Supporting Information

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SI Materials and Methods

Six-Choice Oviposition Assay. Six-choice oviposition assays were performed like two-choice oviposition assays with the exception of food preparation. Food substrate was made by mixing the appropriate volume of ethanol or water into molten fly food cooled to 50 °C. Food was poured into 35-mm Petri dishes and then separated into six equal sections. Sections of different concentrations of ethanol were fitted next to each other into 35-mm Petri dishes, and assay was performed as per two-choice oviposition assay. Oviposition index was calculated as number of eggs laid on × percentage of food/number of total eggs.

Two-Choice Position Assay. Food substrate was made, and flies were collected and treated as described in two-choice oviposition assay above. For position analysis, the two-choice plate was placed in the center of a 60-mm Petri dish. Flies were gently added and the clear plastic 60-mm Petri dish was placed over the top. Flies were filmed using infrared light in the dark at 25 °C for 3 h using a Sony NightShot 0 Lux Digital Handicam. The position of the flies and eggs was recorded every minute and later grouped into 10-min intervals.

Immunohistochemistry. Four-day-old adult female brains were dissected in a PBS solution and fixed for 45 min at room temperature with 4% (vol/vol) formaldehyde. Tissue was left overnight at 4 °C with 1:500 rabbit anti-tyrosine hydroxylase (TH), 1:200 goat rabbit anti-GFP (Invitrogen Molecular Probes), 1:200 mouse anti-CD8 (Invitrogen Molecular Probes), or 1:50 goat anti-mouse nc82 (The Jackson Laboratory), washed four times, and left overnight in 1:500 Alexa-Fluor 594 goat anti-mouse (Invitrogen Molecular Probes) or 1:200 Alexa-Fluor 488 goat anti-rabbit (Invitrogen Molecular Probes). Brains were mounted in Fluoromount-G (Southern Biotech). Samples were imaged using a Zeiss LSM 710 (Janelia Farm Research Campus Imaging Center). Images were acquired using a 20× objective and scanned at a resolution of 1024 × 1024 pixels. Adobe Photoshop CS and Illustrator were used to tile images.

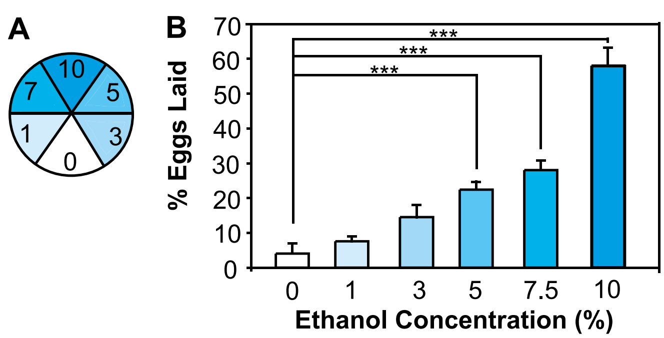

Fig. S1. Oviposition preference for higher concentrations of ethanol in a six-choice test. (*A*) Flies were given a choice between six different ethanol concentrations ranging from 1% to 10% in a "pie" orientation. (*B*) There was a dose-dependent preference for ethanol when flies were given a choice between six different ethanol concentrations with the highest number of eggs being laid on 10% ethanol and lowest number of eggs on 0% ethanol [n = 27 per group; ANOVA: $F_{(5,161)} = 50.47$, $P < 0.0001$; Tukey's post hoc: 0% vs. 1%, $P = 0.94$; 0% vs. 3%, $P = 0.08$; 0% vs. 5%, 7.5%, or 10%, $P < 0.0001$].

