Figure S1, related to Figure 1. Copulation advantage of older males requires Or47b odorant receptor: comparison between wild-type and *Or47b*^{2} mutant.

(A) Courtship competition assay. Two naïve male flies and one virgin female were loaded into a chamber (bottom). (B-C) Copulation distribution, ratio and percentage for the competition between 7-day and 2-day old wild-type males (B) or between 7-day and 2-day old *Or47b*^{2} mutant males (C). 7-day old wild-type males, but not *Or47b*^{2} mutant males, had a copulation advantage over 2-day old males. The identity of the copulating males and the time when copulation first occurred in the 2-hr observation period were recorded. Results are shown in 20-min bins. The copulation distribution plot denotes the copulation events for a given time bin of a given group divided by the total copulation events. (D) Average copulation percentage of 7-day old males pitted against 2-day old males. The copulation advantage of older males is significant in wild-type flies but not in the *Or47b*^{2} mutants. Each data point indicates the result from an experiment, which consisted of 17-40 matches. Lines connect results from parallel experiments. Red bars indicate average copulation percentage from 6 experiments. Statistical significance was determined by paired *t*-test. (E) Copulation distribution, ratio and percentage for the competition between 7-day old wild-type and *Or47b*^{2} mutant males. (F) Same as (E), experiments conducted with 2-day old males. The copulation advantage of wild-type males over *Or47b*^{2} mutant males was observed in older but not in young males. (G) Average copulation percentage of wild-type males competing against *Or47b*^{2} mutants. The copulation advantage of wild-type males is significant in 7-day old males but not in 2-day ones. Each experiment consisted of 18-25 matches. Statistical significance of copulation percentage was determined by *Chi*-square test. Statistical significance of the copulation distribution between the two competing male genotypes or age groups for a given 20-min time bin was determined by *t*-test. Error bars indicate s.e.m. *, $p<0.05$; **, $p<0.01$.

Figure S2, related to Figure 2. Sizes of experimental flies.

(A) The size of experimental flies was approximated by the lengths of thorax and wing, respectively. (B) Comparison of the lengths of thorax and wing between 2-day and 7-day old wild-type males. There is no significant size difference between the two age groups (n= 20).

Figure S3, related to Figure 3. Quantification of cuticular fatty acids.

(A) Female cuticular extract was separated into 8 fractions (F1~F8) by thin layer chromatography. “X” indicates a gap between F6 and F7 fractions, collected as a negative control. Three types of synthetic standards were loaded as markers: FAs (fatty acids), FAMEs (fatty acid methyl esters) and glycerol trolein. (B) Two-photon calcium imaging at the axon terminals of Or47b ORNs expressing *GCaMP3*. Pseudocolored images show representative fraction-evoked responses (left). Odor stimulus: 2 sec. Fraction 3 (F3), corresponding to fatty acids, elicited the strongest calcium responses. Right: Mean $\Delta F/F$ is plotted against time, with line width indicating s.e.m. (n=6). (C) Quantity of cuticular fatty acids extracted from 15 female (red bar) or male (blue bar) flies, both 2-day old. Pentadecanoic acid (C15:0) was added as an internal GC-MS standard for quantification. Except for lauric acid (C12:0), which is more abundant in females but does not excite Or47b ORNs (Fig. 3B), all of the detectable fatty acids are present at similar levels between the two sexes. Statistical differences were determined by Mann-Whitney test. Error bars indicate s.e.m. **, $p<0.01$. (n=5). (D) Representative GC-MS chromatograms from the same female cuticular extract with (black) or without (red) esterification. Fatty acids are not amenable to gas chromatography using standard columns due to their high polarity. Cuticular fatty acids were therefore analyzed after derivatization into methyl esters using methanolic HCl. Fatty acid methyl esters (FAMEs) were not detected without esterification, indicating that FAME level was below the detectable level in the hexane cuticular extract.

Figure S4, related to Figure 4. Activation of at4A by palmitoleic acid requires close-range stimulation.

(A-B) Single-sensillum recording from the at4A ORNs that express Or47b. Palmitoleic acid was delivered at a close range (~4 mm) or further away (~11 mm) (n=6). (C) Comparison of the corresponding spike responses (binned at 50 ms, smoothed peri-stimulus time histograms). (D) Comparison of the corresponding average spike responses. The responses of at4A to palmitoleic acid drop markedly as the stimulus distance increases.

Figure S5, related to Figure 5. **Palmitoleic acid is a stimulatory pheromone.**

(A) Single-pair mating assay with a 7-day old naïve male and a 2-day old virgin female. Linoleic acid (LA) does not activate the Or47b ORNs and perfuming the mating chamber with the compound had no effect on copulation. Ethanol was used as a solvent to dilute LA. Cumulative copulation rates are plotted against time (middle panel). For comparison, final copulation rates are shown with lines connecting results from parallel experiments (right panel). Perfuming the mating chamber with palmitoleic acid enhanced copulation in both 2-day and 7-day old wild-type males (B) but not in *Or47b*^{2} mutant males (C). Each data point indicates result from an individual experiment, which consisted of 24-25 fly pairs. Results are from parallel experiments. Red bars indicate average copulation rates from 5 experiments. Statistical significance was determined by paired *t*-test (A) and ANOVA followed by Tukey's test (B-C) (n=5).

Figure S6, related to Figure 6. Sensitivity of male Or47b ORNs increases with age.

(A-B) Single-sensillum recording from the at4A ORNs that express Or47b. Palmitic acid-evoked spike responses in 2-day old (A) and 7-day old (B) males (n=7). (C) Comparison of palmitic acid-evoked spike responses. Corresponding peri-stimulus histograms are shown (binned and smoothed).

