Inventory of Supplemental Materials

Avoiding DEET through insect gustatory receptors

Youngseok Lee, Sang Hoon Kim and Craig Montell

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Figure S1 (related to Figure 1)

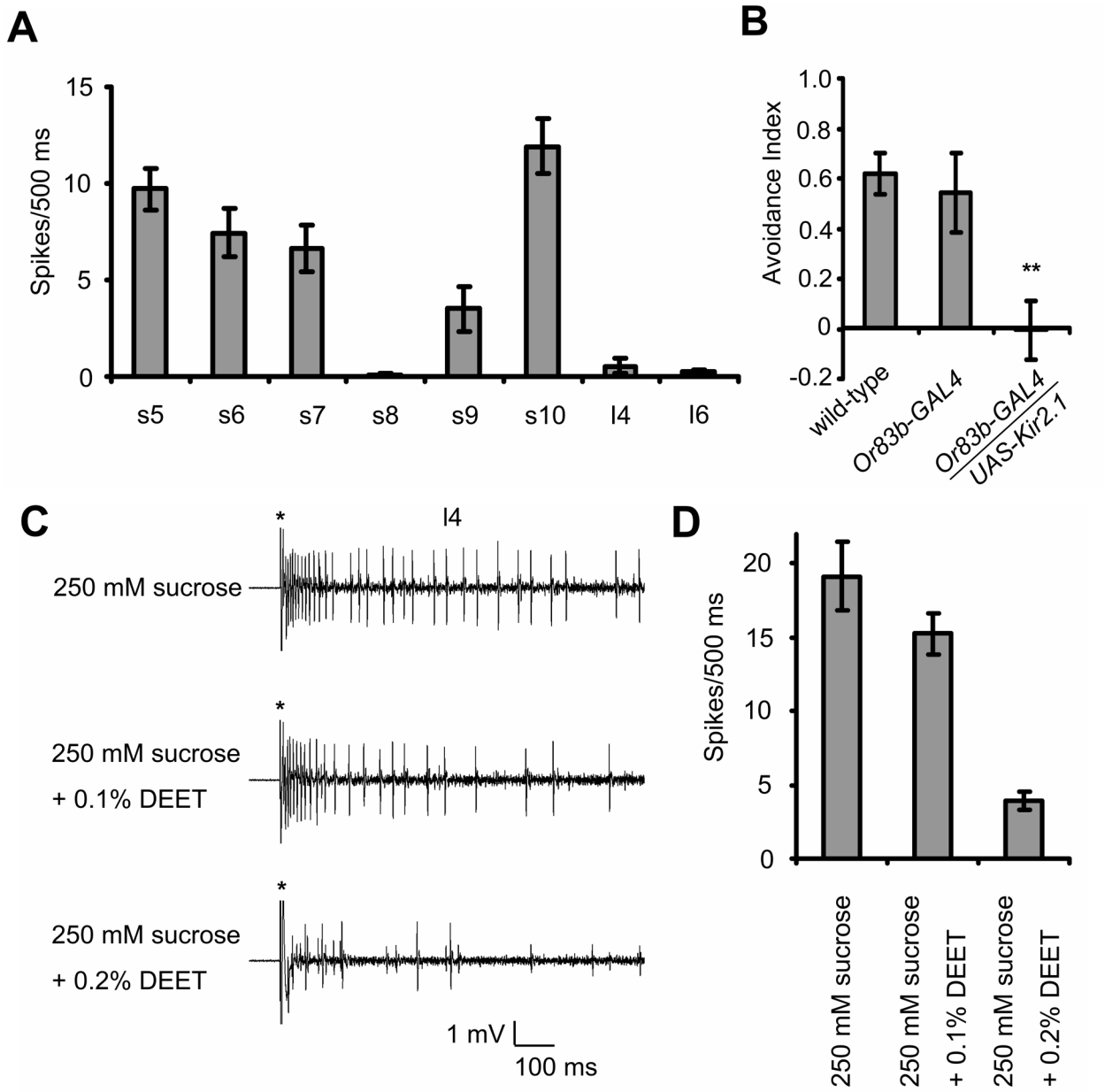

Figure S1. Assaying direct activation of s-type and l-type sensilla by DEET. (A) Tip recordings were performed using 0.2% DEET and the indicated sensilla. To facilitate the ease of accessing sensilla with the recording pipets, we used s-type sensilla situated near the middle of the labella. Number of successful recordings/number of attempted recordings: s5, 20/28; s6, 23/28; s7, 15/26; s8, 0/18; s9, 6/9; s10, 13/18; l4, 0/13; l6, 0/13. (B) *Or83b-GAL4*/*UAS-Kir2.1* flies did not avoid 0.1% benzaldehyde using the DART (direct airborne repellent test) assay. The DART assay (Kwon, Kim, Lee, Atkitake, Woodward, Guggino and Montell, submitted) is a two-way choice test, which is performed by placing 100 flies in a chamber consisting of two connected tubes. One chemical (benzaldehyde) is placed at the bottom of one tube and the negative control chemical (DMSO) at the bottom of the other test tube. The chemicals are separated from the flies by insertion of screens. Olfactory avoidance indexes (means ±S.E.M.s): wild-type control (w^{1118}), 0.62±0.08; *Or83b-GAL4*, 0.54±0.16; *Or83b-GAL4*/*UAS-Kir2.1*, 0.01±0.12. The *p* values for wild-type versus *Or83b-GAL4*/*UAS-Kir2.1* was <0.001. (C) Sample tip recordings using l4 sensilla exposed to sucrose only or sucrose with either 0.1% or 0.2% DEET. (D) DEET inhibited sucrose response from l-type (l4 and l6) sensilla. 250 mM sucrose (n=14 total: l4, n=7; l6, n=7), 250 mM sucrose + 0.1% DEET (n=16: l4, n=8; l6, n=8), and 250 mM sucrose + 0.2% DEET (n=23: l4, n=12; l6, n=11). The error bars indicate S.E.M.s.

Figure S2 (related to Figure 2)

