Supplementary Materials

Supplementary Figures

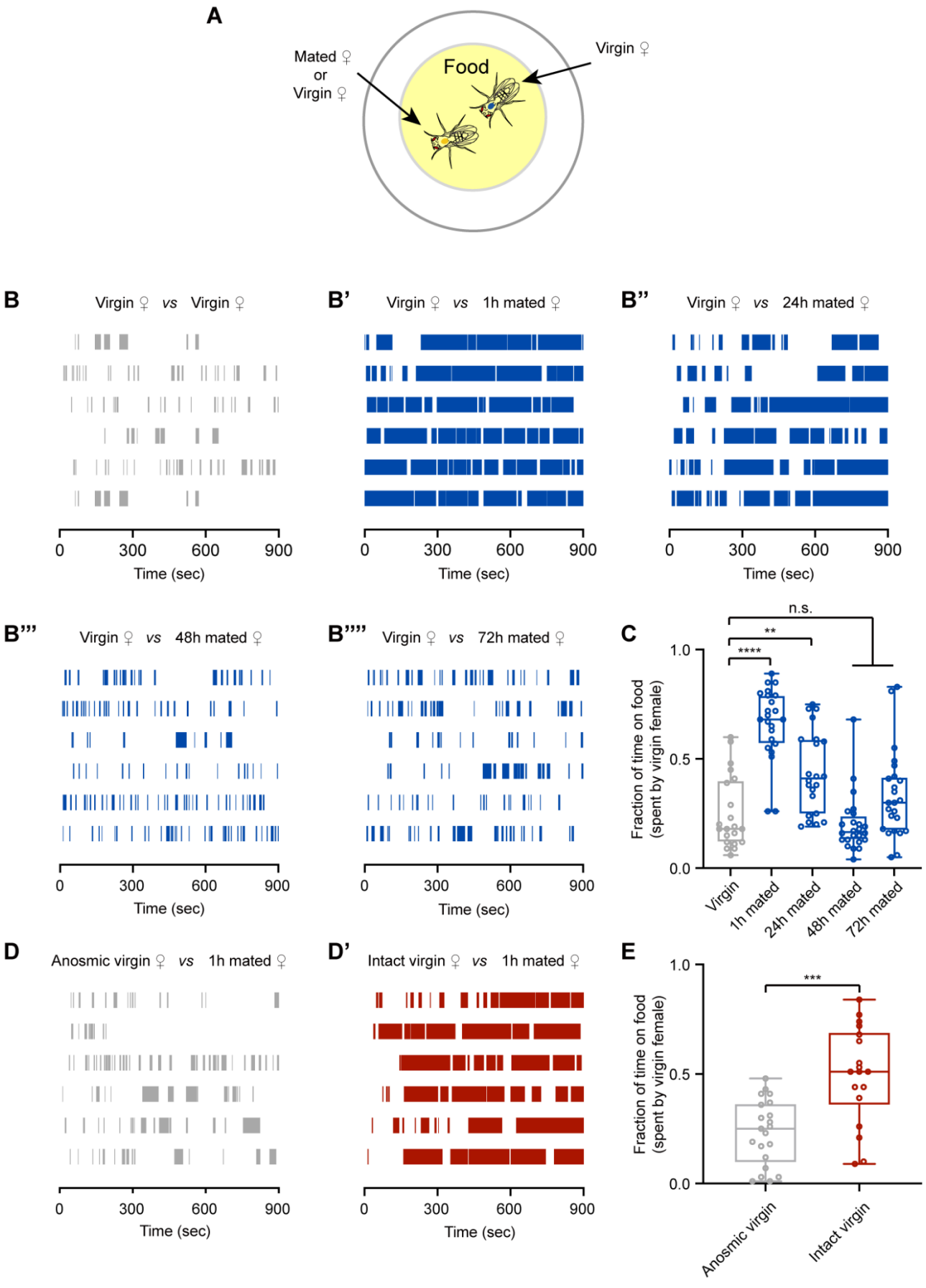

Fig. S1 Virgin females spend more time on food when paired with mated females than with virgin females. **A** Experimental schematic. A virgin female is paired with another virgin female or a mated female. **B–B’’’’** Simultaneous monitoring of representative individual virgin females on food plates for 15 min when they are paired with virgin, 1 h mated, 24 h mated, 48 h mated, or 72 h mated females (*n* = 6). **C** Fraction of time each virgin female spent on the food plate. Competitors of virgin females are indicated under the plot (*n* = 21-23). **D, D’** Simultaneous monitoring of representative individual intact or anosmic virgin females on the food plate for 15 min when they were paired with 1 h mated females (*n* = 6). **E** Fraction of time each virgin female spent on the food plate. Competitors of 1 h mated females are indicated under the plot (*n* = 18-21). ***P* <0.01, ****P* <0.001, *****P* <0.0001, otherwise no significant difference (Kruskal-Wallis with Dunn’s multiple-comparison *post hoc* test for **C**; Mann-Whitney U test for **E**)**.** Error bars, ± SEM.