Figure S7, related to Figure 7. Age or methoprene treatment does not alter the sensitivity of Or67d ORNs.

Two-photon calcium imaging at the axon terminals of Or67d ORNs, which respond to a male pheromone, *cis*-vaccenyl acetate (*c*VA). (A-B) *c*VA-evoked calcium responses in 2-day (A) and 7-day (B) old males. cVA dosage: 0.43 ng to 43 μg. (C) Dose-response curves comparing calcium responses of 2-day and 7-day old males. There is no significant difference between the two age groups. Mean± s.e.m. (n=7-8). (D) Methoprene treatment enhanced Or47b olfactory responses without affecting the sensitivity of Or67d ORNs to *c*VA. Left: sample traces of single-sensillum recordings performed in 2-day old WT (black) or *Or47b* mutant males (red). Right: average spike responses (smoothed and binned at 50 ms, line width represents s.e.m.). (E) Dose-response curves, mean± s.e.m. (n=10), results are from Or67d ORNs and Or47b ORNs in the same antennae. ***, $p<0.001$, *t*-test.

Figure S8, related to Figure 8. Methoprene-tolerant (Met) is required for age-dependent sensitization of Or47b ORNs.

(A) Raster plots and peri-stimulus time histograms of Or47b spike responses of WT control and *Met* mutant males. (B) Comparison of the average spike responses to palmitoleic acid (4.5 μg) between 7-day old WT and *Met* mutant males. Traces are smoothed and binned at 50 ms; line width represents s.e.m. (C) Dose-response curves, mean± s.e.m. (n=10). Results are from parallel experiments.

Table S1, related to Experimental Procedures. Fly genotypes.

Figure 1	(A)	Female:	Wild-type Canton-S
			Orco^{2}
		Male:	Wild-type Berlin
			Orco^{2} (RRID:BDSC_23130)(Larsson et al., 2004)
			Or47b^{2} (RRID:BDSC_51306) and *Or47b*^{3} (RRID:BDSC_51307)(Wang et al., 2011) were backcrossed to *w*$^{-}$, Berlin
	(B)	Female:	Wild-type Canton-S
		Male:	Wild-type Berlin
			Or47b^{2}
			Or47b^{2}*;UAS-Or47b/+* (Hallem and Carlson, 2006; Hallem et al., 2004)
			Or47b^{2}*;Or47b-GAL4/+* (Bloomington #9984, RRID:BDSC_9984)(Fishilevich and Vosshall, 2005)
			Or47b^{2}*;Or47b-GAL4/UAS-Or47b*
			Or47b-GAL4 and UAS-Or47b were backcrossed to w^{-}*, Berlin*
	(C)	Female:	Wild-type Canton-S
		Male:	*Or47b-GAL4/+*
			UAS-dTrpA1/+ (Bloomington #26263, RRID:BDSC_26263)
			Or47b-GAL4/UAS-dTrpA1
			All files were backcrossed to *w*^{1118}
Figure 2	(A-C)	Female:	Wild-type Canton-S
		Male:	Wild-type Berlin
			Or47b^{2}
Figure 3	(A)	Female:	Wild-type Canton-S
	(B)		*UAS-GCaMP3* (Tian et al., 2009) (Bloomington #32234, RRID:BDSC_32234)*;Or47b-GAL4/+*
Figure 4	(A)		Wild-type Berlin
			Or47b^{2}
	(B)		*Or47b-GAL4/UAS-GCaMP3* (GCaMP, Bloomington #32236, RRID:BDSC_32236)
			Or47b3;Or47b-GAL4/UAS-GCaMP3
			Or47b^{3}*;Or47b-GAL4/UAS-GCaMP3,UAS-Or47b*
	(C)		*UAS-GCaMP3;GH146-GAL4/+* (Bloomington #30026, RRID:BDSC_30026)
			UAS-GCaMP3;GH146-GAL4/+,Or47b^{3}
Figure 5	(A-D)	Female:	Wild-type Canton-S
	(A)		Wild-type Berlin
			Or47b^{2}
			Or47b^{3}
	(B)		*Or47b*^{2}*;UAS-Or47b/+*
			Or47b^{2}*;Or47b-GAL4/+*
			Or47b^{2}*;Or47b-GAL4/UAS-Or47b*
	(C)		Wild-type Berlin
	(D)		*Or47b*^{2}
Figure 6	(A, B, E)		Wild-type Berlin
	(C, D, F, G)		*UAS-GCaMP3;Or47b-GAL4/+*

Figure 7	(A, B, D, E)		*UAS-GCaMP3;Or47b-GAL4/+*
	(C, F)	Female:	Wild-type Canton-S
		Male:	Wild-type Berlin
			Or47b^{2}
Figure 8	(A-C)		*UAS-Met-RNAi/+* (VDRC #100638)
			Or47b-GAL4/+
			UAS-Met-RNAi;Or47b-GAL4
	(D)	Female:	Wild-type Canton-S
		Male:	*UAS-Met-RNAi/+*
			Or47b-GAL4/+
			UAS-Met-RNAi;Or47b-GAL4
Figure S1		Female:	Wild-type Canton-S
	(A, B)	Male:	Wild-type Berlin
	(C, D)	Male:	*Or47b*^{2}
	(E-G)	Male:	Wild-type Berlin
			Or47b^{2}
Figure S2		Male:	Wild-type Berlin
Figure S3	(A, C, D)		Wild-type Canton-S
	(B)		*UAS-GCaMP3;Or47b-GAL4/+*
Figure S4			Wild-type Berlin
Figure S5		Females:	Wild-type Canton-S
	(A, B)	Male:	Wild-type Berlin
	(C)	Male:	*Or47b*^{2}
Figure S6			Wild-type Berlin
Figure S7	(A-C)		*UAS-GCaMP3;Or67d-GAL4/+* (Bloomington #9998, RRID:BDSC_9998)
	(D, E)		Wild-type Berlin
Figure S8			Wild-type Berlin
			Met^{1} (Bloomington #3472, RRID:BDSC_3472) (Wilson and Fabian, 1986)

