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Original Article

A Drosophila Gustatory Receptor Required for Strychnine Sensation

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Abstract

Strychnine is a potent, naturally occurring neurotoxin that effectively protects plants from animal pests by deterring feeding behavior. In insects, such as the fruit fly, *Drosophila melanogaster*, bitter-tasting aversive compounds are detected primarily through a family of gustatory receptors (GRs), which are expressed in gustatory receptor neurons. We previously described multiple GRs that eliminate the behavioral avoidance to all bitter compounds tested, with the exception of strychnine. Here, we report the identity of a strychnine receptor, referred to as GR47a. We generated a mutation in *Gr47a* and found that it eliminated strychnine repulsion and strychnine-induced action potentials. GR47a was narrowly tuned, as the responses to other avoidance compounds were unaffected in the mutant animals. This analysis supports an emerging model that Drosophila GRs fall broadly into two specificity classes—one class is comprised of core receptors that are broadly required, whereas the other class, which includes GR47a, consists of narrowly tuned receptors that define chemical specificity.

Key words: bitter, chemosensation, feeding, fruit fly, taste

Introduction

Plants produce nonvolatile repellent compounds, such as alkaloids, to ward off pests. Among the best-characterized alkaloids is strychnine. In mammals, strychnine is detected through taste receptors, which are G-protein coupled receptors (Bufe *et al.* 2002; Meyerhof *et al.* 2010). However, in insects, the taste receptors that enable the behavioral decision to avoid strychnine-containing foods are not known.

Most taste receptors in Drosophila belong to the 68-member gustatory receptor (GR) family (Clyne *et al.* 2000; Dunipace *et al.* 2001; Scott *et al.* 2001; Robertson *et al.* 2003). GRs are unrelated to mammalian taste receptors but are distantly related to Drosophila olfactory receptors. Thirty-one hair-like bristles (sensilla) are distributed on each of two bilaterally symmetrical labella and are grouped based on length: short (S), intermediate (I), and long (L) (Liman *et al.* 2014). Each sensillum contains two or four gustatory receptor neurons (GRNs). Sugar sensation occurs through one GRN per sensillum (Hiroi *et al.* 2002). Detection of bitter compounds is also mediated primarily through one GRN in I-type and S-type sensilla (Meunier *et al.* 2003; Weiss *et al.* 2011).

Based on reporter expression in I- and S-type sensilla on the labellum, >33 genes encode GRs that are likely to function in bitter sensation, and they appear to compartmentalize into groups corresponding to the functional classes (Weiss *et al.* 2011). Currently, only five bitter-responsive GRs have been characterized through analyses

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of loss-of-function mutations. Three (GR32a, GR33a, and GR66a) are required broadly for responding to most aversive compounds and may be coreceptors (Moon *et al.* 2006; Moon *et al.* 2009; Lee *et al.* 2010). In addition to these "core-bitter GRs" (Weiss *et al.* 2011), two GRs (GR93a and GR8a) are narrowly tuned and are required for the responses to caffeine and the toxic amino acid, L-canavanine, respectively (Lee *et al.* 2009; Lee *et al.* 2012).

In insects, the receptor requirement for strychnine is enigmatic. With the exception of strychnine, the behavioral repulsion and electrophysiological responses to other deterrent tastants depend on GR32a, GR33a, and GR66a (Moon et al. 2006, 2009; Lee *et al.* 2010). However, the effects on the strychnine response resulting from mutations in the core-bitter *Gr*s are complex. Loss of any of these receptors virtually eliminates strychnine-induced action potentials. Surprisingly, strychnine repulsion is normal in either *Gr33a* or *Gr66a* mutants and only moderately reduced in *Gr32a* mutants. Thus, no GR has been defined that is essential for strychnine rejection.

Here, we found that mutation of *Gr47a* profoundly reduced both the behavioral and electrophysiological responses to strychnine. *Gr47a*^{1} mutants responded normally to other repellent compounds tested, indicating that GR47a is a relatively narrowly tuned strychnine receptor. We propose that GR47a is a receptor that imparts strychnine specificity.

Materials and methods

Drosophila stocks

We reported the following mutants previously and deposited them in the Bloomington Stock Center: *Gr33a*^{1}, *UAS-Gr33a*, *UAS-Gr66a*, and *Gr66*^{ex83} (Moon *et al.* 2006, 2009). H. Amrein provided the *ΔGr32a* and *UAS-Gr32a* (Miyamoto and Amrein 2008) and the P[*Gr66a-GAL4*] flies (Thorne *et al.* 2004). K. Scott provided the P[*Gr47a*-I-*GFP*] and P[*Gr47a-GAL4*] flies (Wang *et al.* 2004). J.Y. Kwon provided the P[*Gr8a-GAL4*], P[*Gr36a-GAL4*], P[*Gr39b-GAL4*], P[*Gr59c-GAL4*], P[*Gr93a-GAL4*], and P[*Gr98b-GAL4*] flies (Weiss *et al.* 2011). We obtained the *Gr47a* deficiency line, Df(2R)12, from the Bloomington Stock Center (stock 5425). We used *w*^{1118} as the "wild-type" control.

Generation of mutant and transgenic fly lines

We used ends-out homologous recombination (Gong and Golic 2003) to generate the deletion in *Gr47a*^{1}, which removed residues −166 to +291. To obtain the knockout construct, we amplified two 3-kb genomic fragments by polymerase chain reaction (PCR), and then subcloned the DNAs into the pw35 vector (Gong and Golic 2003). We generated the original transgenic insertion lines using germline transformation (BestGene Inc.) and obtained homologous recombinants as described (Gong and Golic 2003). We confirmed the homologous insertions by PCR using primer pairs P1/P2 and P3/P4 (Figure 1A): P1, 5′-TGGCCTGACCCAAAGGCCTATAAA-3′; P2, 5′-TCAGAA CAGTCACACTCACACGCA-3′; P3, 5′-TGAACTGGAATATGGGC GAACCCT-3′; P4, 5′-GCGCTTGTTTGTTTGCTCAGCTTG-3′.