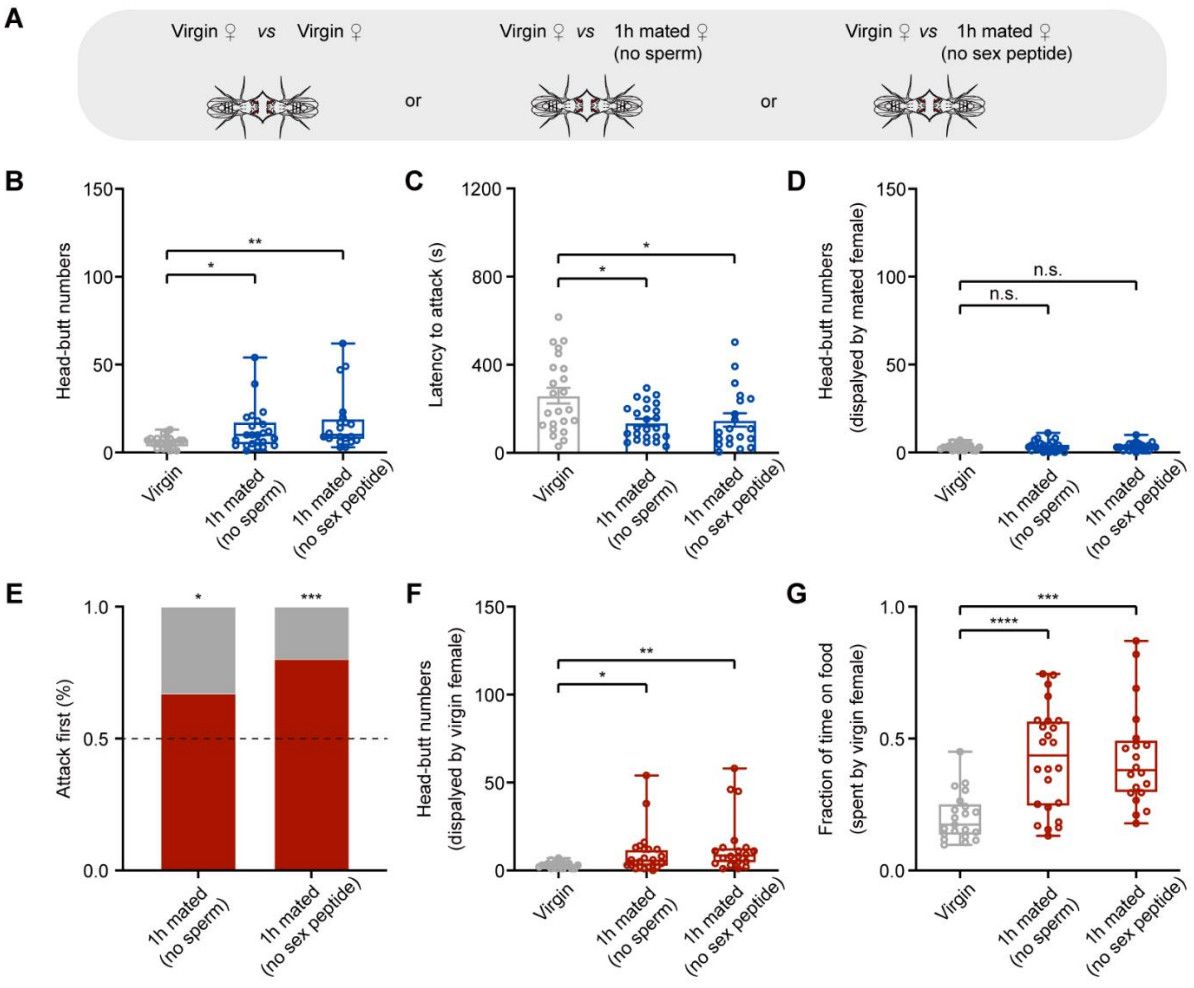

Fig. S2 Sex peptide and sperm do not influence virgin female aggression. **A** Experimental schematic. A virgin female is paired with another virgin female or a mated female without sperm or sex peptide. **B, C** Head-butt numbers and latency by a pair of female flies. Competitors of virgin females are indicated under the plot. **D** Head-butt numbers by 1 h mated females. **E** Percentages of initiating attack by virgin females (gray bars) and mated females (red bars). **F** Head-butt numbers by virgin females. **G** Fraction of time each virgin female spent on the food plate (*n* = 20-24). Competitors of virgin females are indicated under the plot. **P* <0.05, ***P* <0.01, *****P* <0.0001, otherwise no significant difference (Kruskal-Wallis with Dunn's multiple-comparison post hoc test for **B**, **C**, **D**, **F,** and **G**; χ^2 test for **E**)**.** Error bars, ± SEM.