References

1. Fishilevich, E., and Vosshall, L.B. (2005). Genetic and functional subdivision of the *Drosophila* antennal lobe. Curr. Biol. *15*, 1548–1553.
2. Hallem, E.A., and Carlson, J.R. (2006). Coding of Odors by a Receptor Repertoire. Cell *125*, 143–160.
3. Hallem, E.A., Ho, M.G., and Carlson, J.R. (2004). The molecular basis of odor coding in the *Drosophila* antenna. Cell *117*, 965–979.
4. Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H., and Vosshall, L.B. (2004). Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. Neuron *43*, 703–714.
5. Tian, L., Hires, S.A., Mao, T., Huber, D., Chiappe, M.E., Chalasani, S.H., Petreanu, L., Akerboom, J., McKinney, S.A., Schreiter, E.R., et al. (2009). Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat. Methods *6*, 875–881.
6. Wang, L., Han, X., Mehren, J., Hiroi, M., Billeter, J.-C., Miyamoto, T., Amrein, H., Levine, J.D., and Anderson, D.J. (2011). Hierarchical chemosensory regulation of male-male social interactions in *Drosophila*. Nat. Neurosci. *14*, 757–762.
7. Wilson, T.G., and Fabian, J. (1986). A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. Dev. Biol. *118*, 190-201.