To obtain the *UAS-Gr47a* transgene, we amplified the full-length *Gr47a* cDNA by reverse transcription polymerase chain reaction (RT-PCR) using fly labellar mRNA. We subcloned the cDNA into the pUAST vector (Brand and Perrimon 1993) and verified the cDNA by DNA sequencing. The transformation vector was injected into *w*^{1118} embryos (BestGene Inc.). The *Gr47a* genomic transgene, P[g*Gr47a*], was 19639-bp long and extended to 3330 and 14799 bp 5′ and 3′ of the predicted transcribed region. To generate this transgene, we subcloned the genomic region from P[*acman*] CH322-152A22 (www.pacmanfly.org) into the insertion site of the *attP154* on the 3rd chromosome (BestGene Inc.).

RT-PCR analyses

We used TRIzol (Invitrogen) to extract mRNA from the labella, wings, abdomens, and legs and AMV reverse transcriptase to generate the cDNAs (Promega). To perform the RT-PCR, we used the following *Gr47a* primers: 5′-ATGGCCTTTACCAGCTCGCA-3′; 5′- GCGAACATGGAGAGCAAACG-3′. The following were the *tubulin* primers: 5′-TCCTTCTCGCGTGTGAAACA-3′; 5′- CCGAA CGAGTGGAAGATGAG-3′. The *Gr47a* RT-PCR products were obtained after 40 cycles.

Immunohistochemistry

The labella of *Gr66a-GAL4*/*Gr47a*-I-*GFP*;*UAS-DsRed*/+ flies were dissected and fixed using 4% paraformaldehyde with 0.2% Triton X-100 in phosphate buffered saline (PBS-T) for 15 min at room temperature. We then washed the labella three times with PBS-S (1× PBS and 0.2% saponin), cut them in half with a razor blade, and blocked the samples with blocking buffer (1× PBS, 0.1% saponin and 5 mg/ml bovine serum albumin) for 4 h at 4 °C. Primary antibodies, anti-GFP green fluorescent protein (GFP) (Molecular Probe, mouse anti-GFP, cat. no. A11120, 1:1 000) and anti-DsRed (Clontech, rabbit anti-DsRed, catalogue no. 632496, 1:1 000), were added to fresh blocking buffer and incubated with the labella overnight at 4 °C. The labella were washed three times with PBS-T at 4 °C and incubated with secondary antibodies (goat anti-mouse Alexa488 and goat anti-rabbit Alexa568, 1:200) for 4 h at 4 °C. The tissues were washed three times with PBS-T and mounted in mounting buffer (37.5% glycerol, 187.5 mM NaCl, 62.5 mM Tris pH8.8). The samples were viewed using a Zeiss LSM700 confocal microscope.

Chemicals

Sucrose, caffeine, denatonium, lobeline, papaverine, quinine, strychnine, theophylline, umbelliferone, sulforhodamine B, and KCl were purchased from Sigma-Aldrich Co. Berberine sulfate trihydrate and Brilliant Blue FCF were obtained from Wako Pure Chemical Industries Ltd.

Two-way choice behavioral assays

We performed the two-way choice assays as described previously (Meunier *et al.* 2003; Moon *et al.* 2006). Briefly, we starved 50–70 flies (3–6 days old) for 18 h in a humidified chamber, and then introduced the animals into 72-well microtiter dishes. We filled alternating wells with 1% agarose combined with one of two types of test mixtures: 1-mM sucrose or 5-mM sucrose plus an avoidance chemical. To monitor food intake, one test mixture contained a blue dye (brilliant blue FCF, 0.125 mg/ml), whereas the other contained a red dye (sulforhodamine B, 0.2 mg/ml). We allowed the flies to feed for 90 min at room temperature in the dark and froze the animals at −20 °C. The numbers of flies that were blue (N^B), red (N^R), or purple (N^P) were determined in a blind fashion based on the colors of the abdomen. The preference index (PI) values were calculated according to the following equation: $(N^B + 0.5N^P) / (N^R + N^B + N^P)$ or $(N^R + 0.5N^P)/(N^R + N^B + N^P)$, depending on the dye/tastant combinations. PIs equal to 1.0 and 0 indicated complete preferences for either 1 or 5 mM sucrose plus an avoidance chemical, respectively. A PI = 0.5 indicated no bias between the two food alternatives.

Proboscis extension response assays

We performed the proboscis extension response (PER) assays as described (Shiraiwa and Carlson 2007) with minor modifications. We starved the flies by placing them in an empty vial for 18–24 h