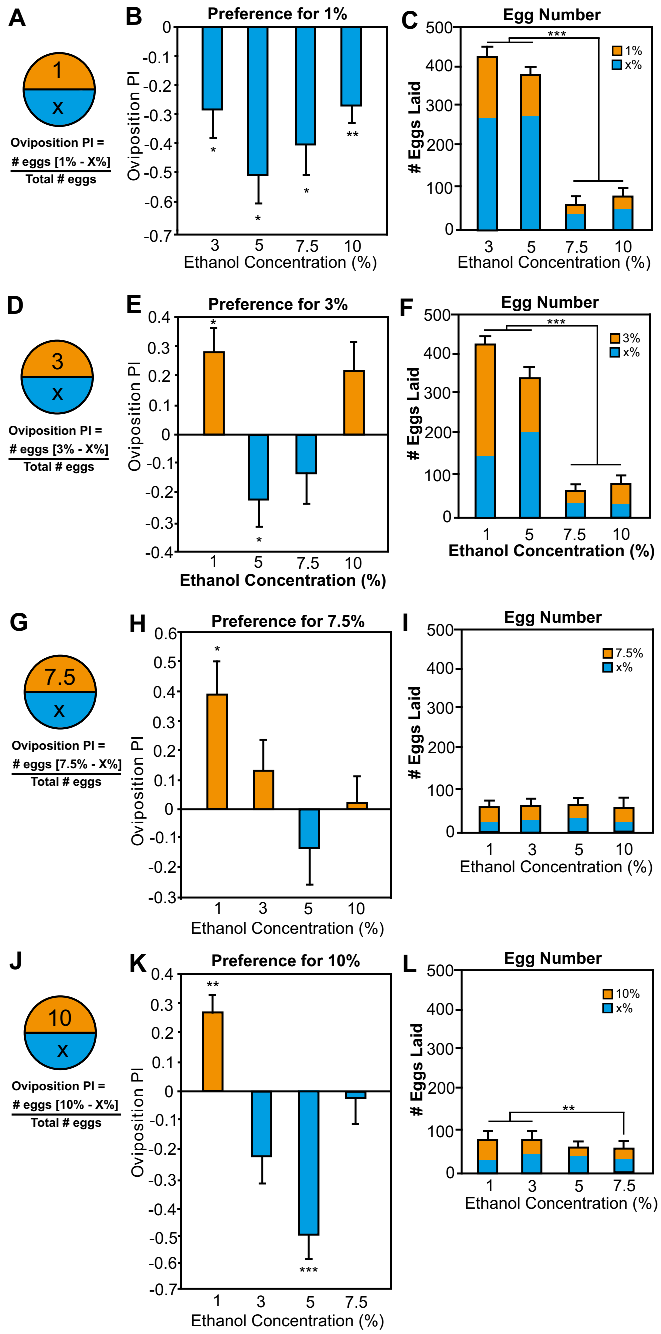

Fig. S2. Flies prefer to lay eggs on concentrations of 5% ethanol in a forced choice between concentrations. (*A*, *D*, *G*, and *J*) Oviposition preference index (PI) was calculated by subtracting the number of eggs on the stated (X) concentration of food from the number of eggs on 1% (*A*), 3% (*D*), 7.5% (*G*), and 10% (*J*) ethanol-food respectively and dividing by the total number of eggs. (*B*) Flies preferred to lay their eggs on 3%, 5%, 7.5%, or 10% ethanol food compared with 1% ethanol food [Wilcoxon PI against zero: 3%, $\chi^2_{(1,8)} = 6.05$, $P = 0.01$; 5%, $\chi^2_{(1,8)} = 6.05$, $P = 0.01$; 7.5%, $\chi^2_{(1,12)} = 4.93$, $P = 0.03$; 10%, $\chi^2_{(1,28)} = 7.56$, $P = 0.006$]. (*C*) The number of eggs laid on ethanol concentrations under 5% was significantly greater than the number of eggs laid on ethanol concentrations over 5% [ANOVA: $F_{(3,35)} = 277.86$, $P < 0.0001$; Tukey's post hoc comparisons: 1%:3% vs. 1%:7.5%, $P < 0.0001$; 1%:3% vs. 1%:10%, $P < 0.0001$; 1%:5% vs. 1%:7.5%, $P < 0.0001$; 1%:5% vs. 1%:10%, $P < 0.0001$]. (*E*) Flies laid significantly more eggs on 3% ethanol food compared with 1% ethanol food but not compared with 5%, 7.5%, or 10% ethanol food [Wilcoxon PI against zero: 1%, $\chi^2_{(1,8)} = 6.05$, $P = 0.01$; 5%, $\chi^2_{(1,8)} = 6.05$, $P = 0.01$; 7.5%, $\chi^2_{(1,26)} = 3.17$, $P = 0.07$; 10%, $\chi^2_{(1,20)} = 2.61$, $P = 0.11$]. (*F*) The number of eggs laid on ethanol concentrations under 5% was significantly greater than the number of eggs laid on ethanol concentrations over 5% [ANOVA: $F_{(3,27)} = 219.83$, $P < 0.0001$; Tukey's post hoc comparisons: 1%:3% vs. 3%:7.5%, $P < 0.0001$; 1%:3% vs. 3%:10%, $P < 0.0001$; 3%:5% vs. 3%:7.5%, $P < 0.0001$; 3%:5% vs. 3%:10%, $P < 0.0001$]. (*H*) Flies laid significantly more eggs on 7.5% ethanol food compared with 1% ethanol food but not 3%, 5%, or 10% ethanol food [Wilcoxon PI against zero: 