Figure S1

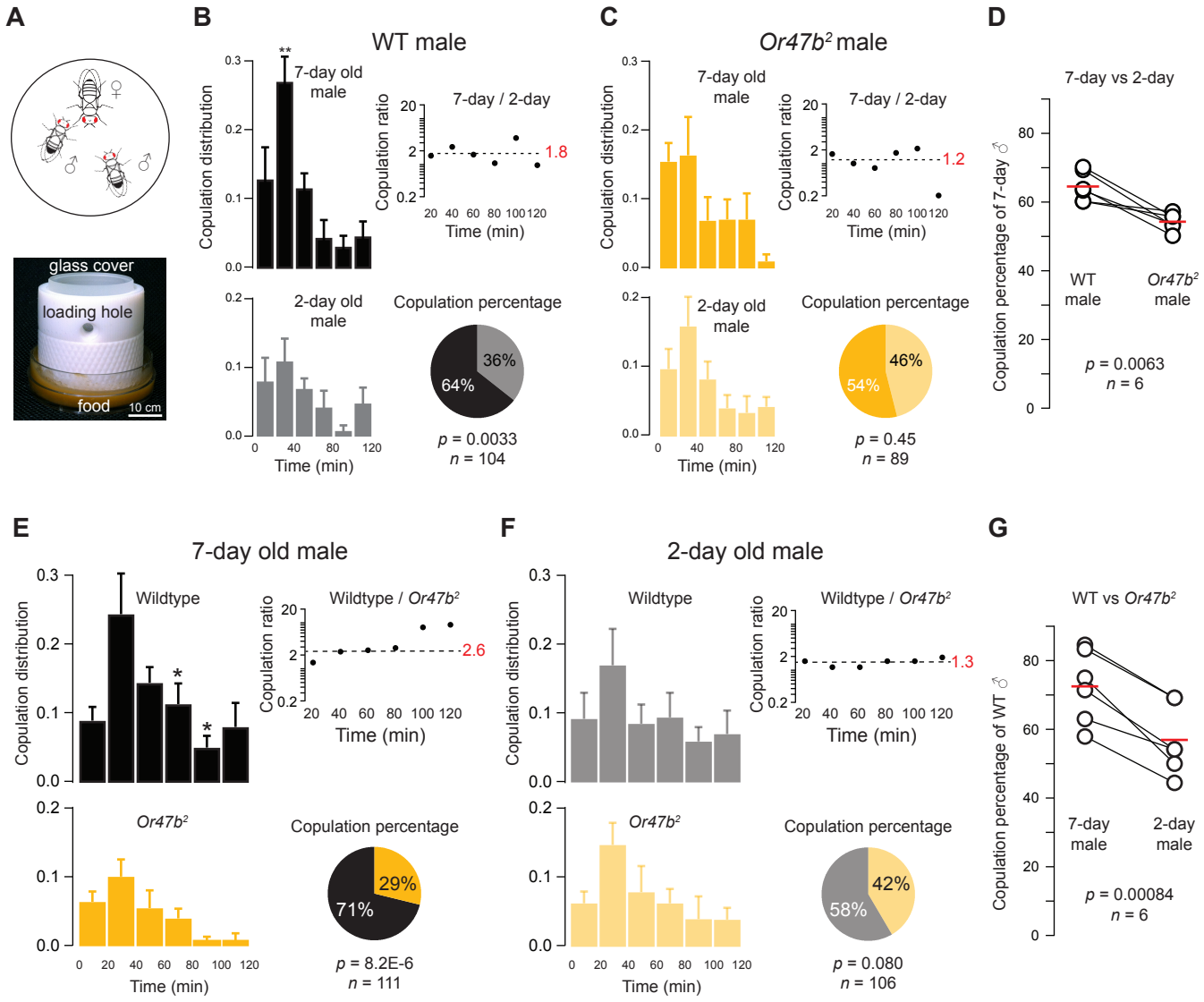

A
glass cover
loading hole
food
10 cm
B
WT male
7-day old male
Copulation distribution
Copulation ratio
7-day / 2-day
1.8
Time (min)
2-day old male
Copulation percentage
36%
64%
p = 0.0033
n = 104
C
Or47b² male
7-day old male
7-day / 2-day
1.2
2-day old male
Copulation percentage
46%
54%
p = 0.45
n = 89
D
7-day vs 2-day
Copulation percentage of 7-day ♂
WT male
Or47b² male
p = 0.0063
n = 6
E
7-day old male
Wildtype
Wildtype / Or47b²
2.6
Or47b²
Copulation percentage
29%
71%
p = 8.2E-6
n = 111
F
2-day old male
Wildtype
Wildtype / Or47b²
1.3
Or47b²
Copulation percentage
42%
58%
p = 0.080
n = 106
G
WT vs Or47b²
Copulation percentage of WT ♂
7-day male
2-day male
p = 0.00084
n = 6

Figure S2

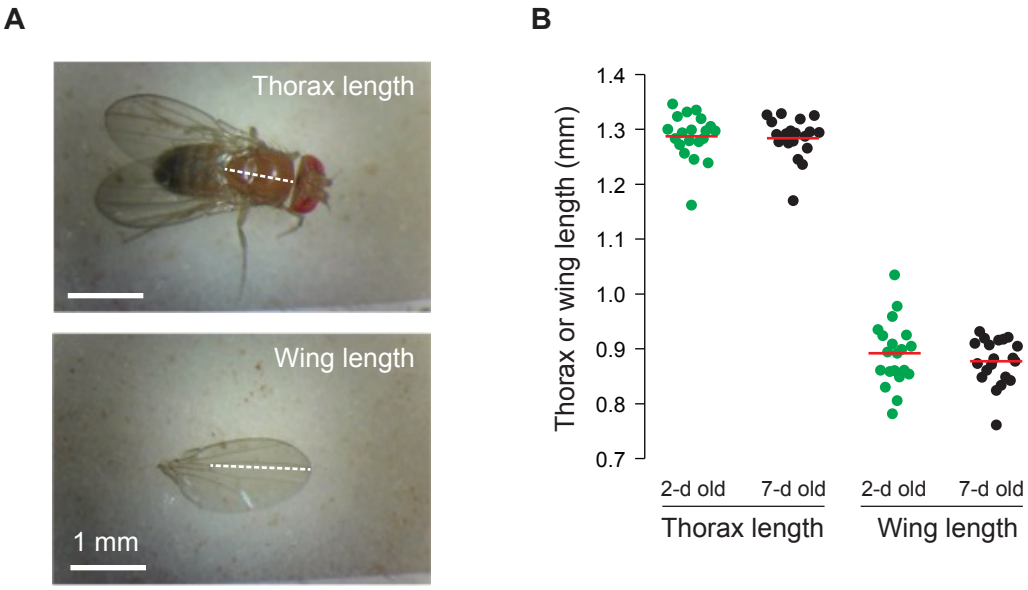

A
Thorax length
Wing length
1 mm
B
Thorax or wing length (mm)
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
2-d old
7-d old
2-d old
7-d old
Thorax length
Wing length

