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Molecular sensor of nicotine in taste of *Drosophila melanogaster*

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ABSTRACT

Nicotine is an alkaloid and potent parasympathomimetic stimulant found in the leaves of many plants including *Nicotiana tabacum*, which functions as an anti-herbivore chemical and an insecticide. Chemoreceptors embedded in the gustatory receptor neurons (GRNs) enable animals to judge the quality of bitter compounds and respond to them. Various taste receptors such as gustatory receptors (GRs), ionotropic receptors (IRs), transient receptor potential channels (TRPs), and pickpocket channels (PPKs) have been shown to have important roles in taste sensation. However, the mechanism underlying nicotine taste sensation has not been resolved in the insect model. Here we identify molecular receptors to detect the taste of nicotine and provide electrophysiological and behavioral evidence that gustatory receptors are required for avoiding nicotine-laced foods. Our results demonstrate that gustatory receptors are reasonable targets to develop new pesticides that maximize the insecticidal effects of nicotine.

1. Introduction

Bees cannot taste neonicotinoids and are not repelled by them, so unfortunately, these important insect pollinators are exposed to the neonicotinoids that are sprayed on crops (Kessler et al., 2015). In order to develop insecticides that target only harmful insects, it is important to understand the mechanisms of how nicotine is sensed. Nicotine is most commonly known as the substance in cigarettes that lends them their addictive properties (Changeux, 2010). In addition, nicotine has an anorexic effect and can elicit weight loss in mouse models (Mineur et al., 2011). Interestingly, abuse of nicotine reduces taste sensitivity (Bigiani, 2015; Enoch et al., 2001); however, despite several studies focused on the taste sensation of nicotine (Bigiani, 2015; Enoch et al., 2001; Mineur et al., 2011), the molecular sensor for nicotine remains enigmatic.

The chemosensors embedded in the gustatory receptor neurons (GRNs) impart the sensation of chemicals in the environment, which enables the animals to judge the quality of foods in nature. Chemicals are sensed via transduction of stimuli in a channel gated mechanism, which allows the organism to promptly respond to the chemicals. *Drosophila* chemosensors are distributed all over the body including labellum, legs, ovipositor, and wing margin (Lee and Poudel, 2014). The major taste organ, the labellum, is enriched with 31 stratified taste sensilla which are denominated according to their lengths, such as long L-type, intermediate I-type, and short S-type. Generally, L and S-type sensilla harbor 4 GRNs, while I-type sensilla harbor 2 GRNs (Hiroi et al., 2002, 2004; Meunier et al., 2003). Chemoreceptors, such as gustatory receptors (GRs), odorant receptors (ORs), ionotropic receptors (IRs), transient receptor potential channels (TRPs), and pickpocket channels (PPKs), are expressed in the GRNs/ORNs and have defined ligand response profiles (Fowler and Montell, 2013; Lee and Poudel, 2014; Rimal and Lee, 2018; Rimal et al., 2019; Vosshall et al., 1999). The *gustatory receptor* (*Gr*) family comprises 60 genes and encodes 68 proteins via alternative splicing in the *Drosophila* genome (Lee and Poudel, 2014). The receptors that are expressed in bitter cells are generally considered bitter receptors, and the genes that are expressed in the sweet cells are considered to encode sweet receptors.

Type II taste receptor cells (Type II TRCs) respond to sweet, bitter, or umami taste stimuli via downstream activation of the TRPM5 channel (Zhang et al., 2003). Thus, type II TRCs are involved in attractive as well as aversive pathways, depending on which taste receptors are expressed. Mammalian TRCs sense bitter tastants via T2Rs, and sweet tastants via T1Rs (Chandrashekar et al., 2006). However, it is still unknown which T2R responds to nicotine.

To unravel the mechanism underlying nicotine taste sensation, we adopted behavioral, genetic, and electrophysiology analyses using *Drosophila* as a model organism. We reveal that taste cells perceive nicotine as a bitter tastant and that *Grs* form a multimeric receptor unit that is essential for sensing nicotine. The narrowly tuned receptor GR10a, along with broadly tuned receptors GR32a and GR33a, are essential for the behavioral response to nicotine. These important insights into the mechanism by which nicotine is sensed in fruit flies may serve

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as a beneficial avenue to develop nicotine based insecticidal targets.