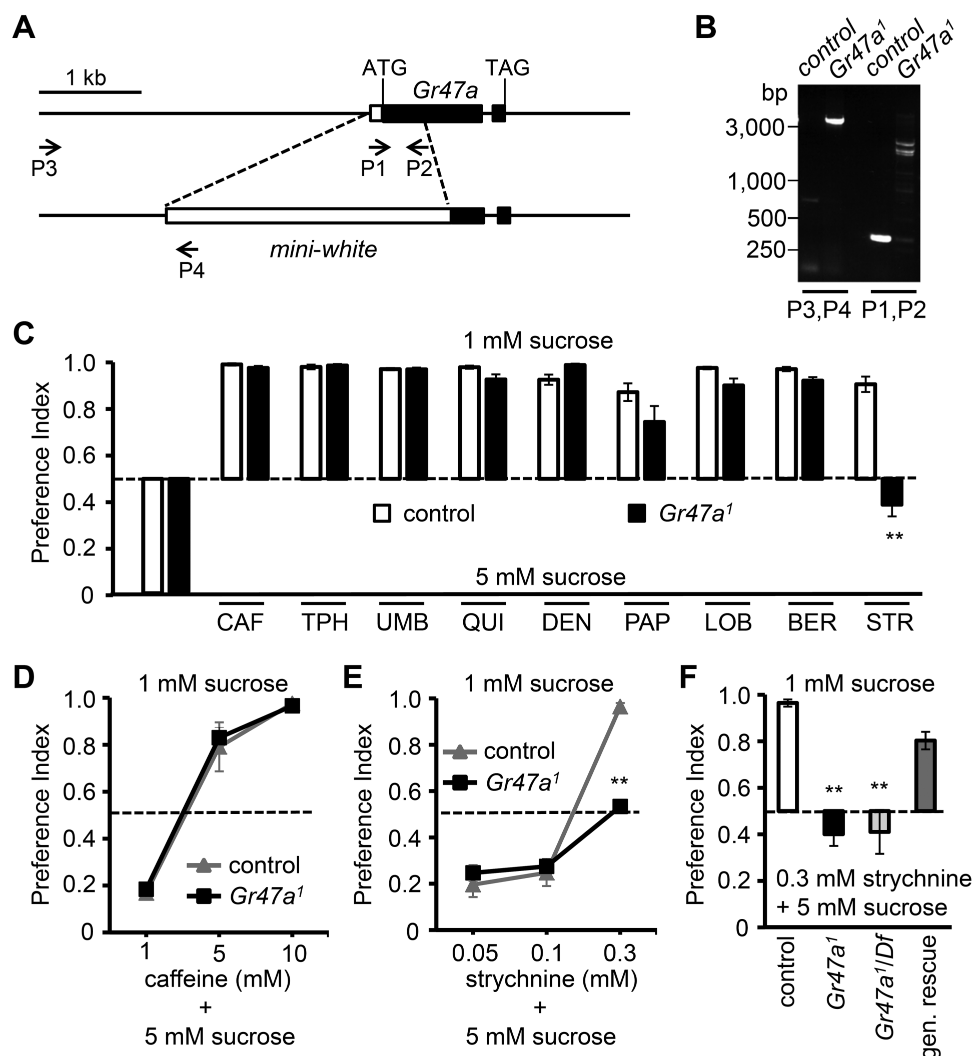

Figure 1. *Gr47a* is required for behavioral avoidance to strychnine. (**A**) Physical map of the *Gr47a* genomic region. *Gr47a[1]* was generated by ends-out homologous recombination. The white box indicates the *mini-white* gene. The arrows indicate the primers used for the PCR analyses in **B**. (**B**) PCR analyses of genomic DNA. The PCR products, which were generated using DNA prepared from control or *Gr47a[1]* flies and the indicated PCR primer pairs, were fractionated on an agarose gel. The 3-kb product produced using the P3 and P4 primers indicated successful targeting. The 300-bp product using the P1 and P2 primers confirms the genomic deletion. (**C**) Avoidance of noxious compounds. The flies were given a choice between 1-mM sucrose and 5-mM sucrose plus the following aversive compounds: 10-mM caffeine (CAF), 10-mM theophylline (TPH), 5-mM umbelliferone (UMB), 1-mM quinine (QUI), 0.2-mM denatonium (DEN), 1-mM papaverine (PAP), 0.3-mM lobeline (LOB), 0.05-mM berberine (BER), and 0.3-mM strychnine (STR). n = 4–8. The control flies used here and throughout this work were w^{1118}. (**D**) Concentration-dependent avoidance of caffeine in control and *Gr47a[1]* flies. n = 4. (**E**) Concentration-dependent avoidance of strychnine in control and *Gr47a[1]* flies. n = 4. (**F**) Strychnine-avoidance behavior exhibited by *Gr47a[1]* homozygous flies, *Gr47a[1]* placed in trans with a deficiency (Df) that uncovered the *Gr47a[1]* mutation, and rescue of the strychnine sensation defect in *Gr47a[1]* using a $Gr47a^+$ genomic rescue transgene. n = 4–7. The error bars represent SEMs. The asterisks indicate significant differences from control flies (P < 0.01) using single factor ANOVA with Scheffe's analysis as a post hoc test to compare two sets of data.

with a piece of Kimwipe soaked with water. We removed the fly from the vial with an aspirator, placed it in a 200-µl yellow tip, and moved it to the end of the tip by applying air. Using a razor blade, we increased the opening of the 200-µl yellow tip. The proboscis extended out of the tip opening. Before testing any bitter tastants, we offered a 2% sucrose solution to the fly. If the fly did not respond to the 2% sucrose, we discarded it. We also tested the flies' response to water, which served as a negative control to make sure that the flies were not responding to water alone. We then offered each fly 2% sucrose (1st exposure), 2% sucrose plus 1-mM strychnine (1st exposure), 2% sucrose (2nd exposure), and 2% sucrose plus 1-mM strychnine (2nd exposure). We applied water to the flies between each for the preceding food offers.

Electrophysiological responses of GRNs to tastants

We performed tip recordings as described (Moon *et al.* 2006) using 10-mM caffeine, 10-mM theophylline, 10-mM umbelliferone, 1-mM denatonium, 1-mM lobeline, 1-mM papaverine, 1-mM quinine, 1-mM strychnine, and 0.1-mM berberine. We immobilized newly eclosed flies by inserting a glass capillary filled with Ringer's solution into the abdomen, which we extended into the head. This electrode also functioned as the indifferent electrode. We stimulated the sensilla with a recording pipet (10- to 20-µm tip diameter) containing the tastants dissolved in 1-mM KCl, which served as the electrolyte in all recordings. The recording electrode was connected to a preamplifier (TastePROBE, Syntech, Hilversum, The Netherlands), and we collected and amplified the signals 10× using

a signal connection interface box (Syntech) in conjunction with a 100- to 3000-Hz band-pass filter. Recordings of action potentials were acquired using a 12-kHz sampling rate and analyzed using Autospike 3.1 software (Syntech).

Statistical analyses

All error bars represent standard error of the means (SEMs). Single factor ANOVA with Scheffe's analysis as a post hoc test was used to compare multiple sets of data. Asterisks indicate statistical significance (* $P < 0.05$, ** $P < 0.01$).