Figure S3

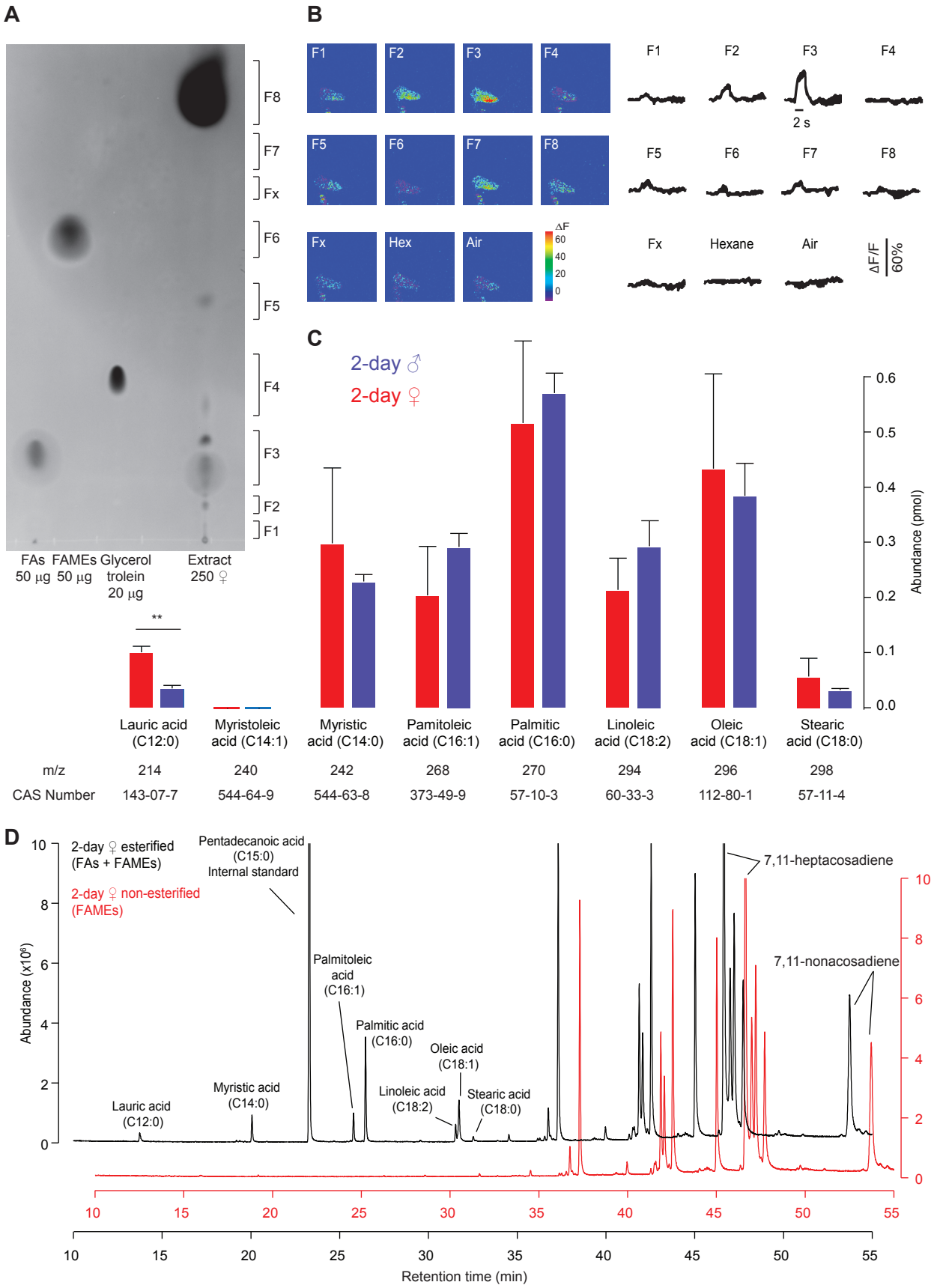

A
F8
F7
Fx
F6
F5
F4
F3
F2
F1
FAs 50 µg
FAMEs 50 µg
Glycerol trolein 20 µg
Extract 250 ♀
B
F1
F2
F3
F4
F5
F6
F7
F8
Fx
Hex
Air
ΔF
60
40
20
0
2 s
Hexane
ΔF/F
60%
C
2-day ♂
2-day ♀
**
Abundance (pmol)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Lauric acid (C12:0)
Myristoleic acid (C14:1)
Myristic acid (C14:0)
Pamitoleic acid (C16:1)
Palmitic acid (C16:0)
Linoleic acid (C18:2)
Oleic acid (C18:1)
Stearic acid (C18:0)
m/z
214
240
242
268
270
294
296
298
CAS Number
143-07-7
544-64-9
544-63-8
373-49-9
57-10-3
60-33-3
112-80-1
57-11-4
D
2-day ♀ esterified (FAs + FAMEs)
2-day ♀ non-esterified (FAMEs)
Pentadecanoic acid (C15:0) Internal standard
Palmitoleic acid (C16:1)
Palmitic acid (C16:0)
Oleic acid (C18:1)
Linoleic acid (C18:2)
Stearic acid (C18:0)
Myristic acid (C14:0)
Lauric acid (C12:0)
7,11-heptacosadiene
7,11-nonacosadiene
Abundance (x10⁶)
Retention time (min)