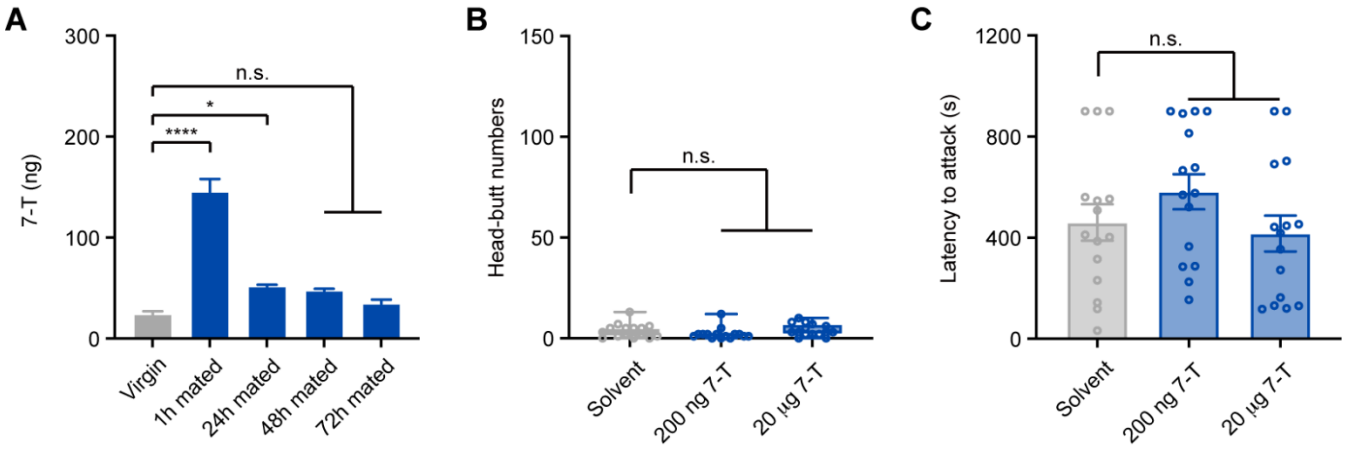

Fig. S3 7-T does not affect virgin female aggression**. A** Quantification of 7-T in extracts of virgin and mated single flies (*n* = 6). **B, C** Head-butt numbers and latency by a pair of virgin female flies by application of 200 ng and 20 µg 7-T, or solvent (*n* = 15). **P* <0.05, *****P* <0.0001, otherwise no significant difference (one-way ANOVA with Tukey's post hoc test for **A**; Kruskal-Wallis with Dunn's multiple-comparison *post hoc* test for **B**, **C**)**.** Error bars, ± SEM.

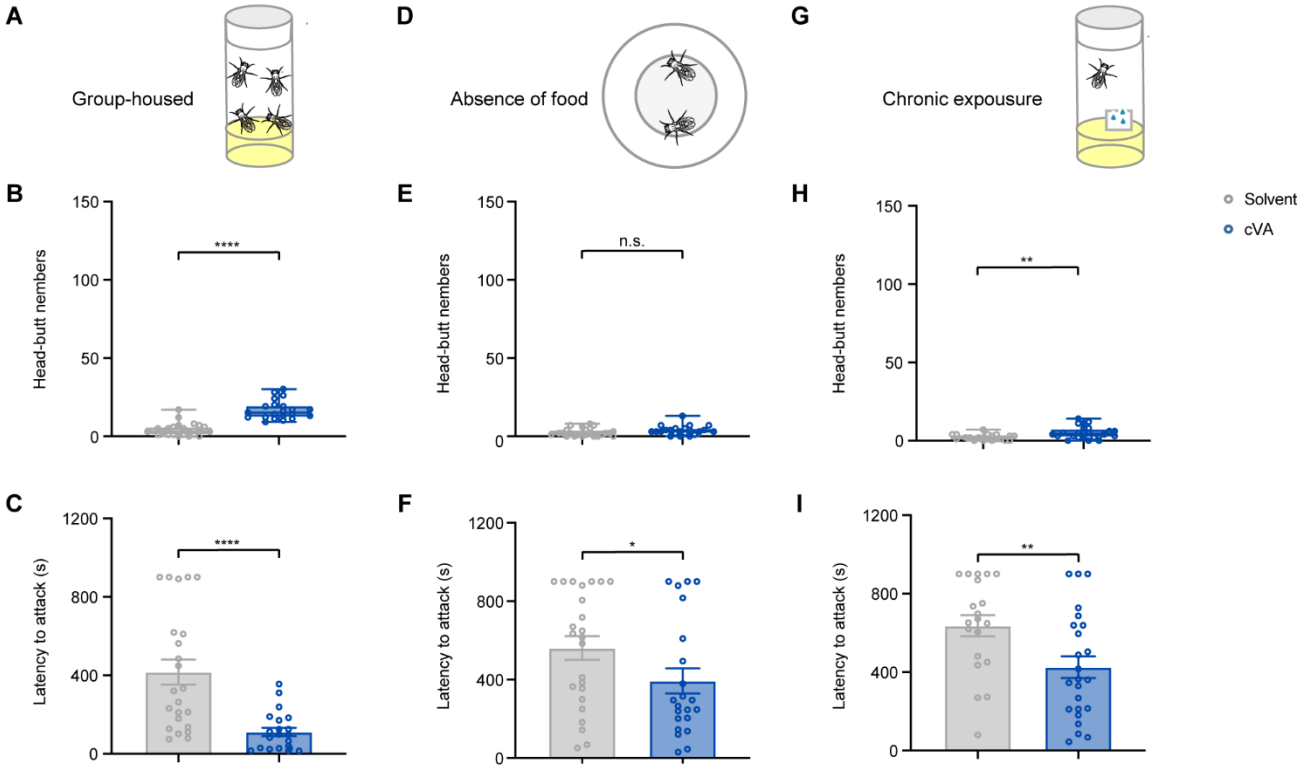

Fig. S4 Other contextual influences on cVA-induced virgin female aggression. **A** Schematic of group-housed treatment. **B, C** Head-butt numbers and latency by two group-housed virgin females by application of 50 μg cVA or solvent (*n* = 21-23). **D** Schematic of aggressive behavior in which two virgin females are paired together on an agarose plate. **E, F** Head-butt numbers and latency by two virgin females on an agarose plate by application of 50 μg cVA or solvent (*n* = 22-24). **G** Schematic of experimental treatment. Virgin females are chronically exposed to solvent or cVA for 24 h. **H, I** Head-butt numbers and latency by virgin females after pre-exposure to solvent or cVA for 24 h (*n* = 20-24). **P* <0.05, ***P* <0.01, *****P* <0.0001, otherwise no significant difference (Mann-Whitney U test for **B**, **C**, **E**, **F**, **H,** and **I**). Error bars, ± SEM.

