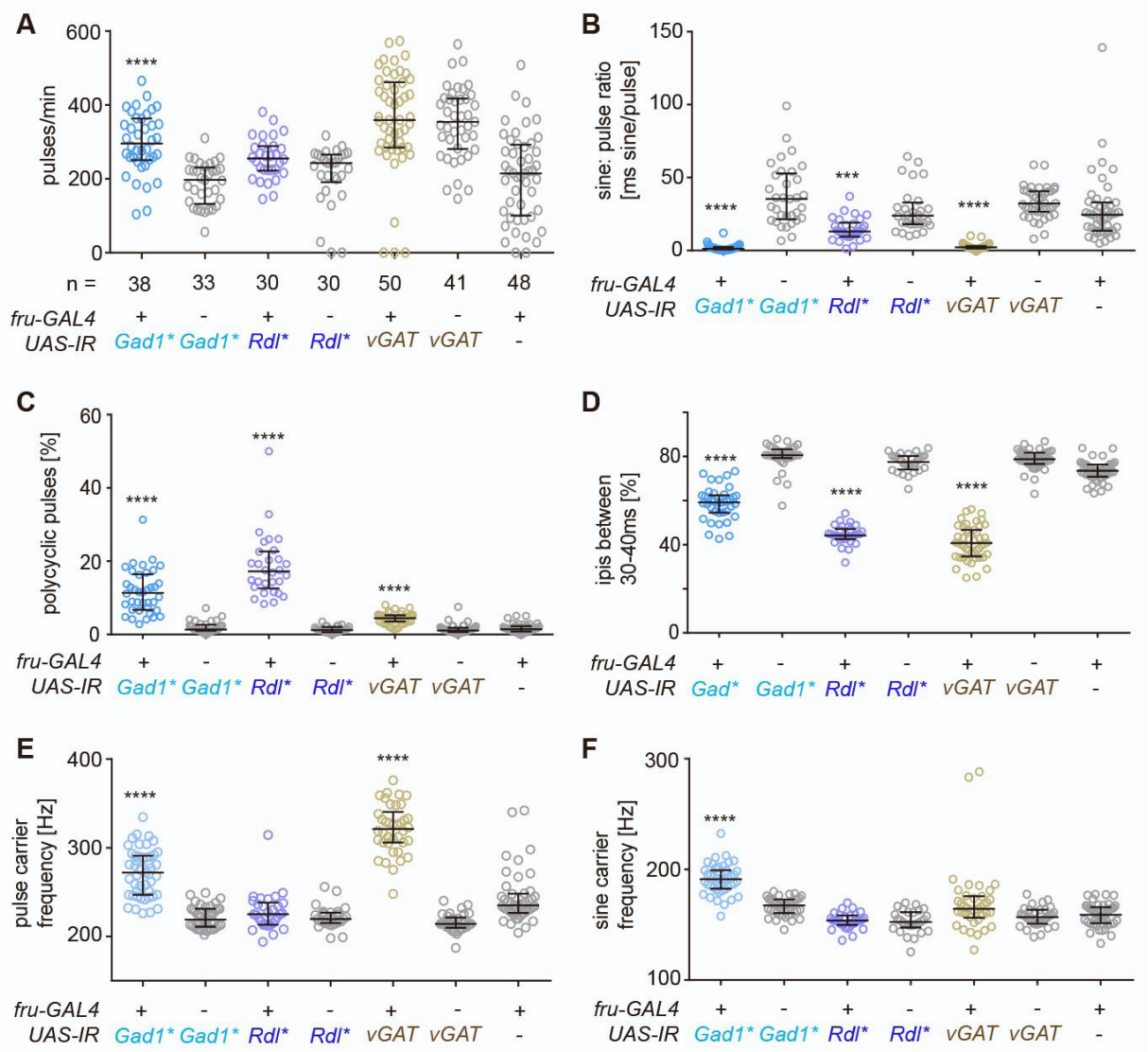

Figure S2. Song patterning defects upon knockdown of GABAergic signaling in *fruitless* neurons with additional RNAi line, related to Figure 2.