Figure S4

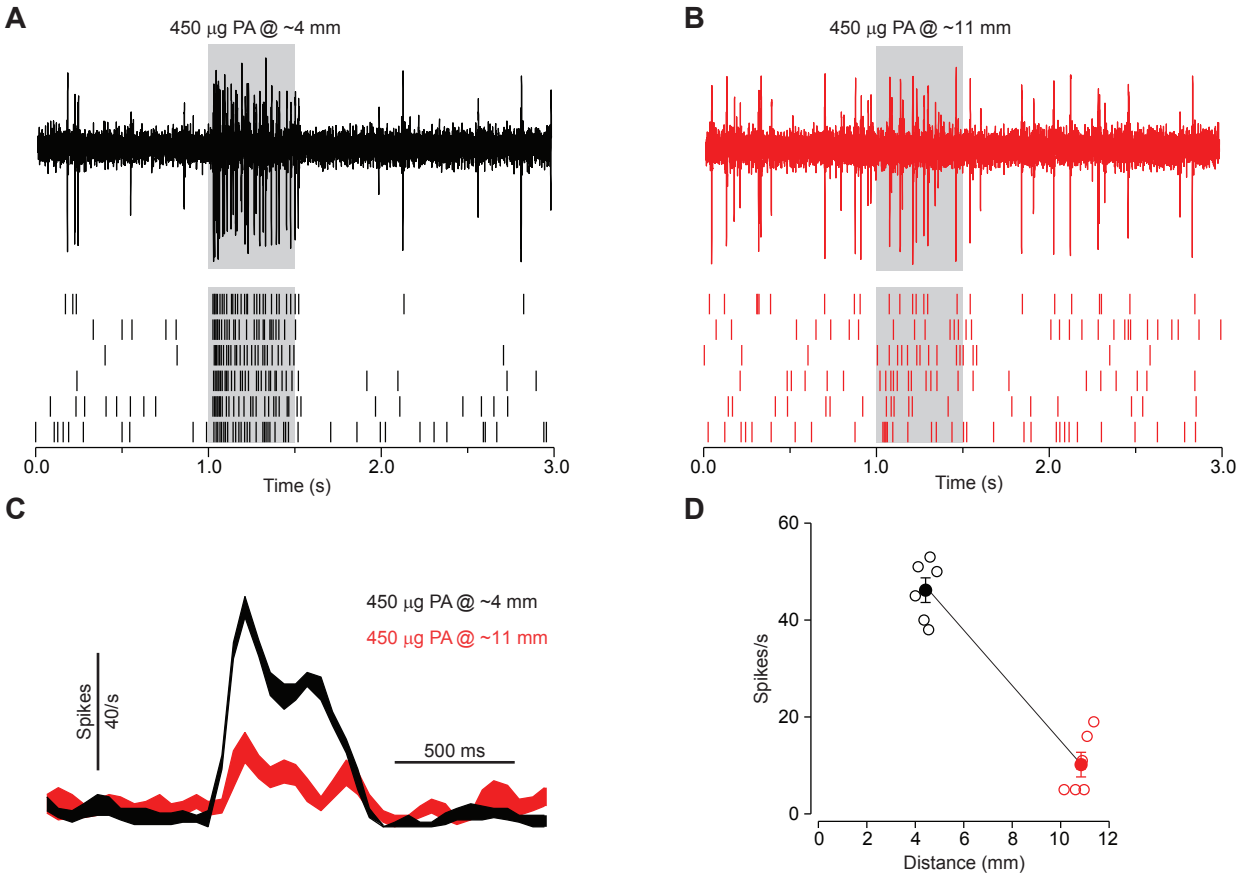

A
450 µg PA @ ~4 mm
0.0
1.0
2.0
3.0
Time (s)
B
450 µg PA @ ~11 mm
0.0
1.0
2.0
3.0
Time (s)
C
450 µg PA @ ~4 mm
450 µg PA @ ~11 mm
Spikes
40/s
500 ms
D
60
40
20
0
Spikes/s
0
2
4
6
8
10
12
Distance (mm)

Figure S5

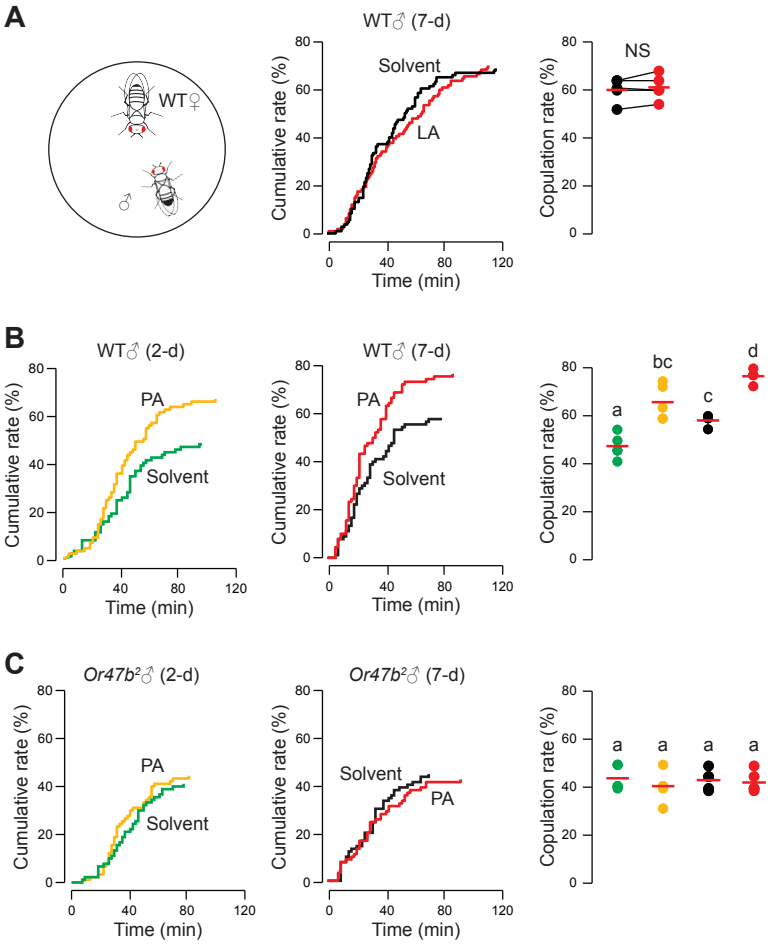

A
WT♀
♂
WT♂ (7-d)
Solvent
LA
Cumulative rate (%)
Time (min)
Copulation rate (%)
NS
B
WT♂ (2-d)
PA
Solvent
WT♂ (7-d)
a
bc
c
d
C
Or47b²♂ (2-d)
Or47b²♂ (7-d)
a
a
a
a