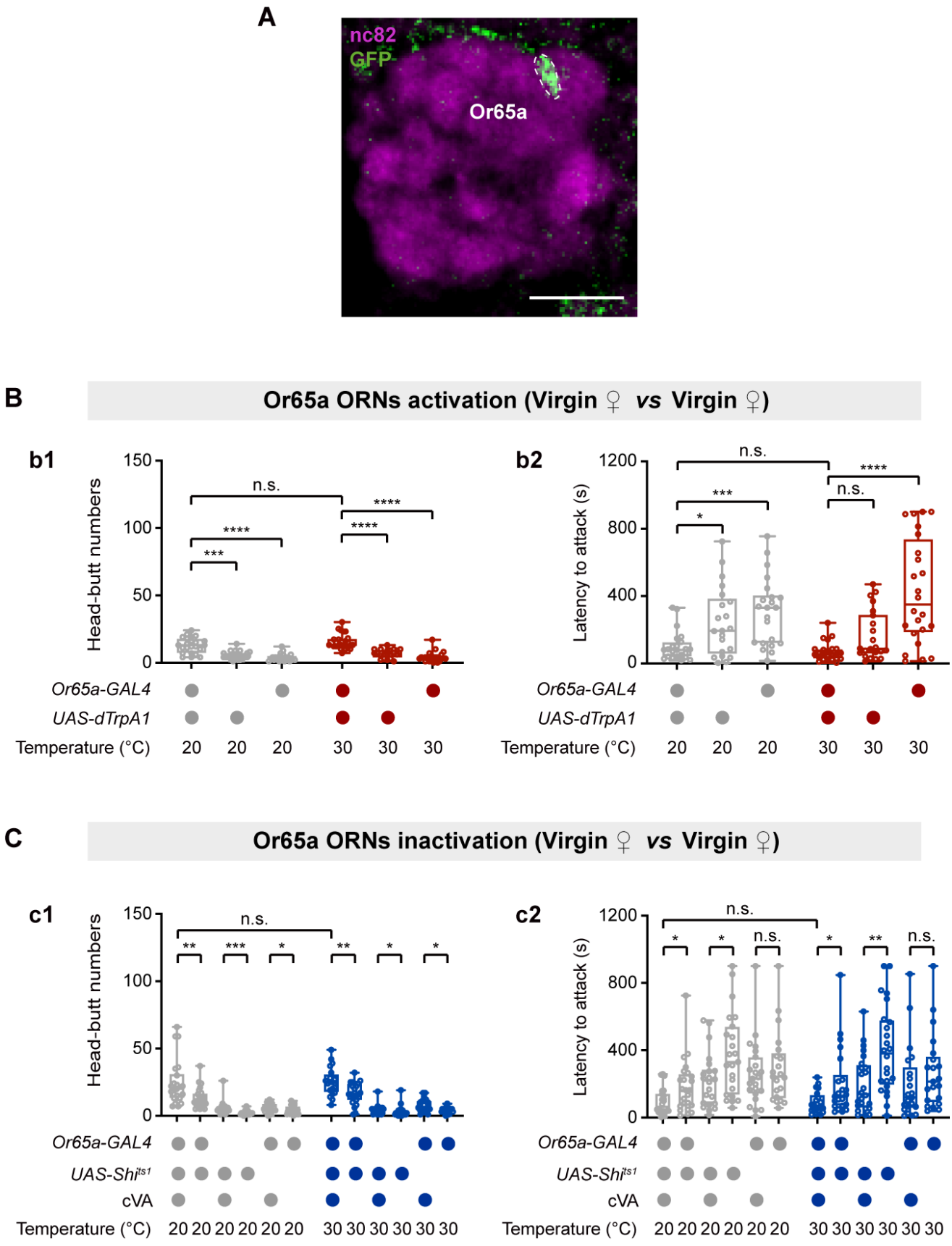

Fig. S5 Or65a ORNs do not regulate cVA-induced virgin female aggression. **A** Confocal image of the antennal lobe. *Or65a-GAL4* labeled Or65a ORNs in the female brain (green: DL3 glomerulus); nc82, neuropil marker (magenta). Scale bar, 20 μm. **B** Head-butt numbers (**b1**) and latency (**b2**) by virgin

females during dTrpA1-mediated thermogenetic activation of Or65a ORNs (n = 21-24). **C** Head-butt numbers (**c1**) and latency (**c2**) by virgin females during *Shibire*^{ts1}-mediated inactivation of Or65a ORNs by application of 50 μg cVA or solvent (n = 20-24). *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001, otherwise no significant difference (Kruskal-Wallis with Dunn's multiple-comparison post hoc test for **B, C**). Error bars, ± SEM.