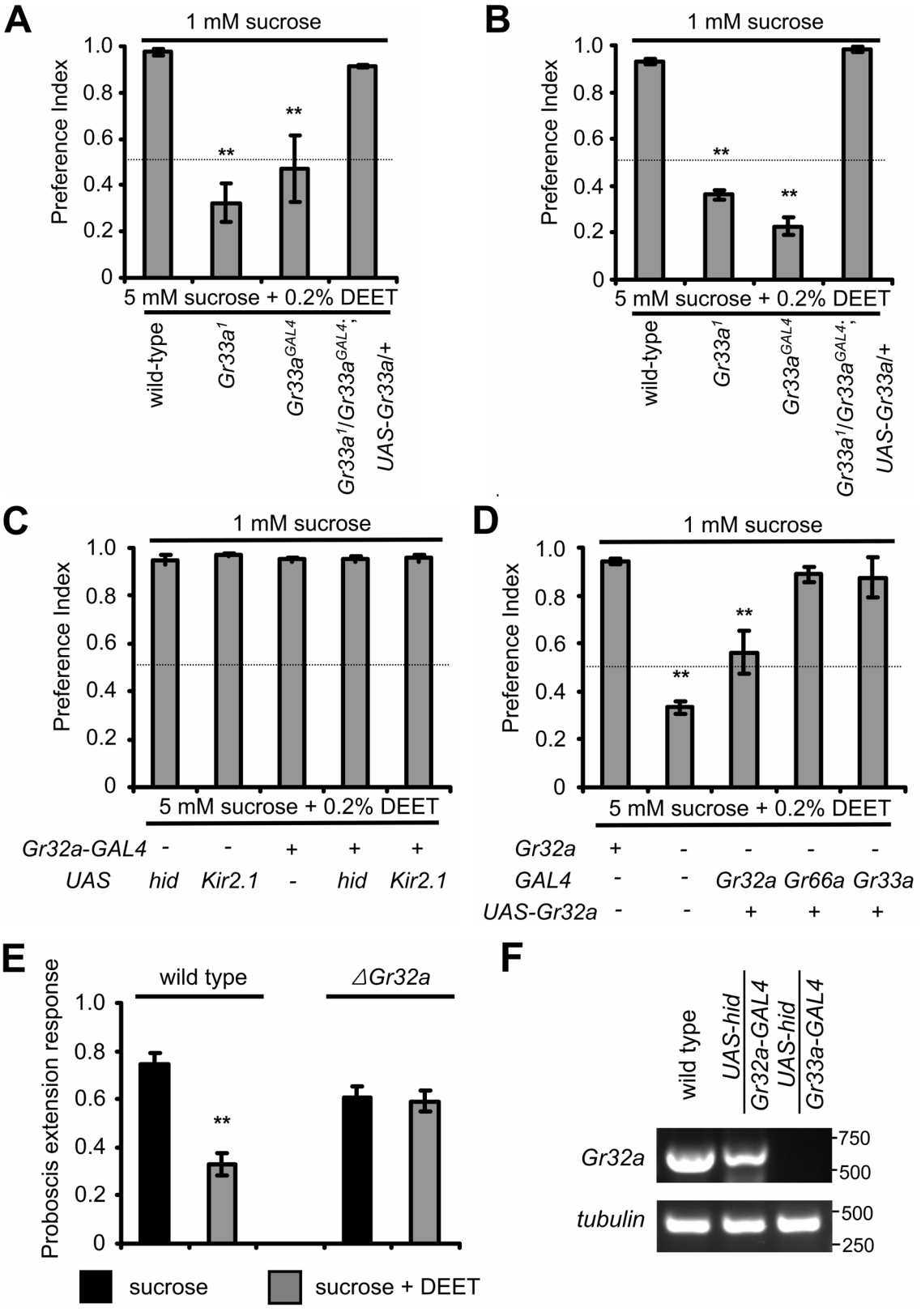

Figure S2. Behavioral gustatory assays using DEET. (A-B) Comparison of the preference indexes obtained when performing the two way choice assays in a blind (A) or non-blind fashion (B). The *p* values for wild-type versus *Gr33a*^{1} or *Gr33A*^{GAL4} were <0.01. (C) The cell death gene (*UAS-hid*) or the Kir2.1 channel (*UAS-Kir2.1*) were expressed under the control of the *Gr32a-GAL4*. (D) Test for rescue of the *Δ Gr32a* phenotype by expressing *UAS-Gr32a* under control of the indicated *GAL4* drivers. ***p* values<0.01. (E) Assaying sensitivity of sensilla in forelegs to DEET avoidance using the proboscis extension response (PER) assay. Either 250 mM sucrose alone or 250 mM sucrose and 0.8% DEET are applied to the forelegs and the fraction of flies that elicited a PER are indicated. Total experiments (7-10 flies per experiment): wild-type, n=9; *ΔGr32a*, n=7. ***p* value=7.3x10^{-6}. (F) Testing whether the *Gr32a-GAL4* is expressed in all cells that express the *Gr32a* RNA. To ascertain whether the *Gr32a-GAL4* fully recapitulated *Gr32a* expression, we expressed the cell death gene (*UAS-hid*) under control of the *Gr32a-GAL4* and then tested whether the *Gr32a* RNA was eliminated. As a control we also expressed *UAS-hid* under control of the *Gr33a-GAL4*. We dissected 50 adult labella from 5 to 7 day old wild-type, *Gr32a-GAL4*/*UAS-hid* and *Gr33a-GAL4*/*UAS-hid* flies, prepared RNA (Stratagene, Absolutely RNA Miniprep Kit), and used AMV reverse transcriptase to generate cDNAs according to the manufacturer's instructions (Promega). To obtain the transcripts, we incubated the cDNA reactions for 60 minutes at 42 ˚C. To conduct the RT-PCR analyses, we used the following primers: *Gr32a*, 5'- AAAATATATGCCGTGCAAGG-3' and 5'-ACTTATCAATGATATTCTGAT -3'; *tubulin*, 5'- TCCTTCTCGCGTGTGAAACA -3' and 5'-CCGAACGAGTGGAAGATGAG -3'. The *Gr32a* and *tubulin* products were amplified after 40 and 27 cycles, respectively.