Figure S6

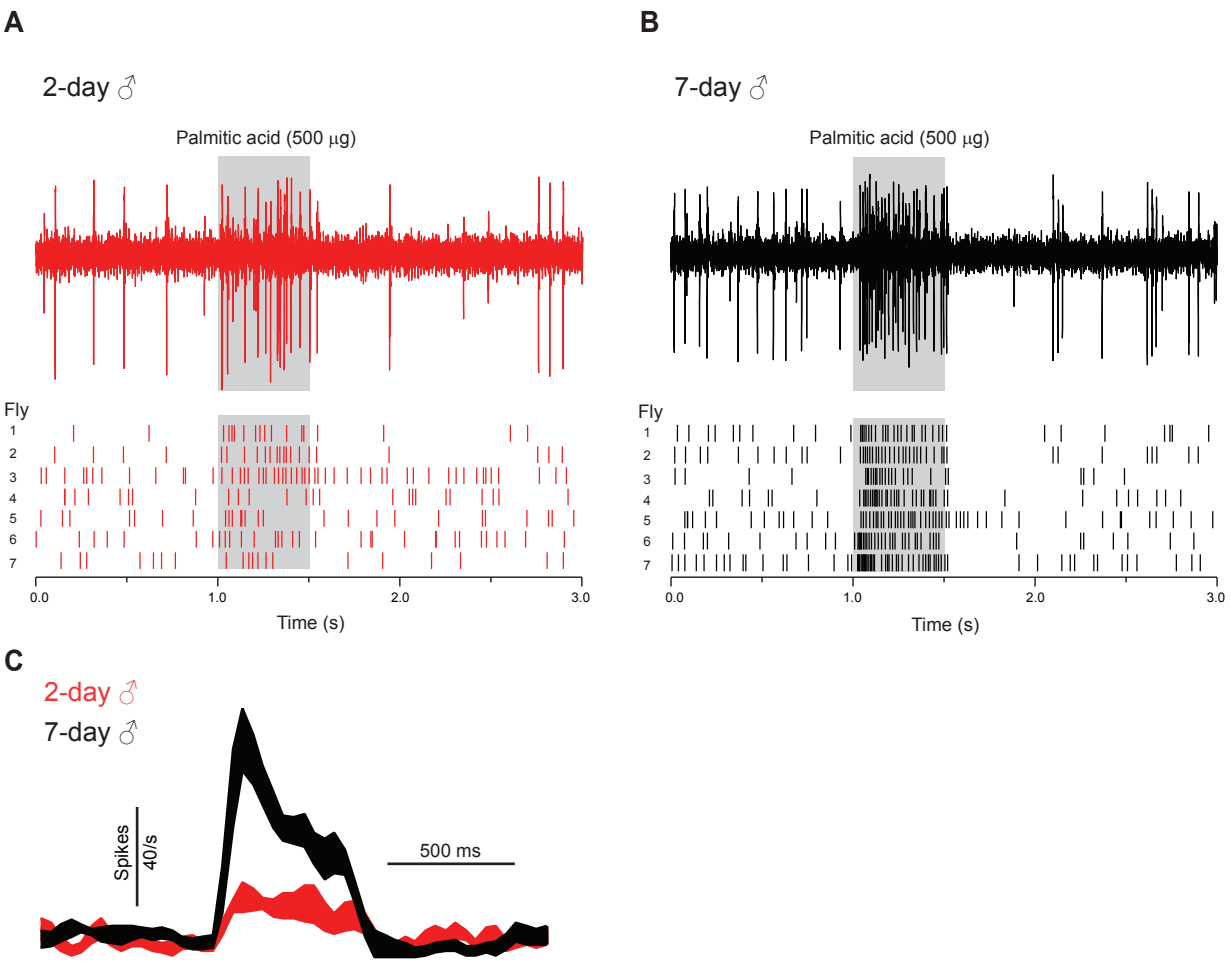

A
2-day ♂
Palmitic acid (500 µg)
Fly
1
2
3
4
5
6
7
0.0
1.0
2.0
3.0
Time (s)
B
7-day ♂
Palmitic acid (500 µg)
Fly
1
2
3
4
5
6
7
0.0
1.0
2.0
3.0
Time (s)
C
2-day ♂
7-day ♂
Spikes
40/s
500 ms