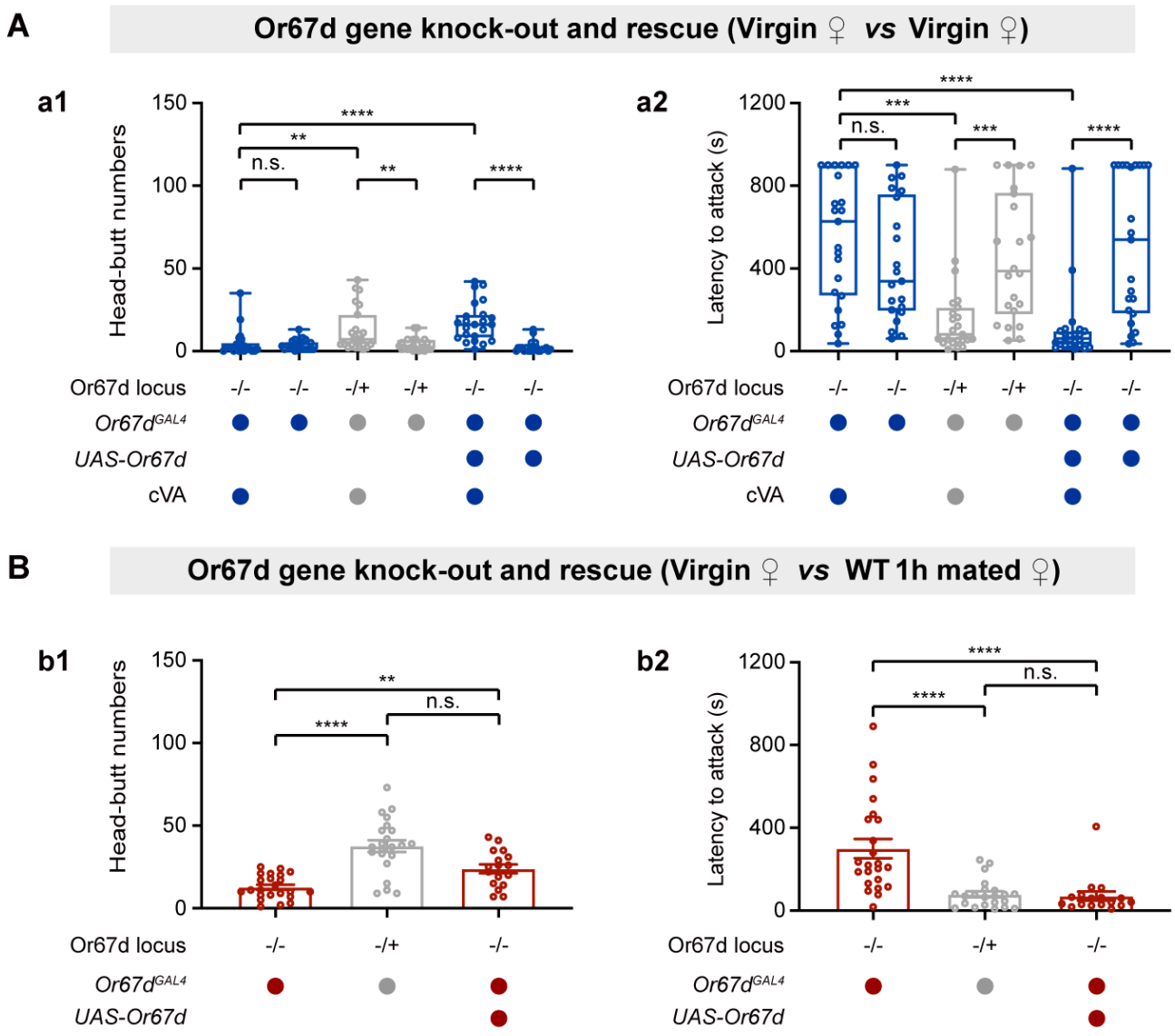

Fig. S6 The *Or67d* gene is required for cVA-induced virgin female aggression. **A** cVA-induced virgin female aggression is suppressed in *Or67d* mutants and is rescued by the expression of *UAS-Or67d* under the control of *Or67d-GAL4* in *Or67d* mutants. (**a1**) Head-butt numbers; (**a2**) latency (n = 21-

23). **B** Head-butt numbers (**b1**) and latency (**b2**) by virgin females toward wild-type 1 h mated females (n = 17-23). Genotypes of virgin females are indicated below the plot. *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001, otherwise no significant difference (Kruskal-Wallis with Dunn's multiple-comparison post hoc test for **A, B**). Error bars, ± SEM.