Figure S3 (related to Figure 4)

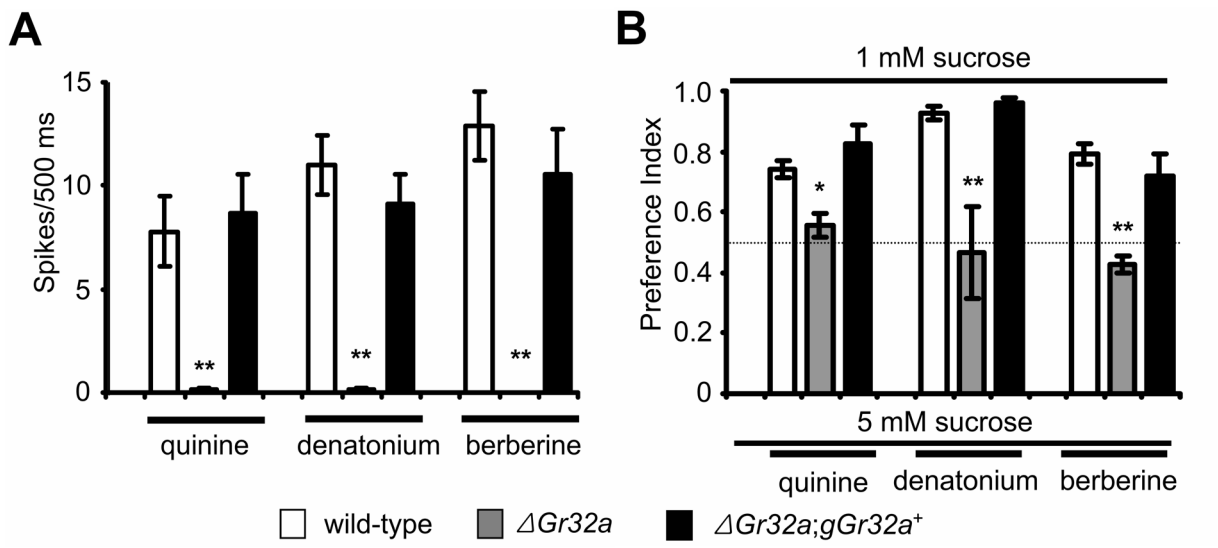

Figure S3. *ΔGr32a* flies displayed defects in sensing quinine, denatonium and berberine. (A) Tip recordings. The reductions in action potentials in *ΔGr32a* flies were rescued with the wild-type genomic transgene, *gGr32a*$^{+}$. (B) Two-way choice assays. The behavioral impairments in *ΔGr32a* were reversed by the introduction of *gGr32a*$^{+}$. The error bars indicated S.E.M.s.

Table S1

(A) Statistics related to Figure 1B.		
DEET conc. (%)	wild-type	
	PI	p values
0	0.09±0.02	
0.05	0.37±0.03	2.1×10^{-9}
0.1	0.64±0.37	1.7×10^{-17}
0.2	0.94±0.01	9.4×10^{-26}
0.4	0.99±0.01	2.5×10^{-24}

(B) Statistics for the data shown in Figure 1C.			
Genotype	PI	p values	p values
UAS-hid /+	0.95±0.02		
UAS-Kir2.1/+	0.97±0.01		
Gr33a^{GAL4}/+	0.91±0.01		
Gr33a^{GAL4}/*UAS-hid*	0.34±0.05	[a] 2.2×10^{-8}	[c] 3.7×10^{-9}
Gr33a^{GAL4}/*UAS-Kir2.1*	0.42±0.11	[a] 9.8×10^{-6}	[d] 7.7×10^{-7}
Or83b-GAL4/+	0.91±0.03		
Or83b-GAL4/*UAS-hid*	0.90±0.02	[b] 1.0	[c] 1.0
Or83b-GAL4/*UAS-Kir2.1*	0.93±0.01	[b] 1.0	[d] 1.0
[a]Relative to *Gr33a*^{GAL4}/+ [b]Relative to *Or83b-GAL4*/+ [c]Relative to *UAS-hid* /+ [d]Relative to *UAS-Kir2.1*/+			