Results

Strychnine behavioral avoidance depended on *Gr47a¹*

To dissect the roles of GRs, we generated a mutation in the *Gr47a* gene, which encodes a protein (GR47a) that belongs to one of the remaining uncharacterized clades within the GR phylogenetic tree. This branch contains GRs that are distantly related to any of the functionally analyzed GRs, including receptors required for the responses to sugars and bitter compounds. To disrupt *Gr47a*, we used ends-out homologous recombination. We created *Gr47a¹* by

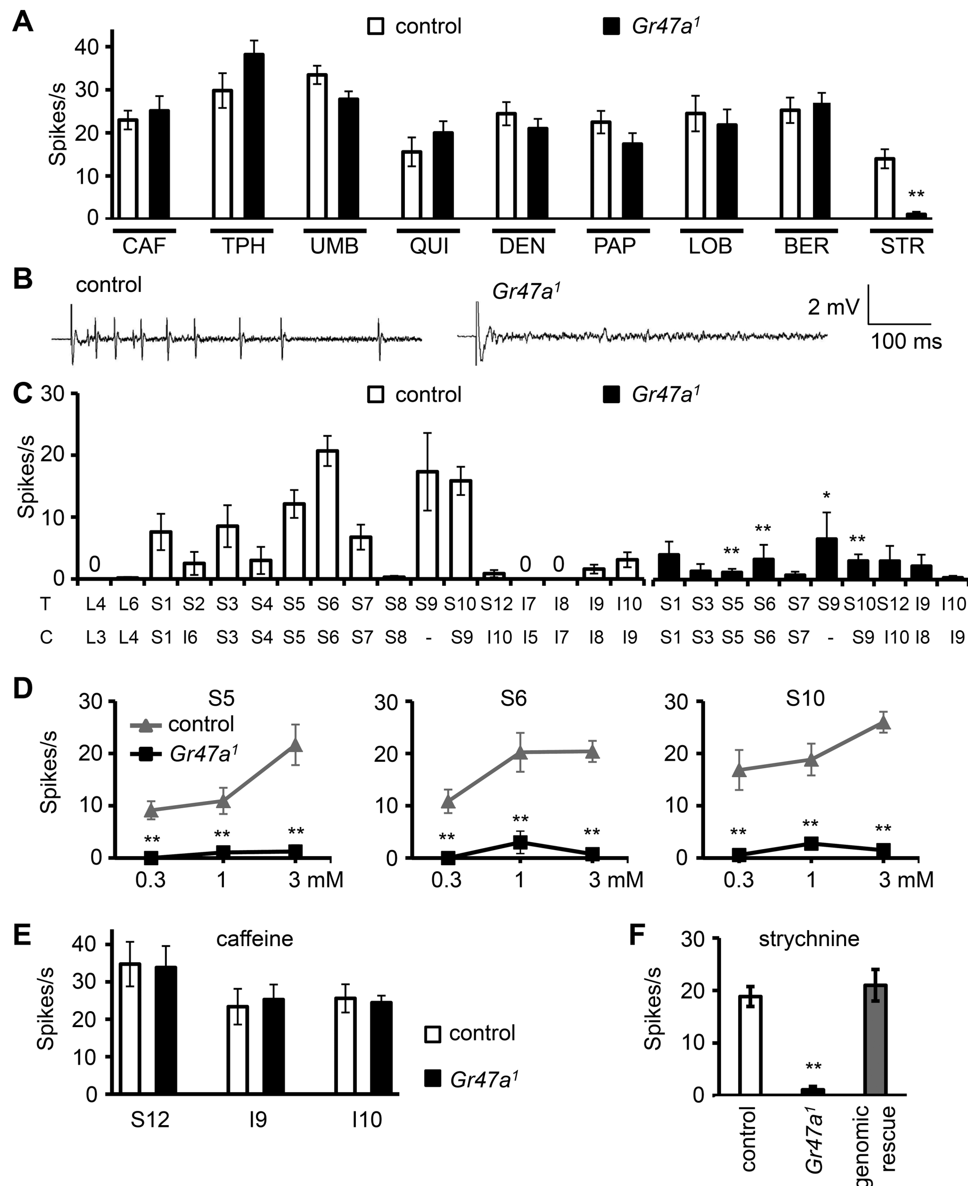

Figure 2. *Gr47a* was indispensible for strychnine-induced nerve firings. (**A**) Tip recordings were performed on S6 bristles on the labella. Average frequencies of action potentials (spikes/s) to 10-mM caffeine, 10-mM theophylline, 10-mM umbelliferone, 1-mM quinine, 1-mM denatonium, 1-mM papaverine, 1-mM lobeline, 0.1-mM berberine, and 1-mM strychnine are shown. $n = 7–12$. (**B**) Representative traces of strychnine-evoked action potentials from control (w^{1118}) and *Gr47a¹* flies. (**C**) Responses of different sensilla to 1-mM strychnine in control and *Gr47a¹* labella. $n = 6–17$. Two nomenclature systems are used to identify the sensilla: Tanimura study (T) (Hiroi *et al.* 2002) and Carlson study (C) (Weiss *et al.* 2011). (**D**) Tip recordings performed using the indicated concentrations of strychnine on S5, S6, and S10 bristles. $n = 7–22$. (**E**) Average frequencies of action potentials (spikes/s) induced by 10-mM caffeine upon application to the indicated sensilla. $n = 8–11$. (**F**) Average frequencies of action potentials (spikes/s) induced by 1-mM strychnine on S6 sensilla using the indicated fly strains. $n = 8–20$. The error bars represent SEMs. The asterisks indicate significant differences from control flies (** $P < 0.01$, * $P < 0.05$) using single factor ANOVA with Scheffe's analysis as a post hoc test.

deleting ~500 base pairs extending 166 base pairs 5′ of the predicted transcription start site through the region coding the N-terminal 97 residues of the 361 amino acid protein (Figures 1A,B).