1%, $\chi^2_{(1,12)} = 4.93$, $P = 0.03$; 3%, $\chi^2_{(1,26)} = 3.17$, $P = 0.07$; 5%, $\chi^2_{(1,24)} = 0.55$, $P = 0.46$; 10%, $\chi^2_{(1,23)} = 0.16$, $P = 0.69$]. (*I*) There were no significant differences in the number of eggs laid on any of the plates containing 7.5% ethanol [ANOVA: $F_{(3,45)} = 0.83$, $P = 0.49$]. (*K*) Flies laid significantly more eggs on 10% ethanol food compared with 1% ethanol food but not 3% or 10% ethanol food [Wilcoxon PI against zero: 1%, $\chi^2_{(1,28)} = 7.56$, $P = 0.006$; 3%, $\chi^2_{(1,20)} = 2.61$, $P = 0.11$; 10%, $\chi^2_{(1,23)} = 0.16$, $P = 0.69$]. Flies laid significantly less eggs on 10% ethanol food compared with 5% ethanol food [$\chi^2_{(1,22)} = 18.67$, $P < 0.0001$]. (*L*) Flies laid significantly less eggs on food containing 7.5% ethanol [ANOVA: $F_{(3,41)} = 9.01$, $P < 0.0001$; Tukey's post hoc comparisons: 1%:10% vs. 7.5%:10%, $P = 0.0002$; 3%:10% vs. 7.5%:10%, $P = 0.0009$]. Bars on graphs represent means ± SEM. $^*P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

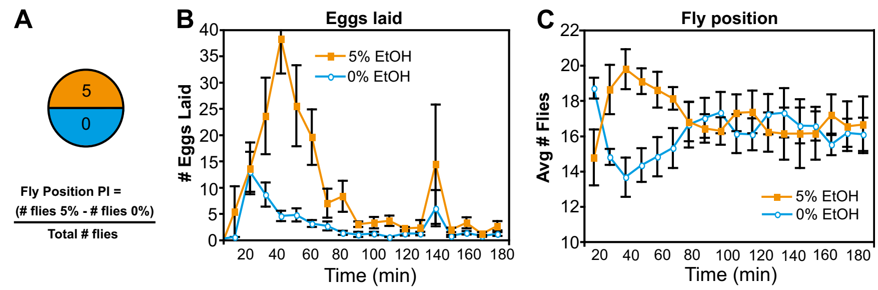

Fig. S3. Flies show ovipositional but not positional preference for ethanol. (*A*) To determine whether flies were averse to staying on ethanol, we measured the position index of flies between 0% and 5% ethanol for 3 h. Position index was measured by subtracting the number of flies on 0% ethanol from the number of flies on 5% ethanol and dividing this by the total number of flies. (*B*) Flies lay most of their eggs in the first hour of exposure to food containing a choice of 0% and 5% ethanol. (*C*) Flies prefer to reside on ethanol-containing food only during egg-laying (Wilcoxon Position PI at 30 min vs. zero; $n = 6$; $P = 0.002$). After flies have completed laying their eggs, there is no preference for position on food containing 0% or 5% ethanol (Wilcoxon Position PI average of 60–180 min vs. zero; $n = 6$; $P = 0.31$). Points on graphs represent means ± SEM.