2. Materials and methods

2.1. Drosophila stock and generation of transgenic flies

We obtained *Gr2a*^{1} (BDSC, 18415), *Gr10a*^{1} (BDSC 29947), *Gr22f*^{1} (BDSC 43859), *Gr23a*^{1} (BDSC, 19287), *Gr28b*^{1} (BDSC 24190), *Gr36b*^{1} (BDSC 24608), *Gr36c*^{1} (BDSC 26496), *Gr58b*^{1} (BDSC 29065), *Gr59a*^{1} (BDSC 26125), *Gr77a*^{1} (BDSC 26374), *Gr93d*^{1} (BDSC 27800), *Gr94a*^{1} (BDSC 17550), *Gr97a*^{1} (BDSC, 18949), and Df (1)BSC287 from the Bloomington stock center. We obtained *Gr22e*^{1} (140936) from Kyoto Drosophila stock center. We previously described *Gr8a*^{1} (Lee et al., 2012), *Gr33a*^{1} (Moon et al., 2009), *Gr47a*^{1} (Lee et al., 2015), *Gr66a*^{ex83} (Moon et al., 2006), *Gr93a*^{3} (Lee et al., 2009), *Gr98b*^{1} (Shim et al., 2015), *UAS-Gr33a* (Moon et al., 2009), and *Gr33a*^{GAL4} (Moon et al., 2009) flies and deposited them into the Bloomington stock center. H. Amrein provided the *ΔGr32a, UAS-Gr32a*, and *Gr5a-GAL4* flies. J.Y. Kwon provided the *Gr10a-GAL4* flies. To obtain the *UAS-Gr10a* transgene, we amplified the full-length *Gr10a* cDNA by RT-PCR from proboscis mRNA using following primer pair: 5′-CGAATTCGGAATGACAT CGCCGGA-3′ and 5′-AACTCGAGCTAGGACTTCTTGCGG-3′ and subcloned the cDNA into the pUAST vector. We verified the correct cDNA sequence by DNA sequencing. The transformation vector was injected into *w*^{1118} embryos. We used *w*^{1118} flies as the "wild-type" control.

2.2. Chemical sources

Sucrose, sulforhodamine B, and nicotine were purchased from Sigma-Aldrich Co. Brilliant blue FCF was purchased from Wako Pure Chemical Industry Ltd.

2.3. RT-PCR

We used TRIzol (Invitrogen) to extract mRNA from the labella, and AMV reverse transcriptase (Promega) to generate the cDNA. To perform the RT-PCR, we used the following *Gr10a* primers: 5′-ATGACATCGCC GGATGAGCGT-3′ and 5′-CCAGAGTTATGAAGTAGGAAAAGGC-3'. The *tubulin* primers were 5′-TCCTTGTCGCGTGTGAAACA-3′ and 5′-CCGAA CGAGTGGAAGATGAG-3'. The RT-PCR products were obtained after 40 cycle.

2.4. Electrophysiology

We performed tip recording assays as previously described (Lee et al., 2009). We immobilized freshly enclosed flies by keeping them on ice and then inserted reference glass electrodes filled with Ringer's solution into the thorax of the flies, extending the electrode towards their proboscis. We stimulated the sensilla with tastants dissolved in buffer solution in recording pipettes (10–20 μm tip diameter). We used 1 mM KCl or 30 mM tricholine citrate as the electrolyte for recording. The recording electrode was connected to a preamplifier (Taste PROBE, Syntech, Hilversum, The Netherlands), and the signals were collected and amplified 10x, using a signal connection interface box (Syntech) in conjunction with a 100–3000 Hz band-pass filter. Recordings of action potentials were acquired using a 12-kHz sampling rate and analyzed using Autospike 3.1 software (Syntech).

2.5. Proboscis extension reflex (PER) assay

The Proboscis Extension Reflex assay was performed as previously described (Poudel and Lee, 2016), with a slight modification. The initial stimuli and positive control used was 2% sucrose. Kim-wipe paper wicks were used as media to provide flies with tastant. Wet wicks with either sucrose or experimental solution were brought in contact to the tarsi. Only flies that showed PER with sucrose were used for further experiments. The test solution contained 2% sucrose with 0.5% nicotine. Flies that showed PER with the experimental solution, i.e. 0.5% nicotine in 2% sucrose, were scored as positive PER. The test was performed with 10–12 flies at a time, and the extension of the proboscis was scored as a PER. The test was repeated four times for each strain, i.e. mutant, control, and rescue fly strains.