A Amount of courtship song (in pulses/min) displayed toward a virgin wild type female of male flies upon RNAi-mediated knockdown of *Gad1*, *Rdl* or *vGAT* with additional *UAS-IR* RNAi lines in *fruitless* neurons and respective genetic controls. n indicates number of flies tested. **B** Amount of sine song (sine: pulse ratio) for knockdown and control males. **C** Amount of polycyclic pulses (% of pulses with more than 2 cycles) in the song of knockdown and control males. **D** Percentage of inter pulse intervals (ipis) within the 30 – 40 ms range in the song of knockdown and control males. **E** Median pulse song carrier frequency for knockdown and control males. **F** Median sine song carrier frequency for knockdown and control males. Each knockdown genotype is compared to its corresponding *UAS- IR* control and the *fru* ctrl, ***p = 0.0007, ****p< 0.0001, Kruskal-Wallis test with Dunn's multiple comparison; n in **B-F** is the same as in **A**. All experiments are performed with wild type virgin females. In all scatterplots, each data point represents one fly, error bars indicate median with interquartile range.

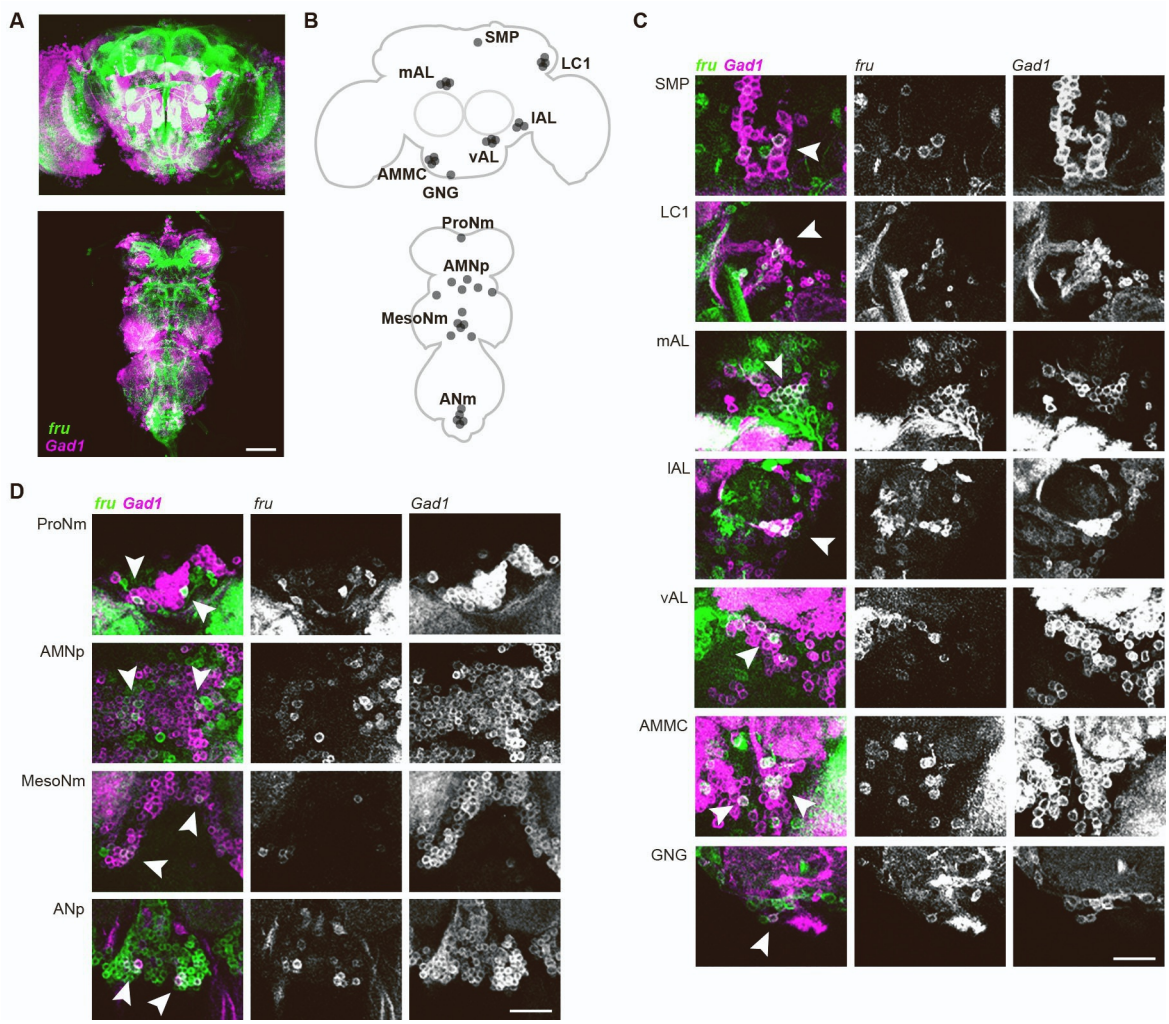

Figure S3. Distribution of *fru*+ GABAergic neurons in brain and VNC, related to Figure 3.
A *fru*+ GABAergic neurons identified by coexpression of *fru-GAL4* (green, labeled with cd8-EGFP) and *Gad1-Lex* transgenes (magenta, labeled with cd8-Tomato) in brain and VNC. **B** Schematic of *fru*+ GABAergic neurons. **C** *fru*+ GABAergic neuronal classes in the brain. **D** *fru*+ GABAergic neuronal classes in the ventral nerve cord. In **C** and **D**, arrowhead point to