To identify a deficit in *Gr47a[1]*, we performed two-way choice tests. Normally, flies choose 5- over 1-mM sucrose (Figure 1C). However, addition of bitter compounds eliminates the preference of control animals for the higher concentration of sugar (Figure 1C). The mutant flies also exhibited normal repulsion to most bitter compounds, including caffeine, quinine, and six others (Figures 1C,D). Among the compounds tested, *Gr47a* was specifically required for inhibiting consumption of strychnine (Figures 1C,E). The impairment in avoiding strychnine was due to mutation of *Gr47a* because we recapitulated the phenotype when we placed *Gr47a[1]* in trans with a deficiency that removed *Gr47a* (Figure 1F). Furthermore, we rescued the phenotype with a wild-type *Gr47a* genomic transgene demonstrating that the defect was due to mutation of *Gr47a* (Figure 1F). Thus, *Gr47a* was narrowly required for strychnine sensation.

Elimination of strychnine-induced action potentials in *Gr47a[1]* flies

We performed tip recordings to assess bitter compound–induced action potentials in *Gr47a[1]* flies. We recorded from S6 sensilla,

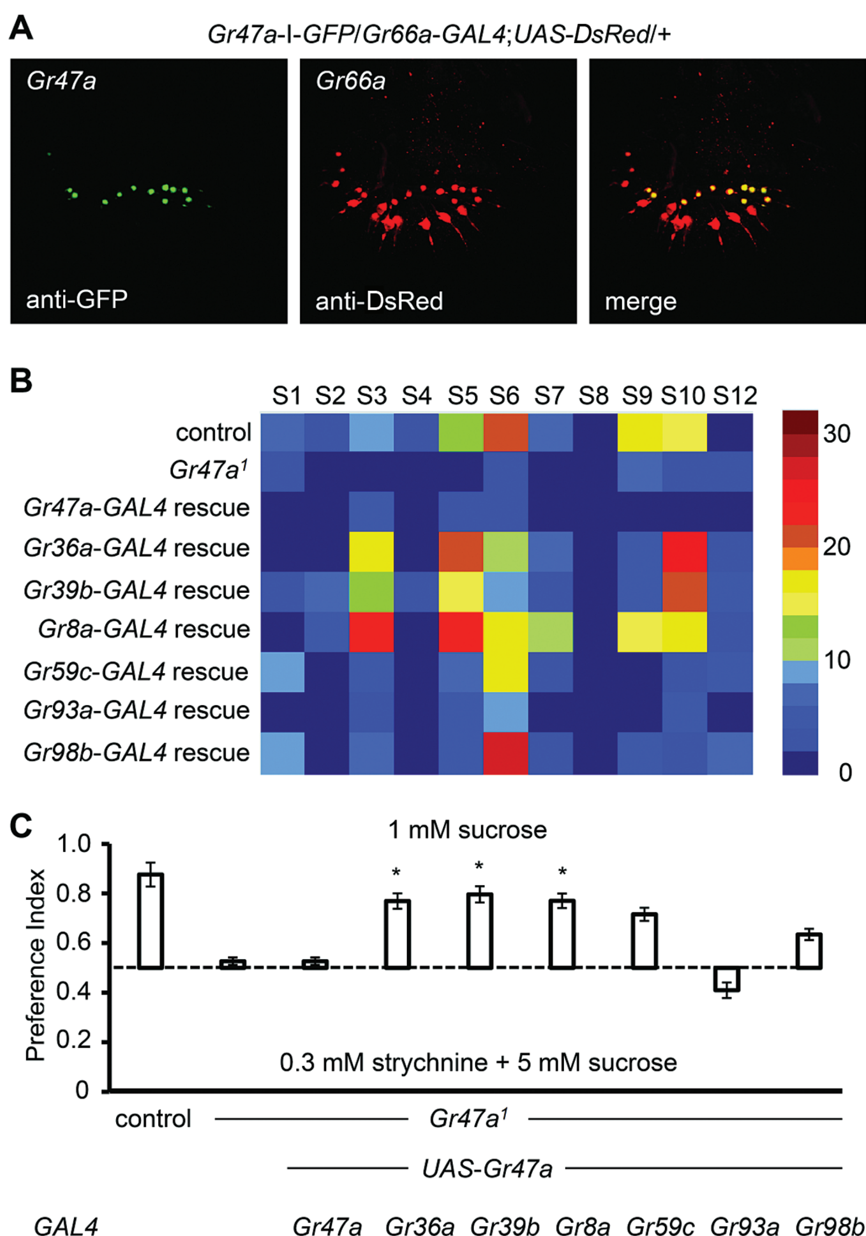

Figure 3. Testing for rescue of strychnine repulsion by expression of *Gr47a* in different sensilla. (**A**) The *Gr47a*-I-*GFP* reporter was expressed in a subset of *Gr66a-GAL4* positive GRNs. Left panel, *Gr47a*-I-*GFP* labeled a subset of bitter GRNs (anti-GFP, green). Middle panel, DsRed was expressed using the *Gr66a-GAL4* and the *UAS-DsRed* transgenes (anti-DsRed, red). The *Gr66a-GAL4* reporter labeled all bitter GRNs. Right panel, merged image of left and middle panels. (**B**) Recovery of the strychnine-induced action potentials in the indicated sensilla in *Gr47a[1]* after expression of *UAS-Gr47a* under control of the indicated *GAL4* drivers. $n = 10$. (**C**) Rescue of the strychnine taste defect in *Gr47a[1]* after expression of *UAS-Gr47a* under control of *GAL4* drivers, which label different sensilla (Weiss *et al.* 2011). $n = 5$. The error bars represent SEMs. The asterisks indicate significant differences from *Gr47a[1]* (*$P < 0.05$) using single factor ANOVA with Scheffe's analysis as a post hoc test.