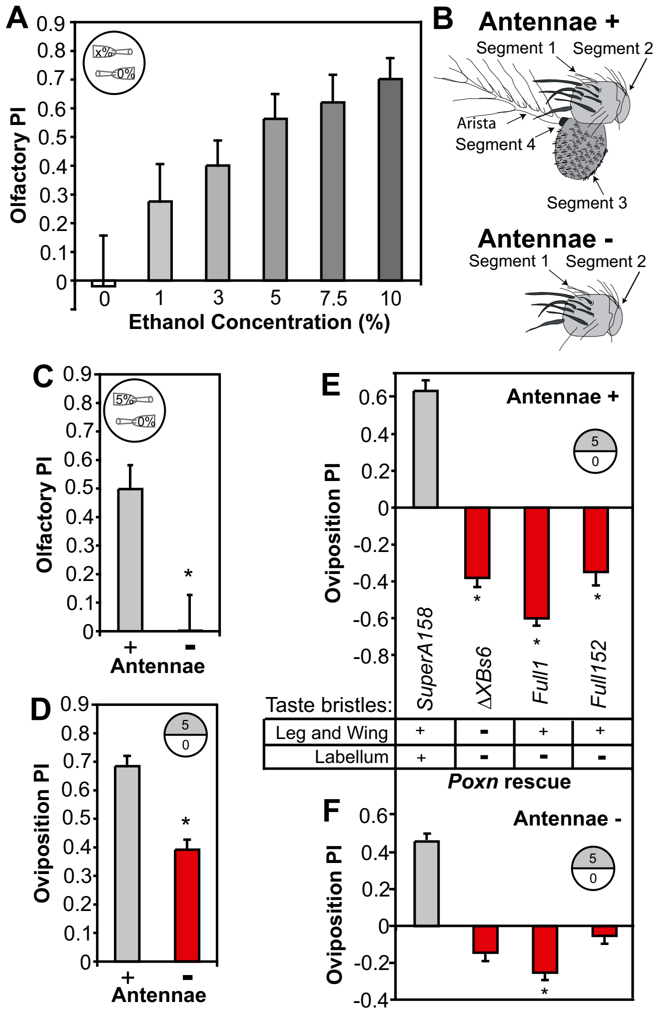

Fig. S4. Smell and taste mediate oviposition preference for ethanol. (*A*) Flies showed a dose-dependent preference for ethanol concentrations up to 10% in an olfactory trap assay. (*B–D*) Removing the third antennal segment abolished the olfactory preference for ethanol [$n = 23$ per group; *t* test, $t_{(1,46)} = 10.94$, $P = 0.002$] (*B* and *C*) but only moderately decreased oviposition preference for 5% ethanol [$n = 16$ per group; *t* test, $t_{(1,32)} = 14.28$, $P = 0.0006$] (*D*). (*E*) *Poxn*-deficiency mutants, in which most chemosensory bristles are replaced by mechanosensory bristles effectively eliminating the ability to taste, show aversion to lay eggs on 5% ethanol (1) [$n = 17–20$ per strain; ANOVA: $F_{(3,70)} = 35.70$, $P < 0.0001$]. Restoration of chemoreceptors in leg bristles (*Full1*, *Full152*) did not affect this response, suggesting the labellum chemoreceptors are required for oviposition preference for ethanol. (*F*) Removing antennae did not completely eliminate oviposition aversion in *Poxn*^{Full1} mutants, suggesting that other sensory modalities such as proprioception may also play a role in oviposition preference for ethanol (Wilcoxon PI vs. zero, $n = 17$, $P = 0.0002$).

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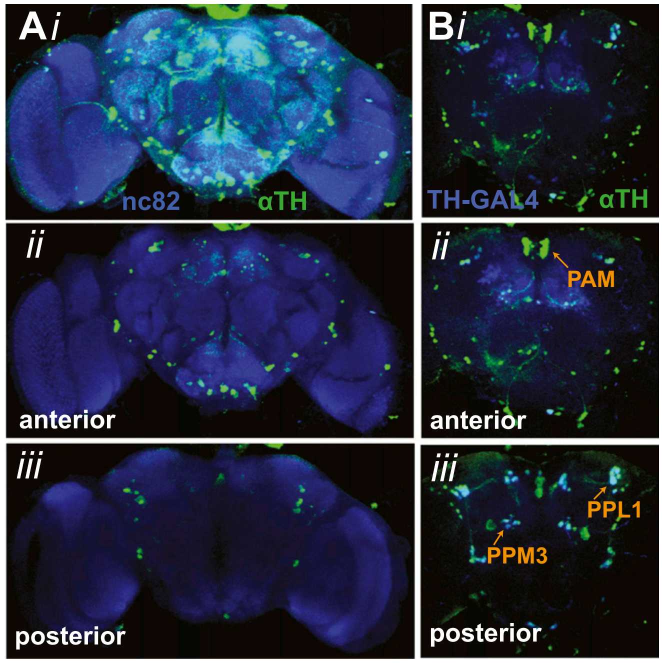