coexpression. Scale bars: 50 µm. SMP, superior medial protocerebrum; LC1, LC1 neurons; mAL, mAL neurons; lAL, lateral antennal lobe; vAL, ventral antennal lobe; AMMC, antennal mechanosensory and motor center; GNG, gnathal ganglia; ProNm, prothoracic neuromere; ANMp, accessory mesothoracic neuropil; MesoNm, mesothoracic neuromere; ANm, abdominal neuromeres. Brain region nomenclature in accordance with [1], VNC region nomenclature in accordance with [2]. See also Table S1 for cell counts and comments.

Abbreviation	*Region/neuronal class*	*Cell count*	*Notes*
Brain		65 ± 13.4 (cells per hemishere, ± s.d., n = 10)	
SMP	superior medial protocerebrum	0.9 ± 1.3	
LC1	LC1 neurons in lateral horn	18.6 ± 5	involved in pheromone processing [3] and regulation of male aggression versus courtship [4], also named aSP8 [5]
mAL	mAL neurons, dorsomedial to the antennal lobe	21.9 ± 7.4	involved in pheromone processing [6, 7] and regulation of male aggression versus courtship [4], also named aDT2 [5]
lAL	lateral antennal lobe	7.4 ± 3.9	might comprise GABA+ iPN neurons [8] and/or fru+ aDT3 neurons [5]
vAL	ventral antennal lobe	8.8 ± 3.2	might comprise fru+, d5-HT1B+ GABA+ neurons implicated in male aggression [9] and/or fru+ aDT6 neurons [5]
AMMC	antennal mechanosensory and motor center	5.8 ± 4.1	might comprise GABA+ fru+ aLN(al) local auditory neurons implicated in courtship song processing [10]
GNG	gnathal ganglia	1.6 ± 1.2	
VNC		89.6 ± 20.5 (cells per entire VNC, ± s.d., n = 8)	
ProNm	prothoracic neuromere	1.9 ± 1.5	
ANMp	accessory mesothoracic neuropil	35 ± 6.6	
MesoNm	mesothoracic neuromere	25.1 ± 13.7	
ANm	abdominal neuromeres	27.6 ± 20.5	