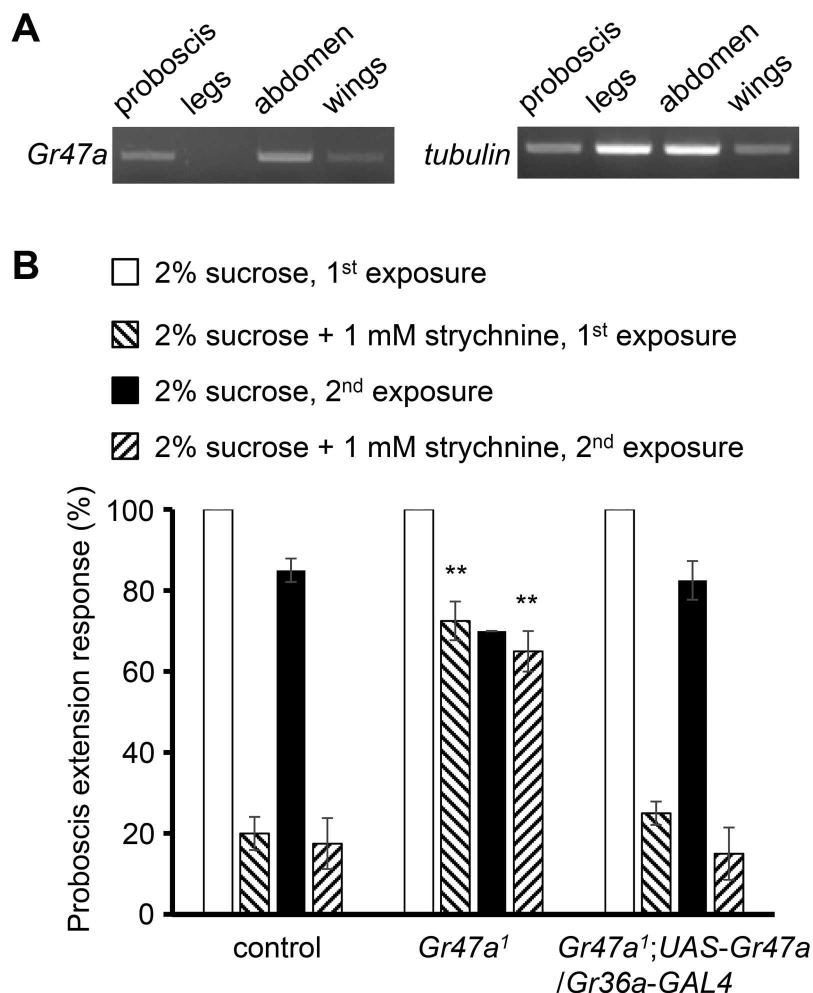

Figure 4. *Gr47a* expression in various body parts and PER assays. (**A**) *Gr47a* expression in the proboscis, legs, abdomen, and wings analyzed by RT-PCR. We also amplified *tubulin* products by RT-PCR, which served as a quality control for the quality of the RNA. (**B**) PER assays performed using the indicated flies. Either 2% sucrose alone or 2% sucrose and 1-mM strychnine were applied to the labella, and the fraction of flies that elicited a PER were determined. Ten flies/experiment were used ($n = 4$). The error bars represent SEMs. The asterisks indicate significant differences from *Gr47a*[1] (**$P < 0.01$) using single factor ANOVA with Scheffe's analysis as a post hoc test to compare two sets of data.

which responded to most aversive tastants including caffeine and strychnine (Weiss *et al.* 2011). Consistent with the behavior, *Gr47a*[1] displayed normal frequencies of action potentials to all bitter compounds tested, except for strychnine (Figures 2A,B).

We surveyed the strychnine responses of S-type sensilla in control and the *Gr47a*[1] flies because S-type but not I- or L-type sensilla are activated by strychnine (Weiss *et al.* 2011). As previously reported, the L- and I-type sensilla were unresponsive to strychnine, whereas multiple S-type sensilla were activated by strychnine (Figure 2C; note that two nomenclature systems are reported and we employ the classical one from the Tanimura group) (Hiroi *et al.* 2002; Weiss *et al.* 2011). S1, S3, and S5 were moderately responsive to strychnine, whereas S6, S9, and S10 were most robustly activated. However, S9 were present in only a subset of labella. Of significance here, we found that strychnine-induced action potentials were severely reduced in *Gr47a*[1] mutant flies (Figures 2A–C), even at the highest concentrations tested (Figure 2D). Three other *Gr47a*[1] sensilla surveyed also showed normal caffeine responses (Figure 2E). We fully rescued this electrophysiological defect with a genomic transgene (Figure 2F).

Rescue of strychnine behavior

There are two *Gr47a* gene reporters, one of which stains just three sensilla (*Gr47a-GAL4*, S12, I8, and I9), whereas the other (*Gr47a*-I-*GFP*) is detected in many more sensilla (Wang *et al.* 2004; Weiss *et al.* 2011). We recapitulated these observations, because we found that 3.2 ± 0.6 ($n = 12$) GRNs were labeled using the *Gr47a-GAL4* driver in combination with the *UAS-GFP* (Weiss *et al.* 2011), whereas the *Gr47a*-I-*GFP* line stained 14.8 ± 0.2 ($n = 7$) GRNs per labellum (Figure 3A). *Gr47a*-I-*GFP* overlapped partially with the *Gr66a-GAL4* reporter, which was ubiquitously expressed in bitter responsive GRNs, and labeled all I-type and half of S-type sensilla. Thus, the limited expression of the *Gr47a-GAL4* driver might not reflect the *bona fide* cellular distribution of *Gr47a*. Consistent with this conclusion, we could not rescue the strychnine deficit in *Gr47a*[1] flies using *Gr47a-GAL4* in combination with *UAS-Gr47a* (Figure 3B; Supplementary Figure 1), and the three *Gr47a-GAL4* positive sensilla (S12, I8, and I9) were unresponsive to strychnine (Figure 2C).