Fig. S5. TH and *TH-GAL4* expression in the adult brain. (*A*) Expression of TH in the adult brain (αTH, green; nc82, blue). TH is expressed in ∼282 neurons organized into 13 clusters of neurons in the protocerebrum (1, 2). (*A*, *i*–*A*, *iii*) Confocal stacks of the entire brain (*A*, *i*), anterior part of the brain (*A*, *ii*), and posterior part of the brain (*A*, *iii*). (*B*) Comparison between *TH-GAL4* and αTH staining in the central brain (αTH, green; αGFP representing *TH-GAL4* expression, blue). There are ∼127 *TH-GAL4*–expressing neurons that can be categorized into 13 different clusters in the brain (1). Five of these clusters show complete expression overlap between *TH-GAL4* and αTH, including the PPL1 and PPM3 neurons (1). Notably, fewer PAM cells are expressed in the *TH-GAL4* pattern compared with the αTH pattern (*B*, *i*). (*B*, *i*–*B*, *iii*) Confocal stacks of the entire central brain (*B*, *i*), anterior part of the brain (*B*, *ii*), and posterior part of the brain (*B*, *iii*). Clusters of dopaminergic neurons are named based on their location in the brain: PAM, protocerebral anterior median; PPL, protocerebral posterior lateral; PPM, protocerebral posterior median.

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2. Nässel DR, Elekes K (1992) Aminergic neurons in the brain of blowflies and *Drosophila*: Dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons. *Cell Tissue Res* 267(1):147–167.

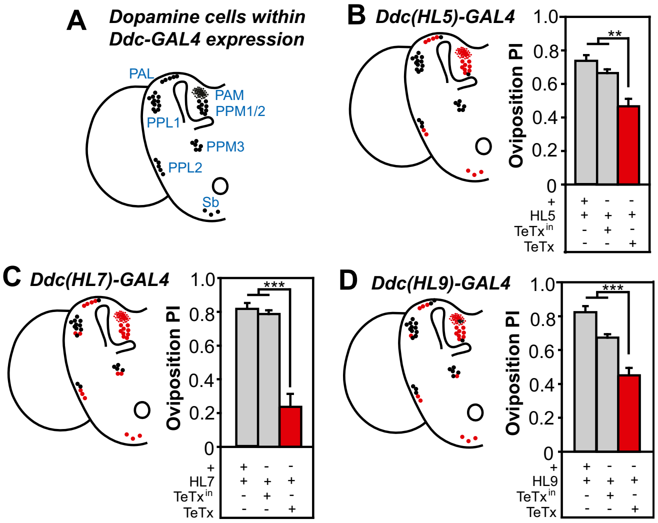

Fig. S6. Dopaminergic neurons affect oviposition preference. (*A*) Schematic showing dopamine-expressing cell bodies within the *Ddc-GAL4* expression pattern. Red neurons in schematics *B–D* represent neurons expressed within each respective GAL4 driver. Black neurons in schematics *B–D* represent other neurons within the *Ddc-GAL4* expression pattern that are not expressed. Nondopaminergic expression is omitted in the schematics. (*B*) We found that disrupting neurotransmission in PAM, PAL, PPM1/2, PPL2, and Sb neurons (1, 2) decreased oviposition preference for ethanol [n = 14–19 per strain; ANOVA: $F_{(2,49)} = 19.39$, $P < 0.0001$; Tukey's post hoc: +/*HL5* vs. *TeTx/HL5*, $P < 0.0001$; *TeTx^in/HL5* vs. *TeTx/HL5*, $P = 0.0002$]. (*C* and *D*) Disrupting neurotransmission in PAM neurons, and subsets of PAL, PPM1/2, PPM3, PPL2, PPL1, and Sb neurons (1–3), decreased oviposition preference for ethanol [*HL7:* n = 15–21 per strain; ANOVA: $F_{(2,48)} = 40.81$, $P < 0.0001$; Tukey's post hoc: +/*HL7* vs. *TeTx/HL7*, $P < 0.0001$; *TeTx^in/HL7* vs. *TeTx/HL7*, $P < 0.0001$; *HL9*: n = 15–19 per strain; ANOVA: $F_{(2,50)} = 33.57$, $P < 0.0001$; Tukey's post hoc: +/*HL9* vs. *TeTx/HL9*, $P < 0.0001$; *TeTx^in/HL9* vs. *TeTx/HL9*, $P < 0.0001$]. Bars on graphs represent means ± SEM. *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$. Clusters of dopaminergic neurons are named based on their location in the brain: PAL, protocerebral anterior lateral; Sb, subesophageal ganglion.