(C) Statistics for the data shown in Figure 1D.					
Genotype	Temperature °C	PI	[a]p values	[b]p values	[c]p values
Gr33a^{GAL4}/+	22	0.91±0.01			
UAS-shi^{ts1}/+	22	0.93±0.01			
Gr33a^{GAL4}/*UAS-shi*^{ts1}	22	0.80±0.05	0.14	0.059	
Gr33a^{GAL4}/+	30	0.93±0.02			
UAS-shi^{ts1}/+	30	0.94±0.01			
Gr33a^{GAL4}/*UAS-shi*^{ts1}	30	0.49±0.03	2.1×10^{-10}	4.6×10^{-11}	5.3×10^{-7}
[a]Relative to *Gr33a*^{GAL4}/+ at the same temperature [b]Relative to *UAS-shi*^{ts1}/+ at the same temperature [c]Relative to *Gr33a*^{GAL4}/*UAS-shi*^{ts1} at different temperature.					

(D) Statistics related to Figure 1F.		
DEET dilution	wild-type	
	spikes/500 ms	[a]p values
$2x10^{-5}$	0.36±0.28	
$2x10^{-4}$	3.73±0.76	0.16
$2x10^{-3}$	9.77±1.10	$9.9x10^{-5}$
$8x10^{-3}$	10.80±0.92	$1.3x10^{-8}$
[a]Relative to $2x10^{-5}$ DEET dilution.		

(E) Statistics for the data shown in Figure 2A.		
Genotype	PI	p values
wild-type	0.94±0.01	
Or83b^{2}	0.88±0.03	0.21
Gr5a$^{\Delta 19}$	0.92±0.02	0.90
Gr63a^{1}	0.90±0.02	0.77
Gr33a^{1}	0.35±0.03	$5.4x10^{-11}$
Gr8a^{2}	0.95±0.01	1.0
Gr47a^{1}	0.90±0.01	0.64
Gr93a^{3}	0.94±0.01	1.0
Gr66a^{ex83}	0.37±0.06	$5.3x10^{-11}$
ΔGr32a	0.33±0.03	$2.4x10^{-8}$

(F) Statistics for the data shown in Figure 2B.				
Genotype	DEET conc. (%)	PI	p values	[a]p values
wild-type	0.1	0.64±0.04		
Gr33a^{1}	0.1	0.23±0.04	$1.0x10^{-4}$	$3.0x10^{-4}$
Gr33a^{GAL4}	0.1	0.20±0.02	$3.9x10^{-5}$	$1.1x10^{-4}$
Gr33a^{1}/*Gr33a*^{GAL4};*UAS-Gr33a*/+	0.1	0.60±0.08	0.97	
wild-type	0.2	0.94±0.01		
Gr33a^{1}	0.2	0.35±0.03	$5.4x10^{-11}$	$2.8x10^{-10}$
Gr33a^{GAL4}	0.2	0.33±0.08	$7.1x10^{-11}$	$3.2x10^{-10}$
Gr33a^{1}/*Gr33a*^{GAL4};*UAS-Gr33a*/+	0.2	0.95±0.02	1.0	
[a]Relative to *Gr33a*^{1}/*Gr33a*^{GAL4};*UAS-Gr33a*/+				

(G) Statistics for the data shown in Figure 2C.				
Genotype	DEET conc. (%)	PI	p values	[a]p values
wild-type	0.1	0.64±0.04		
Δ Gr32a	0.1	0.22±0.03	$1.5x10^{-7}$	
Δ Gr32a;gGr32a^{+}	0.1	0.62±0.01	0.87	$3.0x10^{-7}$
wild-type	0.2	0.94±0.01		
Δ Gr32a	0.2	0.33±0.03	$2.4x10^{-8}$	
Δ Gr32a;gGr32a^{+}	0.2	0.92±0.02	1.0	$6.3x10^{-7}$
[a]Relative to *Δ Gr32a*				