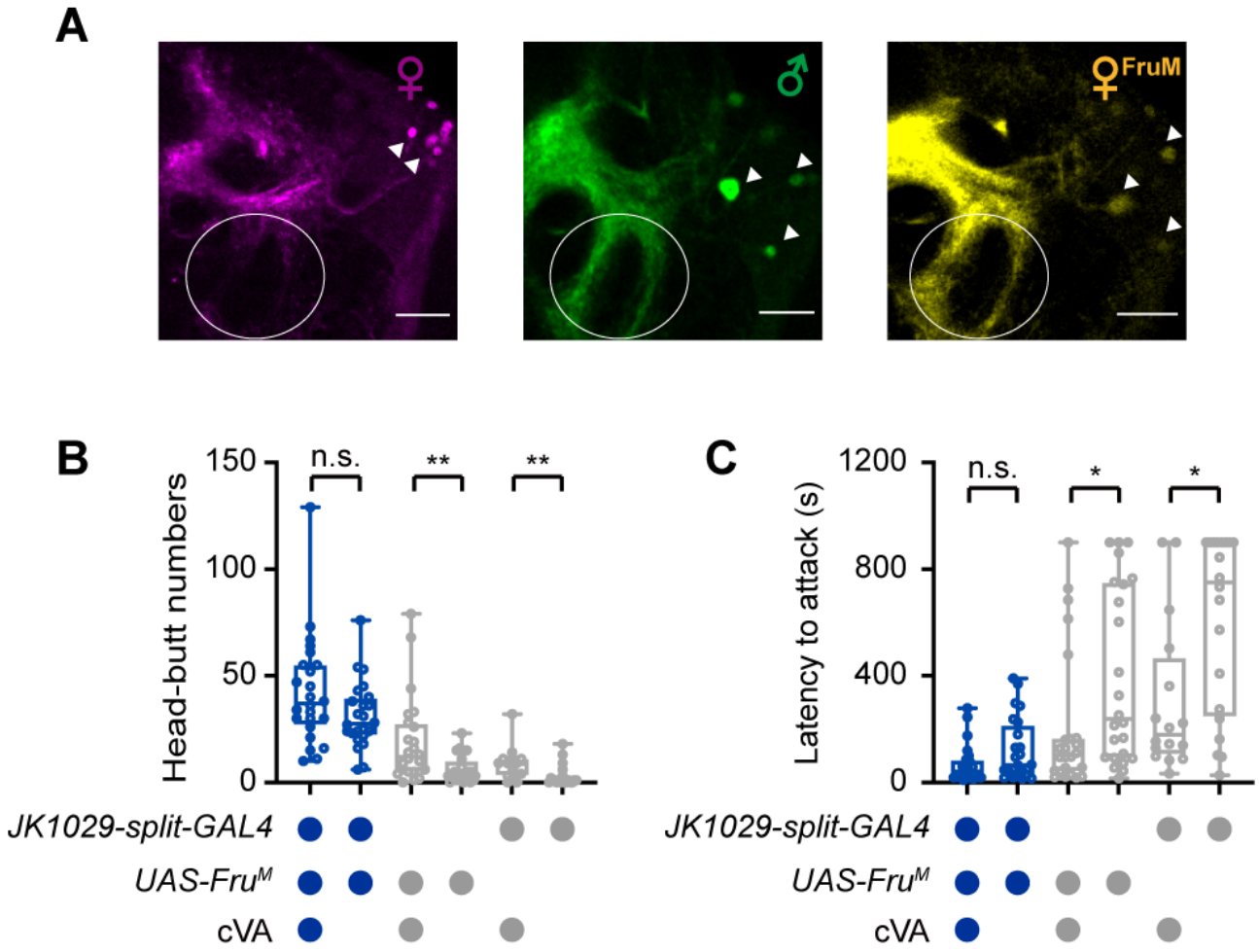

Fig. S7 Expression of Fru^M in aSP-g neurons suppresses cVA-induced virgin female aggression**.**

A Neurites and cell bodies in the lateral horn region of aSP-g neurons in a female (left), a male (middle), and a Fru^M mutant female (right). White circles indicate neurites; white arrowheads indicate cell bodies.

B, C Head-butt numbers and latency by virgin females with masculinized aSP-g neurons by application of 50 μg cVA or solvent (n = 16-24). *P <0.05, **P <0.01, otherwise no significant difference (Mann-Whitney U test for **B, C**). Error bars, ± SEM.