Because *Gr47a*-I-*GFP* is a direct fusion of the *Gr47a* promoter to GFP, we could not use this line for rescue experiments. Therefore, we drove *UAS-Gr47a* using other *GAL4* lines that

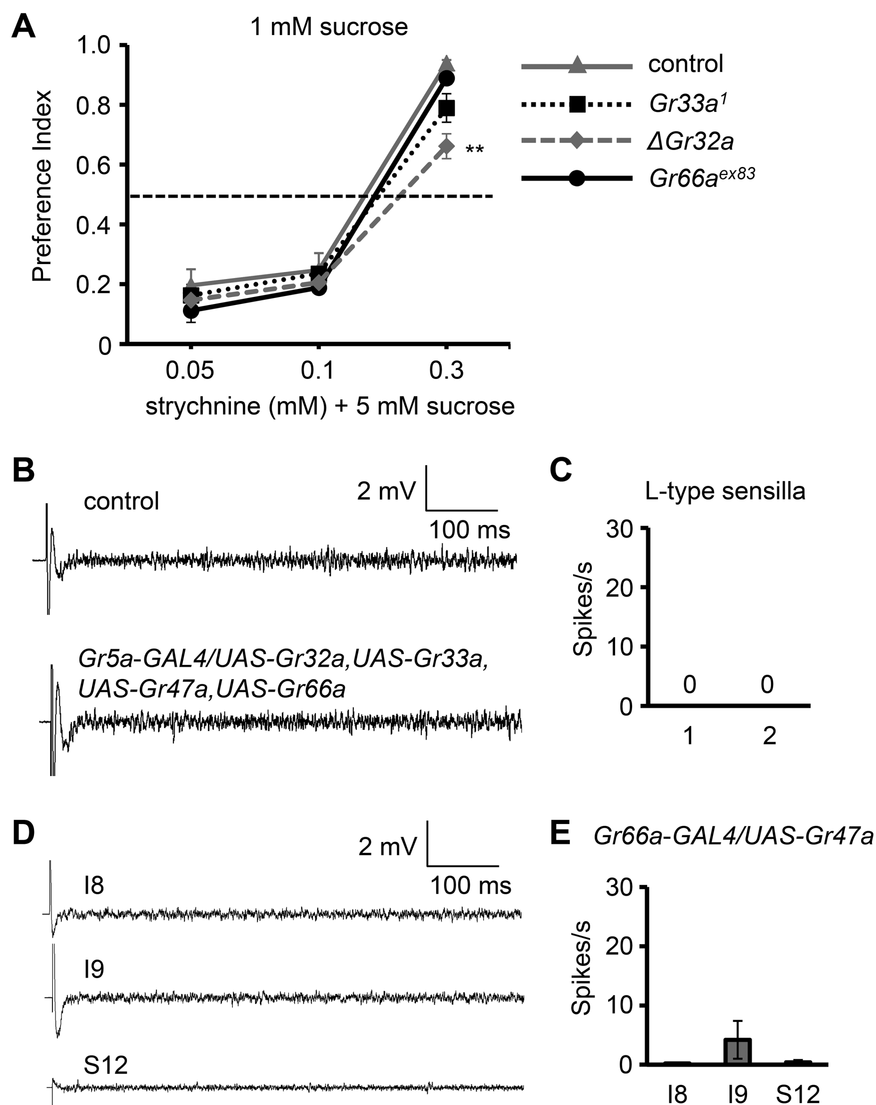

Figure 5. Analyses of strychnine repulsion exhibited by Δ*Gr32a*, *Gr33a¹*, and *Gr66a^ex83^* flies. (**A**) Binary food choice assays using 1-mM sucrose versus 5-mM sucrose laced with different concentrations of strychnine. *n* = 4–7. (**B**) Ectopic expression of *Gr32a*, *Gr33a*, *Gr47a*, and *Gr66a* in *Gr5a*-expressing GRNs using the *GAL4*/*UAS* system was insufficient to produce strychnine-induced action potentials. Shown are sample traces from L4 sensilla in response to 1-mM strychnine. (**C**) Average number of spikes/s in L4 sensilla of control flies (lane 1) or flies expressing *UAS-Gr32a*, *UAS-Gr33a*, *UAS-Gr47a*, and *UAS-Gr66a* under the control of the *Gr5a-GAL4* (lane 2; *n* = 11). (**D**) Expression of *UAS-Gr47a* in I8, I9, and S12 using the *Gr66a-GAL4* was not sufficient to generate strychnine-induced action potentials. Sample traces from the indicated sensilla are shown. (**E**) Average number of spikes/s in the indicated sensilla of flies expressing *UAS-Gr47a* under the control of the *Gr66a-GAL4* (*n* = 10).

were expressed in different subsets of bitter-responsive sensilla. We restored strychnine-induced action potentials and strychnine aversion in the *Gr47a¹* mutant flies using *Gr36a-GAL4*, *Gr39b-GAL4*, or *Gr8a-GAL4* (Figures 3B,C; Supplementary Figure 1). These drivers were detected exclusively in sensilla (S3, S5, and S10) (Weiss *et al.* 2011) that respond to strychnine (Figure 2C). We obtained robust rescue in S6 sensilla only, as a result of expressing *UAS-Gr47a* under the control of *Gr59c-GAL4* and *Gr98b-GAL4* (Figure 3B; Supplementary Figure 1). However, when we used the *Gr93a-GAL4* to perform rescue experiments, we observed few action potentials in S6 sensilla (Figure 3B; Supplementary Figure 1), even though the *Gr93a-GAL4* expression pattern was similar to *Gr98b-GAL4* (Weiss *et al.* 2011). These findings suggested that the *Gr93a-GAL4* was a weak driver relative to the *Gr98b-GAL4*.

Strychnine avoidance dependent on *Gr47a* in the labellum

Drosophila include taste sensilla distributed on multiple body parts other than labella, including the legs, wings, and female oviposter, the latter of which is located at the tip of the female abdomen. To address whether *Gr47a* was expressed in any of these extralabellar portions of the fly, we manually dissected various body parts and performed RT-PCR. As expected, we detected a signal in the proboscis, which included the two bilaterally symmetrical labella (Figure 4A). In addition, we found that *Gr47a* RT-PCRs were produced in the abdomen and wings (Figure 4A). However, we did not detect a signal in the legs (Figure 4A). Consistent with this latter result, the *Gr47*-I-*GFP* reporter did not stain the tarsi.