1. Li H, Chaney S, Roberts IJ, Forte M, Hirsh J (2000) Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in *Drosophila melanogaster*. *Curr Biol* 10 (4):211–214.
2. Kong EC, et al. (2010) A pair of dopamine neurons target the D1-like dopamine receptor DopR in the central complex to promote ethanol-stimulated locomotion in *Drosophila*. *PLoS ONE* 5(4):e9954.
3. Claridge-Chang A, et al. (2009) Writing memories with light-addressable reinforcement circuitry. *Cell* 139(2):405–415.

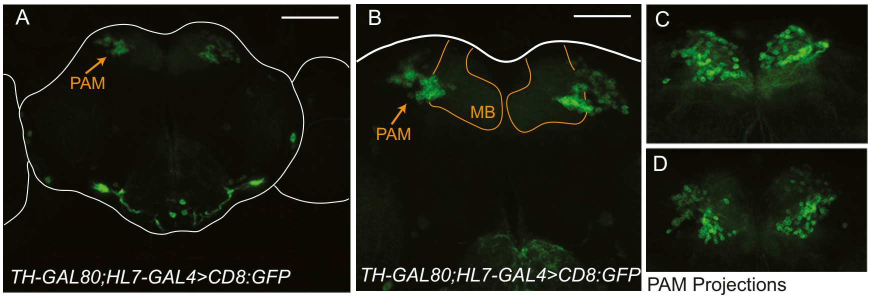

Fig. S7. Expression of PAM and PPL1 GAL4 lines. (*A* and *B*) Expression of GFP (using *UAS-CD8:GFP*) in HL7-expressing neurons that do not also express TH (*HL7-GAL4;TH-GAL80*) (MB, mushroom body; green, CD8:GFP). (*B*) Higher-magnification view of a section of *A*. (*C* and *D*) Projections from the PAM neurons leading to the horizontal lobes of the MB (flies of genotype *TH-GAL80;HL7-GAL4* > *CD8:GFP*; green, anti-GFP).

Table S1. Expression of Dopaminergic GAL4 lines

		Dopaminergic neurons						
GAL4 lines	Ovi pref	PPL1	PPL2	PPM1/2	PPM3	PAM	PAL	Sb
Ddc	Decrease	+	+	+	+	+	+	+
TH	Increase	+	+	+	+	—	+	+
Ddc (HL5)	Decrease	—	+	+	—	+	+	+
Ddc (HL7)	Decrease	+	+	+	+	+	+	+
Ddc (HL9)	Decrease	+	+	+	+	+	+	+
Ddc(HL7);THGAL80	Decrease	—	—	—	—	+	—	—
NP2758	Increase	+	—	—	—	—	—	—
kra;MBGAL80	Increase	+	—	—	—	—	—	—
C346; MBGAL80	Decrease	—	—	—	+	—	—	—

Ovi pref, oviposition preference. Dashes indicate lack of expression.