(H) Statistics for the data shown in Figure 2D.				
Genotype	DEET conc. (%)	PI	p values	[a]p values
wild-type	0.1	0.64±0.04		
Gr66a^{ex83}	0.1	0.26±0.04	$6.5\ x10^{-5}$	
7-Gr66a^{-}*;Gr66a*^{ex83}	0.1	0.20±0.04	$7.0x10^{-6}$	0.75
8-Gr66a^{+}*;Gr66a*^{ex83}	0.1	0.49±0.05	0.14	0.01
wild-type	0.2	0.94±0.01		
Gr66a^{ex83}	0.2	0.37±0.06	$5.3x10^{-11}$	
7-Gr66a^{-}*;Gr66a*^{ex83}	0.2	0.36±0.04	$3.7x10^{-11}$	1.0
8-Gr66a^{+}*;Gr66a*^{ex83}	0.2	0.80±0.03	0.03	$1.3x10^{-7}$
[a]Relative to *Gr66a*^{ex83}				

(I) Statistics for the data shown in Figure 3A.			
Genotype	spikes/500 ms	p values	p values
wild-type	9.77±1.10		
Gr63a^{1}	8.44±1.03	1.0	
Gr93a^{3}	9.55±2.43	1.0	
Gr66a^{ex83}	1.46±0.85	$3.3x10^{-3}$	
7-Gr66a^{-}*;Gr66a*^{ex83}	1.50±0.97	$5.2x10^{-4}$	[a]1.0
8-Gr66a^{+}*;Gr66a*^{ex83}	5.85±0.98	0.68	[a]0.015
Gr33a^{1}	0.50±0.31	$7.8x10^{-4}$	
Gr33a^{1}*/Gr33a*^{GAL4}*;UAS-Gr33a/+*	9.43±0.97	1.0	[b]$3.0x10^{-3}$
Δ Gr32a	0.67±0.47	$2.0x10^{-3}$	
Δ Gr32a;gGr32a^{+}	7.70±1.45	1.0	[c]$3.8x10^{-4}$
[a]Relative to *Gr66a*^{ex83} [b]Relative to *Gr33a*^{1} [c]Relative to *Δ Gr32a*			

(J) Statistics for the data shown in Figure 4A and Figure S3A.				
compounds		wild-type	*⊿Gr32a*	*⊿Gr32a;gGr32a*$^{+}$
caffeine	spikes/0.5 s	11.50±1.09	10.33±1.57	n.d
	p values		0.53	
quinine	spikes/0.5 s	7.80±1.68	0.15±0.10	8.67±1.85
	p values		$4.0x10^{-5}$	0.85
	[a]p values			$6.9x10^{-5}$
denatonium	spikes/0.5 s	11.00±1.41	0.13±0.13	9.11±1.41
	p values		$9.2x10^{-6}$	0.54
	[a]p values			$1.2x10^{-4}$
berberine	spikes/0.5 s	12.88±1.68	0.00±0.00	10.57±2.15
	p values		$6.0x10^{-5}$	0.61
	[a]p values			$4.6x10^{-3}$
lobeline	spikes/0.5 s	12.25±2.06	0.43±0.43	12.55±2.33
	p values		$1.6x10^{-3}$	1.0
	[a]p values			$1.5x10^{-3}$
papaverine	spikes/0.5 s	11.25±1.30	0.00±0.00	9.25±0.92
	p values		$6.0x10^{-8}$	0.72
	[a]p values			$8.5x10^{-5}$
strychnine	spikes/0.5 s	6.20±1.03	6.20±1.03	9.43±1.65
	p values		$1.8x10^{-5}$	0.74
	[a]p values			$1.3x10^{-6}$
[a]Relative to *⊿Gr32a*				