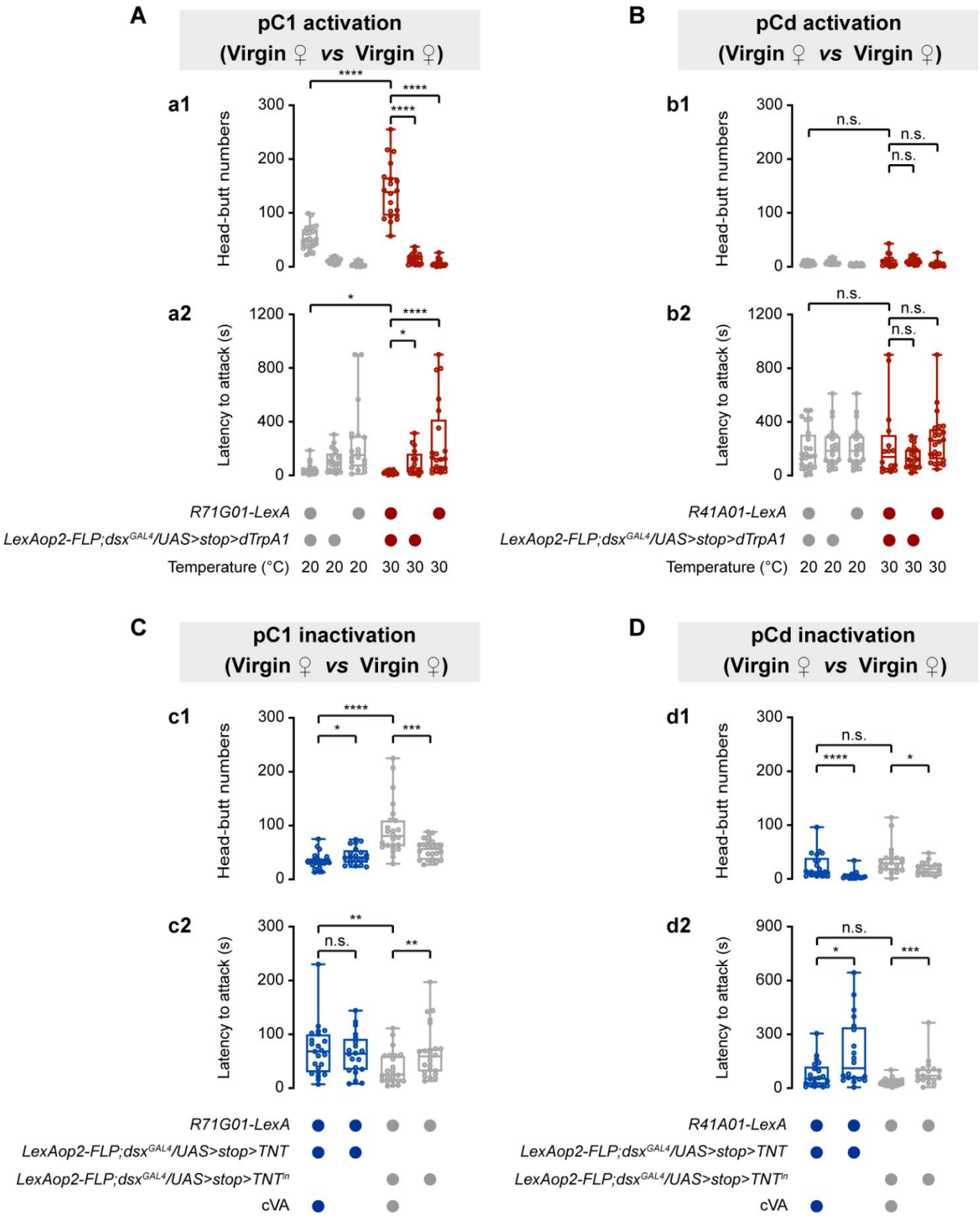

Fig. S8 pC1 neurons, rather than pCd neurons, regulate virgin female aggression. **A** Activation of pC1 neurons labeled by the genetic intersection of *71G01-LexA/LexAop2-FLP; dsx^{GAL4}/UAS > stop > dTrpA1* increases virgin female aggression. (**a1**) Head-butt numbers; (**a2**) latency (n = 17-24). **B** Activation of pCd neurons labeled by the genetic intersection of *41A01-LexA/LexAop2-FLP;*

dsx^{GAL4}*/UAS > stop > dTrpA1* does not increase virgin female aggression. (**b1**) Head-butt numbers; (**b2**) latency (*n* = 16-24). **C** Expressing TNT in pC1 neurons inhibits cVA-induced virgin female aggression. (**c1**) Head-butt numbers; (**c2**) latency (*n* = 20-24). **D** Expression of TNT in pCd neurons does not influence cVA-induced virgin female aggression. (**d1**) Head-butt numbers; (**d2**) latency (*n* = 18-22). **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001, otherwise no significant difference (Kruskal-Wallis with Dunn's multiple-comparison post hoc test for **A-D).** Error bars, ± SEM.