The preceding data suggested that the behavioral avoidance to strychnine resulted from *Gr47a*-dependent sensation in the

labellum. However, taste sensilla in both the legs and labella influence responses using two-way choice assays. Therefore, to address whether strychnine avoidance depended on *Gr47a* expression in the labellum specifically, we assayed PERs, by applying sucrose alone or sucrose plus strychnine to the labellum. Nearly all control flies extended their probosci in response to application of 2% sucrose, and this response was diminished only slightly upon presentation of sucrose a second time (Figure 4B). Addition of 1-mM strychnine greatly suppressed the PER (Figure 4B). The *Gr47a¹* flies also exhibited robust PERs to 2% sucrose (Figure 4B). However, the suppression by 1-mM strychnine was severely impaired in the mutant animals (Figure 4B). We rescued this defect by expressing *UAS-Gr47a* under control of the *Gr36a-GAL4* (Figure 4B).

Behavioral requirement for *Gr47a* is distinct from broadly required *Gr*s

We showed previously that three core-bitter *Gr*s (*Gr32a*, *Gr33a*, and *Gr66a*) are required for strychnine-induced action potentials (Moon *et al.* 2009; Lee *et al.* 2010). However, *Gr33a* mutant flies show normal behavioral repulsion to strychnine over a range of concentrations (Moon *et al.* 2009). Loss of *Gr66a* also does not appear to cause defects in strychnine rejection, whereas mutation of *Gr32a* results in a modest impairment (Lee *et al.* 2010).

Because our previous studies on $Gr66a^{ex83}$ and *ΔGr32a* tested only one concentration of strychnine, we reexamined these latter mutants using a range of strychnine levels. We found that only *ΔGr32a*, but not $Gr33a^{1}$ and $Gr66a^{ex83}$, had a relatively small defect in strychnine avoidance (Figure 5A). Thus, $Gr47a^{1}$ was unique as it was the only mutant that displayed a strong deficit in strychnine aversion. Nevertheless, because four GRs contribute to strychnine-induced action potentials, we considered whether these GRs are sufficient to elicit a strychnine response. We misexpressed the four *Gr*s in sugar-responsive GRNs using the *Gr5a-GAL4* in combination with *UAS-Gr66a*, *UAS-Gr33a*, *UAS-Gr32a*, and *UAS-Gr47a*. However, this manipulation did not induce action potentials in sugar-responsive GRNs upon application of strychnine (Figures 5B,C). Because *Gr66a*, *Gr33a*, and *Gr32a* were present in S12, I8, and I9 sensilla (Weiss *et al.* 2011), we ectopically expressed *UAS-Gr47a* using the *Gr66a-GAL4*. However, introduction of *Gr47a* in these sensilla did not result in significant strychnine-induced action potentials (Figures 5D,E).

Discussion

We found that *Gr47a* was required for the response to strychnine and not any of eight other chemicals tested. This observation, combined with prior genetic analyses of other bitter *Gr*s, supports the emerging model that Drosophila GRs fall into two general specificity classes. The first class is comprised of GRs that are broadly required, and this group of core-bitter receptors includes GR32a, GR33a, and GR66a (Moon *et al.* 2009; Lee *et al.* 2010). The second specificity class consists of GRs that are narrowly tuned and now includes three members: (1) GR47a, (2) GR93a, which is required for sensing caffeine only (Lee *et al.* 2009), and (3) GR8a, which is required for the response to the toxic amino acid derivative, L-canavanine (Lee *et al.* 2012). Nevertheless, it seems very likely that the narrowly tuned receptors, including GR47a, are responsive to more than just one tastant, as the number of GRs expressed in bitter responsive GRNs is limited.

The identification of GRs with broad and narrow specificities is consistent with a comprehensive electrophysiological analysis of the activities of bitter-responsive sensilla (Weiss *et al.* 2011). According to this study, there are two broadly tuned and two narrowly tuned specificity classes. Moreover, the expression patterns of the *Gr* reporters largely support the existence of these functional categories, as expression of many *Gr* reporters is limited to one of the four classes. Exceptions are *Gr32a*, *Gr33a*, and *Gr66a*, which are expressed in all four classes and are broadly required for sensing nearly all bitter compounds.

We conclude that introduction of *Gr47a* in bitter-responsive GRNs in S3, S5, and S10 was sufficient to restore normal strychnine responsiveness, because we rescued both the behavioral and electrophysiological impairments in the $Gr47a^{1}$ mutant by expression of the wild-type *Gr47a* transgene in these sensilla. The *Gr47a-GAL4* did not appear to reflect the normal expression pattern of *Gr47a* because it was not expressed in strychnine-activated sensilla and was not effective in rescuing the $Gr47a^{1}$ phenotype in combination with *UAS-Gr47a*.

With the exception of strychnine, the behavioral avoidance to every deterrent compound tested is greatly impaired by single mutations in at least two, and in most cases, in any of the three core-bitter *Gr*s (*Gr32a*, *Gr33a*, and *Gr66a*). However, strychnine avoidance was normal in flies missing either *Gr33a* or *Gr66a* and only moderately reduced in *Gr32a* mutant flies. Furthermore, misexpression of GR47a with the core receptors, GR32a, GR33a, and GR66a was insufficient to recapitulate a strychnine response in sugar-responsive GRNs. Moreover, introduction of *Gr47a* in bitter-sensing GRNs that express *Gr32a*, *Gr33a*, and *Gr66a*, but do not normally respond to strychnine, was ineffective at conferring strychnine responsiveness. These latter findings further highlight the complexity of most Drosophila GR complexes.

Supplementary Material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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Conflict of interest

The authors have no conflicts of interest to declare.

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