(K) Statistics for the data shown in Figure 4B and Figure S3B.				
compounds		wild-type	*Δ Gr32a*	*Δ Gr32a;gGr32a*$^{+}$
caffeine	PI	0.99±0.01	0.99±0.01	n.d
	p values		0.76	
quinine	PI	0.74±0.03	0.56±0.04	0.83±0.06
	p values		0.03	0.82
	[a]p values			$7.9x10^{-3}$
denatonium	PI	0.93±0.02	0.47±0.15	0.96±0.02
	p values		$9.9x10^{-3}$	1.0
	[a]p values			0.013
berberine	PI	0.79±0.03	0.43±0.03	0.72±0.07
	p values		$6.3x10^{-4}$	0.56
	[a]p values			$3.3x10^{-3}$
lobeline	PI	0.97±0.01	0.47±0.09	0.92±0.03
	p values		$3.7x10^{-6}$	0.67
	[a]p values			$3.3x10^{-5}$
papaverine	PI	0.86±0.04	0.63±0.08	0.90±0.01
	p values		0.014	0.92
	[a]p values			0.013
strychnine	PI	0.96±0.02	0.66±0.08	0.90±0.02
	p values		$1.5x10^{-3}$	0.62
	[a]p values			$8.9x10^{-3}$
[a]Relative to *Δ Gr32a*				

(L) Statistics for the data shown in Figure 4C.					
compounds		wild-type	*Gr66a*^{ex83}	*7-Gr66a*$^{-}$; *Gr66a*^{ex83}	*8-Gr66a*$^{+}$; *Gr66a*^{ex83}
lobeline	spikes/0.5 s	13.21±2.06	0.56±0.56	0.57±0.57	8.75±1.22
	p values		$6.3x10^{-4}$	$1.9x10^{-3}$	0.82
	[a]p values			1.0	$2.8x10^{-6}$
papaverine	spikes/0.5 s	11.62±1.25	0.86±0.70	1.2±0.81	12.78±1.09
	p values		$4.2x10^{-7}$	$6.1x10^{-8}$	0.98
	[a]p values			1.0	$2.0x10^{-7}$
strychnine	spikes/0.5 s	7.33±1.15	0.27±0.27	0.90±0.41	4.75±0.98
	p values		$1.3x10^{-5}$	$1.3 x10^{-4}$	0.49
	[a]p values			0.71	$3.3x10^{-5}$
[a]Relative to *Gr66a*^{ex83}					

(M) Statistics for the data shown in Figure 4D.					
compounds		wild-type	*Gr66a*^{ex83}	*7-Gr66a*$^{-}$; *Gr66a*^{ex83}	*8-Gr66a*$^{+}$; *Gr66a*^{ex83}
lobeline	PI	0.97±0.01	0.54±0.04	0.51±0.04	0.91±0.02
	p values		2.0×10^{-4}	9.9×10^{-5}	0.91
	[a]p values			0.98	1.7×10^{-4}
papaverine	PI	0.86±0.04	0.25±0.05	0.29±0.06	0.54±0.01
	p values		9.0×10^{-7}	2.1×10^{-6}	7.8×10^{-4}
	[a]p values			0.94	6.7×10^{-3}
strychnine	PI	0.96±0.02	0.87±0.01	0.94±0.01	0.91±0.03
	p values		0.021	0.87	0.26
	[a]p values			0.11	0.54
[a]Relative to *Gr66a*^{ex83}					

(N) Statistics for the data shown in Figure 4E. (n≥4, 20 flies per experiment)				
Time (hr)	0% DEET	0.1% DEET	0.2% DEET	0.4% DEET
0	100±0.0	100±0.0	100±0.0	100±0.0
12	100±0.0	100±0.0	96.3±2.4	98.3±1.7
24	100±0.0	100±0.0	75.0±5.4	68.3±4.9
36	100±0.0	100±0.0	63.8±4.3	56.7±5.3
48	100±0.0	100±0.0	50.0±5.8	23.3±3.3
60	100±0.0	100±0.0	35.0±5.8	8.3±3.1
72	98.8±1.3	100±0.0	21.3±3.2	0.8±0.8
84	98.8±1.3	100±0.0	0.0±0.0	0.0±0.0
96	98.8±1.3	100±0.0		
108	98.8±1.3	100±0.0		

The mean PIs and spike frequencies (±SEMs) are indicated. n≥6 (50–100 flies per experiment) for all the behavioral assays using DEET. n≥4 (50–100 flies per experiment) for the behavioral assays using other aversive tastants. n≥7 for the tip recordings. The *p* values are relative to wild-type unless specified otherwise and are based on one way ANOVA with Scheffé post-hoc analysis to test statistical significance.