2.6. Survival assay

We performed a survival assay of flies as we previously described (Lee et al., 2018). We used 10 males and 10 female flies for each experimental set. Flies raised on normal food were considered to be the control group. We performed and observed the viability of flies every 12 h for 6.5 days. For the binary food survival assays, 1% agarose was mixed with 100 mM fructose or 200 mM fructose with 0.1% nicotine, which were placed in a Petri dish on opposite sides. The experiments were terminated after 9 days. Each condition was tested 4–6 times.

2.7. Statistical analysis

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

3. Results

3.1. Flies sense nicotine as an aversive tastant

Nicotine acts as a potential toxin in insects (Fig. 1A). Mice sense nicotine as a bitter tastant, suggesting that flies may also find nicotine aversive (Oliveira-Maia et al., 2009). To uncover the taste response to nicotine in flies, we performed proboscis extension reflex (PER) assays with nicotine across multiple concentrations (Fig. 1B). Wild-type flies showed reduced PER to 0.5% and 1% nicotine compared to lower concentrations such as 0.01% or 0.1% nicotine (Fig. 1B). When we ablated the bitter-sensing GRNs by expressing a pro-apoptotic gene, *hid* (Zhou et al., 1997), in bitter GRNs under the control of a *Gr33a-GAL4* driver line, we found that the nicotine-mediated suppression of the PER is attenuated (Fig. 1C). Conversely, ablating the aversive calcium-sensing GRNs using a *ppk23-GAL4* driver line (Lee et al., 2018) had no effect on the PER response to nicotine. These results suggest that nicotine is sensed by bitter GRNs (Fig. 1C). Furthermore, we found that nicotine induced high frequency action potentials in S-type, but not L-type sensilla, indicating that nicotine directly activates bitter-sensing GRNs, which are housed in the S-type sensilla (Fig. 1 D, E, and F). The increased action potentials are dependent on the dose of nicotine (Fig. 1 E and F). Furthermore, this response is completely suppressed in the S-type sensilla of flies with ablated bitter-sensing GRNs, *UAS-hid/+;Gr33a-GAL4/+* (Fig. 1G). This is consistent with the reduced PER seen in Fig. 1C. The responses to nicotine remain unchanged in flies with ablated sweet sensing (*Gr5a-GAL4*) (Montell, 2009), calcium sensing (*ppk23-GAL4*) (Lee et al., 2018), and water sensing (*ppk28-GAL4*) neurons (Cameron et al., 2010) (Fig. 1G). These results suggest that the mode of action of nicotine in taste is through activation of bitter-sensing GRNs.

3.2. Gustatory receptors are essential for sensing nicotine

Among the 68 GR proteins, only eight sensors participate in the sensation of sweet compounds (Dahanukar et al., 2007; Jiao et al., 2007), while many other receptors are involved in the sensation of bitter chemicals (Lee et al., 2012; Lee and Poudel, 2014; Moon et al., 2006; Sung et al., 2017; Weiss et al., 2011). Due to nicotine's ability to activate bitter-sensing GRNs, we hypothesized that *Grs* are the candidate molecular sensors of nicotine in flies. To identify the peripheral sensor, we tested 21 *Gr* mutants for *Grs* that are primarily expressed in