Figure S7

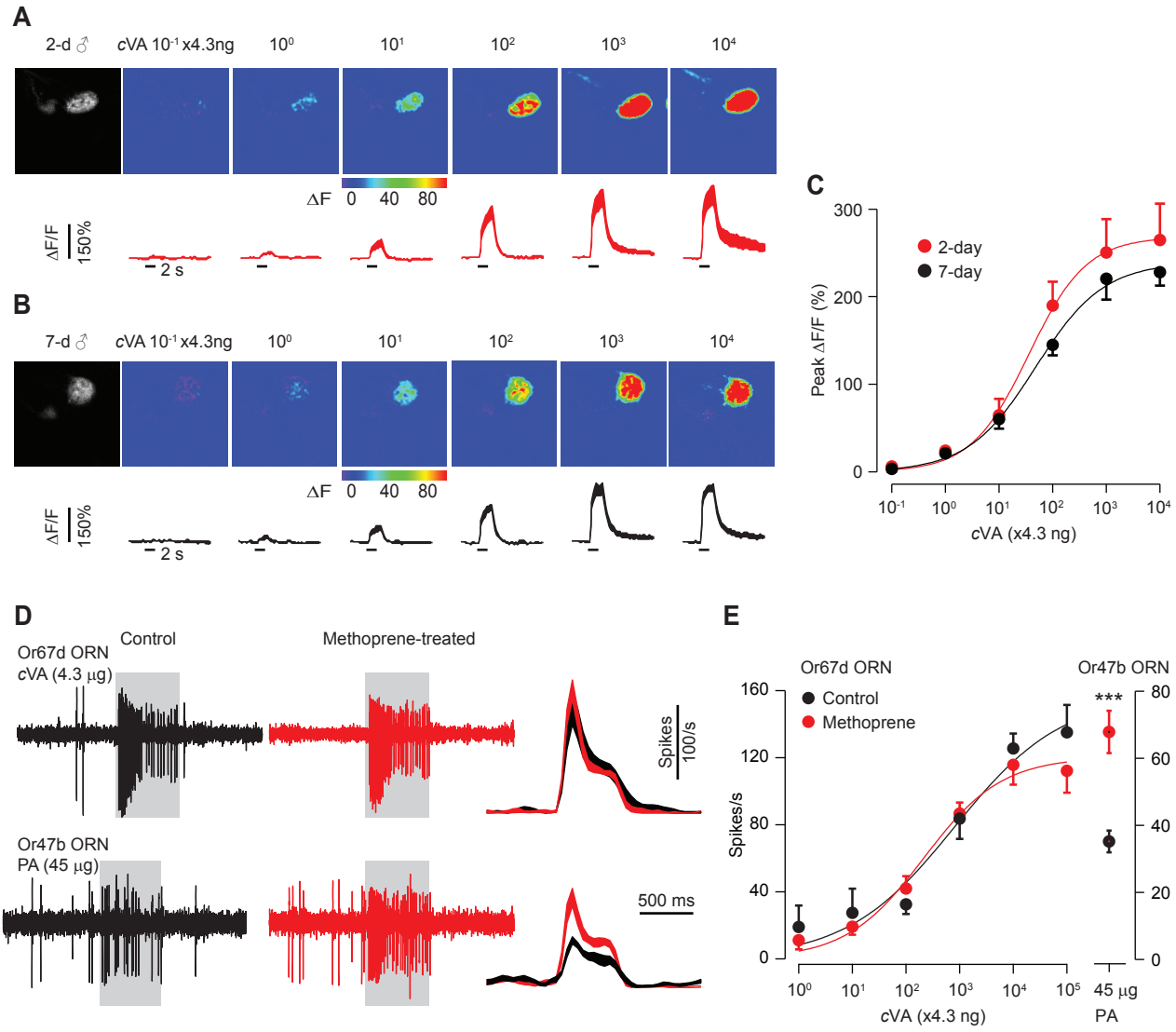
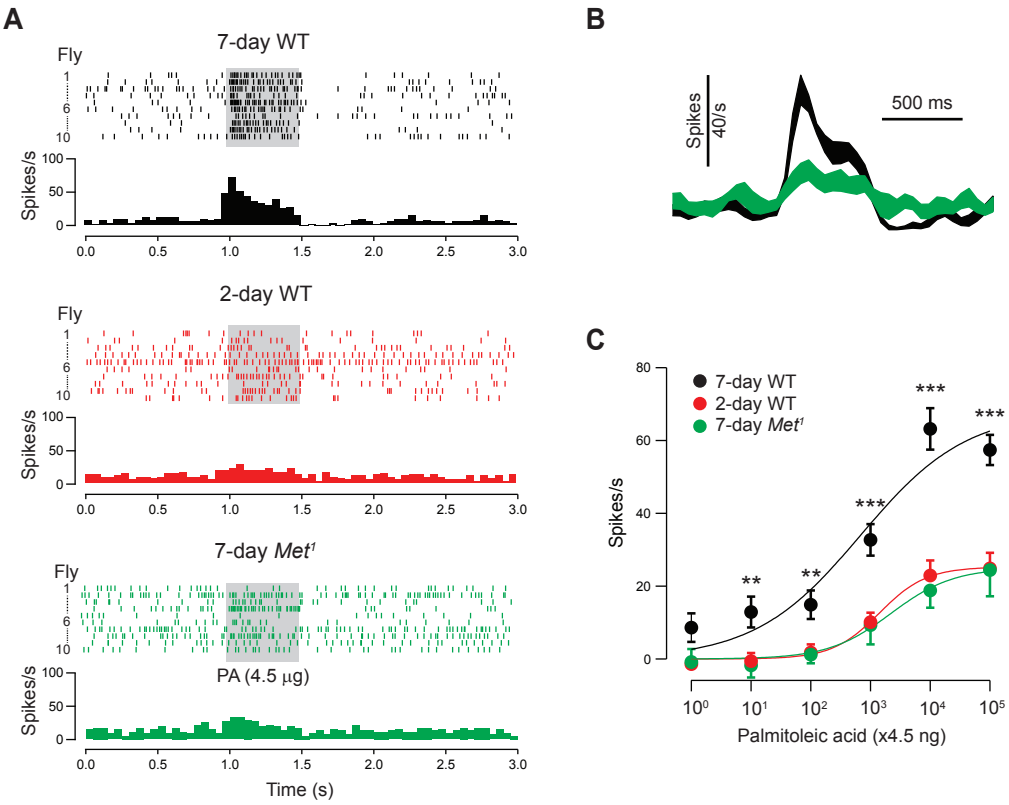

A
2-d ♂
cVA 10⁻¹ x4.3ng
10⁰
10¹
10²
10³
10⁴
ΔF 0 40 80
ΔF/F 150%
2 s
B
7-d ♂
cVA 10⁻¹ x4.3ng
10⁰
10¹
10²
10³
10⁴
ΔF 0 40 80
ΔF/F 150%
2 s
C
Peak ΔF/F (%)
300
200
100
0
2-day
7-day
10⁻¹
10⁰
10¹
10²
10³
10⁴
cVA (x4.3 ng)
D
Control
Methoprene-treated
Or67d ORN
cVA (4.3 μg)
Spikes
100/s
Or47b ORN
PA (45 μg)
500 ms
E
Or67d ORN
Or47b ORN
Control
Methoprene
***
Spikes/s
160
120
80
40
0
80
60
40
20
0
10⁰
10¹
10²
10³
10⁴
10⁵
cVA (x4.3 ng)
45 μg
PA

Figure S8


A
7-day WT
Fly
Spikes/s
2-day WT
7-day Met1
PA (4.5 µg)
Time (s)
B
Spikes
40/s
500 ms
C
7-day WT
2-day WT
7-day Met1
Spikes/s
Palmitoleic acid (x4.5 ng)