Binary Food Choice Assay

A. Preparation of plates

1) Two types of tastants are prepared in 1.5 ml microfuge tubes. Add the following:
   a) 10 μl red (sulforhodamine B, 0.2 mg/ml; Sigma-Aldrich; S9012) or blue dye (brilliant blue FCF, 12.5 mg/ml; Wako Chemical; 027-12842).
   b) 10 μl sucrose (100 mM stock or 500 mM stock) to obtain a final concentration of either 1 mM or 5 mM sucrose.
   c) 0 to 100 μl aversive chemical stock (10 – 100 mM stock solutions).*
   d) 880 – 970 μl 1% agarose (liquefied in the microwave) to bring the final volume to 1 ml.
2) Vortex and place in a 65°C heat block for a maximum of 30 minutes.
3) Vortex again and distribute 11 μl of the two types of tastants in a zigzag pattern into 72 well plates (Nunc 136528).

   *DEET is a volatile compound. Therefore, 0.5 – 8 μl DEET is added just before distributing the agarose cocktail into each well (step 3).

B. Preparation of flies and conducting assays

1) For each assay, transfer 50 – 100 flies (≤4 day old) to a new vial containing standard cornmeal and molasses fly food, and maintain the flies in the fresh vial for two days.
2) To starve the flies before initiating the assays, lightly tap the flies into a vial containing 1% agarose only and maintain the flies for 18 – 24 hr in the dark in a humidified chamber.
3) Transfer the flies to a 72 well plate prepared as described above. Expose the flies to CO_2 for the minimum time necessary (<3 sec) to immobilize them. Immediately insert the flies on top of the wells and place the cover over the plate. The assays are performed between 10 AM to 1 PM, because the circadian cycle can affect food consumption, which will impact on the ease to which the colors of the abdomens can be assessed.
4) To conduct the assays in a blind manner, another investigator relabels the plates and maintains the code until the assays are completed.
5) The plates are incubated for 90 minutes in a dark, humidified chamber.
6) Invert the plates and place them in -20˚C freezer to collect the flies onto the lids.
7) Assess the colors of both the dorsal and ventral parts of abdomen (red, blue or purple). Typically, the colors of 90-100% of the flies can be assessed. The genotypes are not revealed to the investigator performing the color assessments

until after the tabulations are completed.

8) Determine the preference indexes (PIs) according to the following formula. Those flies that do not contain a colored abdomen (~0-10% of total) are not included in the calculation.

$$PI=(N^B+0.5N^P)/(N^R+N^B+N^P) \text{ or } (N^R+0.5N^P)/(N^R+N^B+N^P).$$

blue, N^B; red, N^R; purple, N^P.

Tip recordings

1) Collect 1-7 day-old flies by placing the vials on ice for 5 min to immobilize the flies.
2) To insert the reference electrode into the fly:
   a) Press the ventral side of the fly down onto clay with forceps.
   b) Insert a glass capillary tube (World Precision Instruments,1B150F-3) filled with Ringer's solution through the thorax into the head (Figure 1).

Figure 1

3) Insert the recording electrode (10 – 20 μm diameter) over the sensillum on the labellum.
   a) Recording responses to bitter compounds: Use a small (e.g. s6) sensillum and an electrode containing 1 mM KCl.
   b) Recording responses to sucrose: Use a large sensillum and an electrode containing 30 mM tricholine citrate.
4) Connect the recording electrode to a preamplifier (Taste-PROBE, Syntech, Hilversum, The Netherlands). The signals are collected and amplified 10-fold via a signal connection interface box (Syntech) in conjunction with a 100 to 3,000 Hz band-pass filter. The action potentials are recorded with a 12 kHz sampling rate.
5) Analyze the frequencies of action potentials using Autospike 3.1 software (Syntech) between 50 ms and 550 ms after attaching the recording electrode.