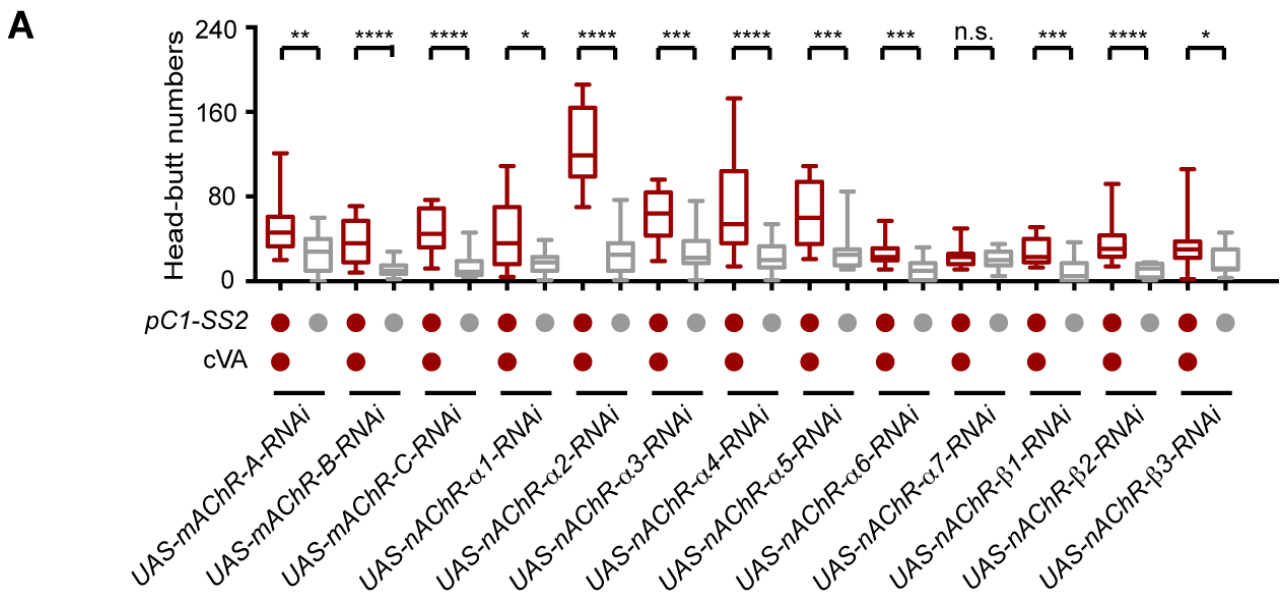

Fig. S9 Screening mAChRs and nAChRs in pC1 neurons. **A** Head-butt numbers by virgin female flies with RNAi-mediated nAChR or mAChR knockdown in the presence of 50 μg cVA or solvent (*n* = 15-21). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, otherwise no significant difference (Mann-Whitney U test for **A).** Error bars, ± SEM.

Supplementary Tables

Table S1 Quantification of cuticular hydrocarbons in virgin and 1–72 h mated females

	Mean ± SEM (ng)						
Compound name	Virgin (6)	1 h mated (6)	24 h mated (6)	48 h mated (6)	72 h mated (6)	*F*	*P* value
cis-Vaccenyl Acetate (cVA)	0	511.3 ± 35.29 (A)	68.82 ± 4.842 (B)	41.77 ± 2.358 (B)	19.31 ± 3.044 (B)	181.2	<0.0001
7-Tricosene	24.16± 2.953 (A)	145.5 ± 12.6 (B)	51.63 ± 1.79 (C)	47.7 ± 1.651 (AC)	34.6 ± 4.138 (AC)	62	<0.0001
n-Tricosene	143.6 ± 15.68	173.2 ± 15.91	162.3 ± 12.94	175.4 ± 7.812	185.2 ± 15.43	1.303	0.2959
n-Tetracosane	35.44 ± 2.639 (A)	32.68 ±5.458 (AB)	49.26 ± 2.017 (AC)	59.59 ±2.395 (CD)	67.96 ±3.778 (D)	18.92	<0.0001
9-Pentacosene	61.36 ± 9.025	60.39 ± 7.412	54.76 ± 2.894	66.69 ± 4.168	66.99 ± 6.375	0.6306	0.6452
7-Pentacosene	46.53 ± 5.532	62.86 ± 6.800	47.4 ± 2.053	52.3 ± 2.197	57.02 ± 6.364	1.849	0.151

n-Pentacosene	147.8 ± 12.7 (A)	150 ± 12.61 (A)	165.5+10.24 (AB)	164 ± 9.327 (AB)	203.3 ± 14.46 (B)	3.446	0.0225
7,11-Heptacosadiene	488 ± 48.83 (A)	499.4 ± 47.17 (A)	682.1 ± 31.68 (AB)	782 ± 52.99 (B)	866.3 ± 102.1 (B)	7.508	0.0004
2-methyl-Hexacosane	91.18 ± 6.541	80.36 ± 8.907	85.05 ± 16.48	96.4 ± 9.396	94.24 ± 10.45	0.3726	0.8259
7,11-Nonacosadiene	128.1 ± 12.21	111.3 ± 7.263	122.7 ± 26.53	91 ± 11.28	99.02 ± 12.67	1.018	0.4172

Females were either virgin, 1 h mated, 24 h mated, 48 h mated, or 72 h mated. Only compounds detected in different mating statuses are listed in this table. If a specific compound was not detectable in a group (indicated by "0") the mating status was not included in the statistical analysis. Compound annotation with colors indicate: blue, produced by males and transferred to females during copulation; yellow, dienes made specifically by females. One-way ANOVA and Tukey's *post-hoc* pairwise comparison values are shown. Within compounds, groups with significantly different means are indicated with different letters. The number of replicates is indicated between brackets next to each group.

Table S2 Synaptic connections identified by EM reconstruction

	post (Cell ID)				
pre (Cell ID)	pC1a_R (5813046951)	pC1b_R (267214250)	pC1c_R (267551639)	pC1d_R (5813063587)	pC1e_R (514850616)
aSP-g1_R (485775679)	0	1	0	0	0
aSP-g1_R (610916994)	0	0	0	0	0
aSP-g1_R (485430434)	0	0	0	0	0
aSP-g1_R (887148641)	0	0	0	0	0
aSP-g1_R (949534412)	0	0	0	0	0
aSP-g2_R (421992069)	0	11	0	0	0
aSP-g2_R (641278400)	0	1	0	0	0
aSP-g2_R (855414220)	0	4	0	0	0

aSP-g2_R (5813115796)	0	1	0	0	0
aSP-g3A_R (329919036)	0	0	0	0	0
aSP-g3B_R (485430336)	0	0	0	0	0
aSP-g3B_R (421650982)	0	1	0	0	0

The number of synaptic connections identified between aSP-g neurons and pC1 neurons. X_R indicates right-hemisphere X cells (X indicates aSP-g cells or pC1 cells).