Table S1. *Gad1+ fru+* neurons in the central nervous system, related to Figure 3 and S3.
Cells coexpressing *fru-GAL4* and *Gad1-LexA* in different brain and VNC regions of male flies. Brain region nomenclature in accordance with [1], VNC region nomenclature in accordance with [2]. Brain neurons were observed in more discrete clusters and counted by hemispheres, VNC neurons had more variable and dispersed positions and were counted per entire tissue. 130 ± 19.8 neurons were counted in the entire brain (n = 5 brains, s.d.). For cell locations and exemplary micrographs, see Figure S3.

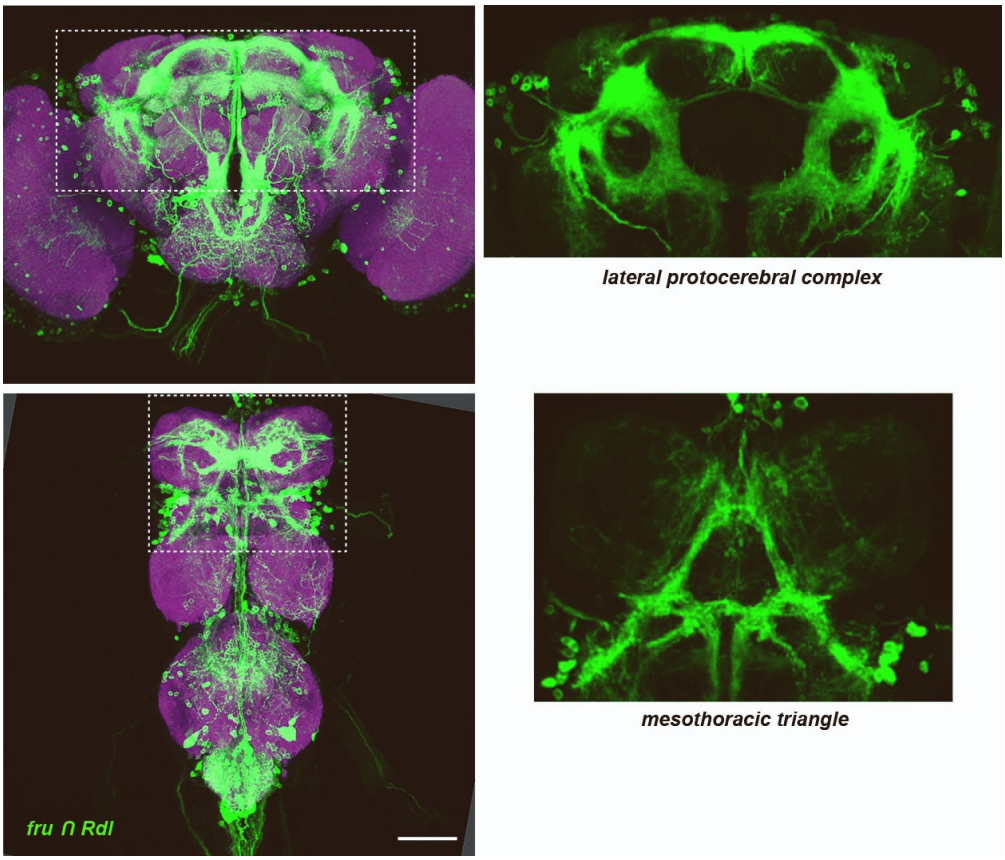

Figure S4. Distribution of *fru*+ *Rdl*+ neurons in brain and VNC, related to Figure 3.
fru+ *Rdl*+ neurons identified by expression of cd8-EGFP (green) in male flies of the genotype *UAS>stop>cd8-EGFP; fru-FLP, Rdl-GAL4*) in brain and VNC. Left side: Maximum intensity projection confocal stacks of full tissues, neuropil anti-bruchpilot staining in magenta, scale bar: 100 µm. Right side: areas indicated with white rectangles in magnification, showing strong expression in the lateral protocerebral complex and the mesothoracic triangle. Maximum intensity projection of subset of z sections corresponding to 21 µm (brain) and 52 µm (VNC).