Table S2. Number of eggs laid during oviposition preference experiments

GAL4 lines	*+/GAL4*	*UAS-TeTx^{in} /GAL4*	*UAS-TeTx /GAL4*
Ddc	289 ± 26	85 ± 20	30 ± 3
TH	260 ± 27	153 ± 28	174 ± 23
TRH	215 ± 14	273 ± 19	68 ± 16
Ddc (HL5)	213 ± 19	167 ± 37	69 ± 17
Ddc (HL7)	307 ± 24	69 ± 6	26 ± 4
Ddc (HL9)	226 ± 23	191 ± 9	49 ± 6
Ddc(HL7);THGAL80	297 ± 12	141 ± 27*	119 ± 16
NP2758	309 ± 26	92 ± 8	65 ± 5
kra;MBGAL80	192 ± 10	127 ± 18	37 ± 6
C346	269 ± 23	151 ± 15	69 ± 9
MB247	151 ± 22	54 ± 8	55 ± 13
NP65	190 ± 19	169 ± 25	44 ± 7
4–59	171 ± 27	83 ± 11	57 ± 12
2–72	234 ± 12	109 ± 23	31 ± 6
11–27	216 ± 21	68 ± 3	46 ± 5
4–67	182 ± 33	99 ± 9	67 ± 7
C232	196 ± 26	125 ± 21	27 ± 5

Numbers represent means ± SEM. Statistical comparison defined by an ANOVA between *+/GAL4*, *UAS-TeTx^{in}/GAL4*, and *UAS-TeTx/GAL4* is represented by black ($P > 0.05$) and red ($P < 0.05$).
*Tested line: +/TH-GAL80, UAS-TeTx.

Table S3. Tukey's post hoc statistical comparisons

Figure	Comparison	*P*
Fig. 2*B*	*+/Ddc* vs. *TeTx/Ddc*	<0.0001
	$TeTx^{in}$*/Ddc* vs. *TeTx/Ddc*	<0.0001
	+/TeTx vs. *TeTx/Ddc*	<0.0001
Fig. 2*C*	*+/TH* vs. *TeTx/TH*	0.0001
	$TeTx^{in}$*/TH* vs. *TeTx/TH*	0.0007
	+/TeTx vs. *TeTx/TH*	0.09
Fig. 2*D*	*+/TRH* vs. *TeTx/TRH*	0.97
	$TeTx^{in}$*/TRH* vs. *TeTx/TRH*	0.96
	+/TeTx vs. *TeTx/TRH*	0.06
Fig. 3*A*	*+/HL7;THGAL80* vs. *TeTx/HL7*	0.001
	$TeTx^{in}$*/HL7;THGAL80* vs. *TeTx/HL7;THGAL80*	0.007
Fig. 3*B*	*+/c346* vs. *TeTx/c346*	<0.0001
	$TeTx^{in}$*/c346* vs. *TeTx/c346*	<0.0001
	+/TeTx;THGAL80 vs. *c346;MBGAL80/TeTx;THGAL80*	0.06
	+/TeTx;THGAL80 vs. *c346;MBG80'TeTx*	0.01
Fig. 3*C*	*+/NP2758* vs. *TeTx/NP2758*	0.01
	$TeTx^{in}$*/NP2758* vs. *TeTx/NP2758*	0.06
	+/TeTx;THG80 vs. *NP2758/TeTx;THG80*	0.78
Fig. 3*D*	*+/kra-MBGAL80* vs. *TeTx/kra-MBGAL80*	0.06
	$TeTx^{in}$*/kra-MBGAL80* vs. *TeTx/kra-MBGAL80*	0.03
	+/TeTx vs. *kra;MBGAL80/TeTx;THG80*	0.59
Fig. 3*E*	*+/NP2758* vs. *UAS-dTrpA1/NP2758*	0.0004
Fig. 3*F*	*+/kra-MBGAL80* vs. *UAS-dTrpA1/kra-MBGAL80*	<0.0001
Fig. 4*B*	*+/NP65* vs. *TeTx/NP65*	0.04
	$TeTx^{in}$*/NP65* vs. *TeTx/NP65*	0.002
	+/4–59 vs. *TeTx/4–59*	0.01
	$TeTx^{in}$*/4–59* vs. *TeTx/4–59* $P = 0.02$	0.02
Fig. 4*D*	*+/2–72* vs. *TeTx/2–72*	<0.0001
	$TeTx^{in}$*/2–72* vs. *TeTx/2–72* $P = 0.0008$	0.0008
	+/11–27 vs. *TeTx/11–27*	<0.0001
	$TeTx^{in}$*/11–27* vs. *TeTx/11–27*	<0.0001
	+/4–67a vs. *TeTx/4–67a*	0.004
	$TeTx^{in}$*/4–67a* vs. *TeTx/4–67a*	0.05
Fig. 4*F*	*2–72/DopR2-IR*	<0.0001
	2–72/DopR1-IR	0.0002