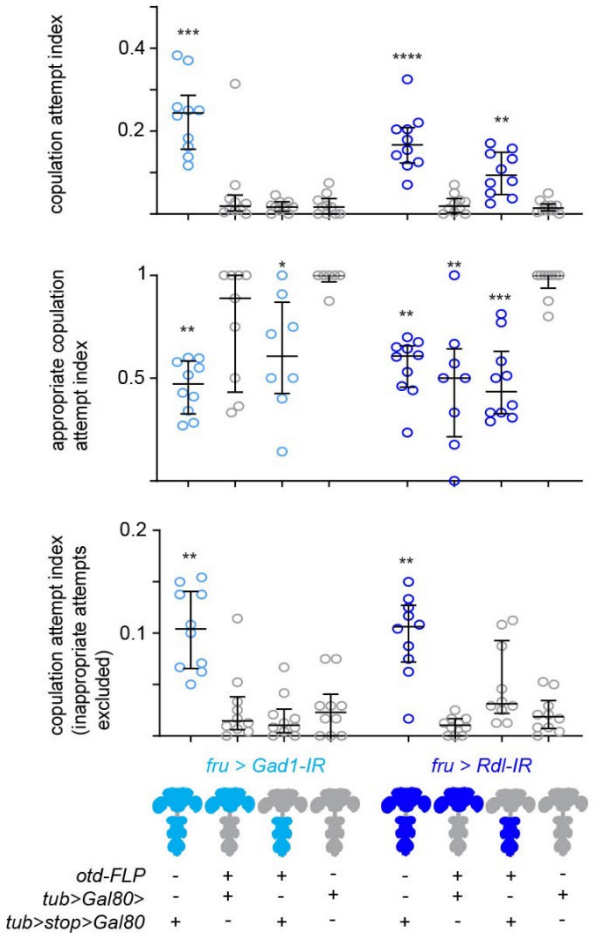

Figure S5. Control of copulation attempts by *fru*+ GABAergic neurons in brain and VNC, related to Figure 3.

Effects on copulation attempts of brain or VNC-specific RNAi-mediated knockdown of *Gad1* or *Rdl* in *fruitless* neurons and respective genetic controls. Copulation attempt frequency is given as Copulation attempt index (fraction of video frames in which the male attempted copulation) and appropriate direction of attempts as Appropriate copulation attempt index (fraction of copulation attempts which were directed at and in reach of the female) for knockdown and control males. In the graph at the bottom, the Copulation attempt index excluding all inappropriate copulation attempts is shown for the same set of experiments. *p<0.05, **p<0.006, ***p<0.003, Kruskal-Wallis test with Dunn's multiple comparison, n = 10 males per genotype, flies that did not attempt copulation were omitted for determination of the appropriate copulation attempt index. In all scatterplots, each data point represents one fly, error bars indicate median with interquartile range.

Figure	Short name	Genotype
1, 2, S1	*fru > Gad1-IR*	*+; P{TRiP.HMC03350}attP40/+; fruitless-GAL4/+*
	Gad1-IR ctrl	*+; P{TRiP.HMC03350}attP40/+; +/+*
	fru > Rdl-IR	*+; P{TRiP.HMC03643}attP40/+ ; fruitless-GAL4/+*
	Rdl-IR ctrl	*+; P{TRiP.HMC03643}attP40/+ ; +/+*
	fru ctrl	*+; +/+ ; fruitless-GAL4/+*
S2	*fru > Gad1-IR**	*w1118, P{UAS-dicer2, w[+]}; P{GD8508}v32344/+; fruitless-GAL4/+*
	*Gad1-IR** ctrl	*w1118, P{UAS-dicer2, w[+]}; P{GD8508}v32344/+; +/+*
	*fru > Rdl-IR**	*w1118, P{UAS-dicer2, w[+]}; P{GD4609}v41103/+; fruitless-GAL4/+*
	*Rdl-IR** ctrl	*w1118, P{UAS-dicer2, w[+]}; P{GD4609}v41103/+; +/+*
	fru > vGAT-IR	*w1118, P{UAS-dicer2, w[+]}; P{KK101500}VIE-260B /+; fruitless-GAL4/+*
	vGAT-IR ctrl	*w1118, P{UAS-dicer2, w[+]}; P{KK101500}VIE-260B /+; +/+*
S3	*fru, Gad1*	*w1118; UAS-mCD8-Gfp/LexAop-mCD8-tomato; Gad1-LexA/fruitless-GAL4*
S4	*fru ∩ Rdl*	*w1118; UAS-FRT-stop-FRT -mCD8-Egfp/+; Rdl-GAL4/fruitless-FLP*
3A	*fru > cd8-EGFP tub>stop>Gal80*	*w1118; UAS-mCD8-Gfp/+; tubP-FRT-stop-FRT-GAL80/fruitless-GAL4*
	fru > cd8-EGFP otd-FLP, tub>Gal80>	*w1118; Otd-nlsFLPo.attp40/UAS-mCD8-Gfp; tubP-FRT-GAL80-FRT/fruitless-GAL4*
	fru > cd8-EGFP otd-FLP, tub>stop>Gal80	*w1118; Otd-nlsFLPo.attp40/UAS-mCD8-Gfp; tubP-FRT-stop-FRT-GAL80/fruitless-GAL4*
	fru > cd8-EGFP tub>Gal80>	*w1118; UAS-mCD8-Egfp/+; tubP-FRT-GAL80-FRT/fruitless-GAL4*
3B-F, S5	*fru > Gad1-IR tub>stop>Gal80* (brain, vnc)	*w1118; P{TRiP.HMC03350}attP40/+; tubP-FRT-stop-FRT-GAL80/fruitless-GAL4*
	fru > Gad1-IR otd-FLP, tub>Gal80> (brain)	*w1118; Otd-nlsFLPo.attp40/P{TRiP.HMC03350}attP40; tubP-FRT-GAL80-FRT/fruitless-GAL4*
	fru > Gad1-IR otd-FLP, tub>stop>Gal80 (vnc)	*w1118; Otd-nlsFLPo.attp40/P{TRiP.HMC03350}attP40; tubP-FRT-stop-FRT-GAL80/fruitless-GAL4*
	fru > Gad1-IR tub>Gal80> (control)	*w1118; P{TRiP.HMC03350}attP40/+; tubP-FRT-GAL80-FRT/fruitless-GAL4*
	fru > Rdl-IR tub>stop>Gal80 (brain, vnc)	*w1118; P{TRiP.HMC03643}attP40/+; tubP-FRT-stop-FRT-GAL80/fruitless-GAL4*
	fru > Rdl-IR otd-FLP, tub>Gal80> (brain)	*w1118; Otd-nlsFLPo.attp40/P{TRiP.HMC03643}attP40; tubP-FRT-GAL80-FRT/fruitless-GAL4*
	fru > Rdl-IR otd-FLP, tub>stop>Gal80 (vnc)	*w1118; Otd-nlsFLPo.attp40/P{TRiP.HMC03643}attP40; tubP-FRT-stop-FRT-GAL80/fruitless-GAL4*
	fru > Rdl-IR tub>Gal80> (control)	*w1118; P{TRiP.HMC03643}attP40/+; tubP-FRT-GAL80-FRT/fruitless-GAL4*

Table S2. Genotypes of experimental flies, related to STAR Methods.

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