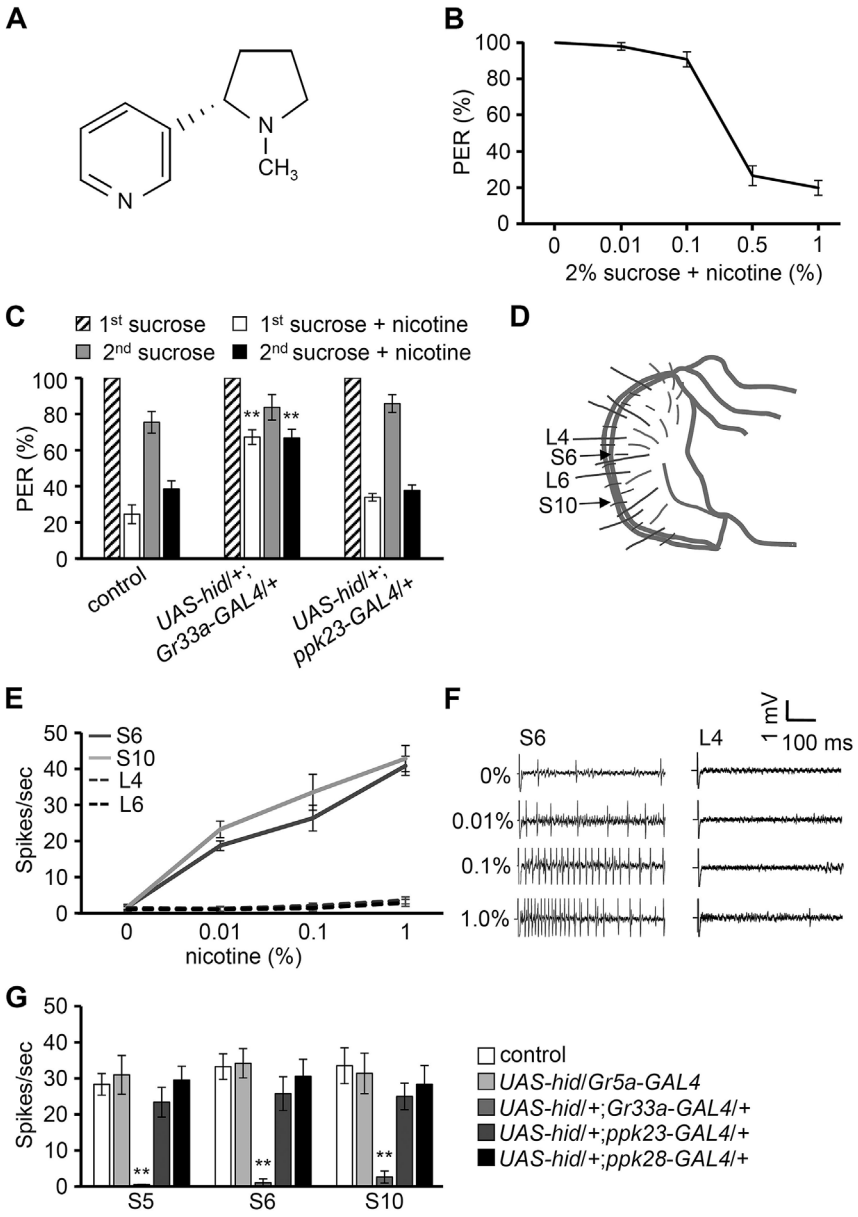

Fig. 1. Nicotine is avoided by the activation of bitter GRNs. (A) Structure of nicotine. (B) Average percentage of proboscis extension reflex (PER) in response to 2% sucrose or 2% sucrose with the indicated concentrations of nicotine. n = 4–6. (C) Average percentage of proboscis extension by control and bitter- or calcium-sensing GRN ablated flies in response to 2% sucrose or 2% sucrose with 0.5% nicotine. n = 4–6. (D) Schematic illustration of taste sensilla decorated on the fly labellum. (E) Average frequency of action potentials elicited from control flies. Four sensilla indicated in D were recorded. n = 12–14. (F) Representative traces obtained from S6 and L4 sensilla. (G) Average frequency of action potentials elicited from control and each GRN ablated flies on three S-type sensilla. n = 12–14. All error bars represent SEMs. Single factor ANOVA with Scheffe's analysis was used as a *post hoc* test to compare multiple sets of data. Asterisks indicate statistical significance (**$P < 0.01$).

bitter-sensing GRNs (Weiss et al., 2011) for their PER to nicotine (Fig. S1A). From these studies, we identified two broadly required *Gr* mutants, Δ*Gr32a* and *Gr33a*^{1}, and a newly characterized *Gr10a*^{1} mutant with greatly impaired PER to nicotine (Fig. 2A, taken from Fig. S1A). We confirmed that *Gr10a* is expressed in the labellum by reverse transcription-polymerization chain reaction (RT-PCR) analysis, and this signal was undetectable in the flies with ablated bitter-sensing GRNs (Fig. 2B). When we reduced levels of *Gr10a* by expressing *Gr10a* RNA interference in pan-neurons using *elav-GAL4* or by examining a trans-heterozygote with deficiency line (*Gr10a*^{1}*/Df*), we found that the flies no longer showed reduced PER to nicotine (Fig. 2C). Finally, we restored normal PER to nicotine by expressing wild-type cDNA of *Gr10a* in all bitter-sensing GRNs driven by *Gr33a-GAL4* (Fig. 2D). However, *Gr10a-GAL4* is known to be expressed specifically in antenna, but not in labellum (Delventhal and Carlson, 2016); therefore, the expression of *UAS-Gr10* driven by *Gr10a-GAL4* did not recover the defects in the physiological and behavioral responses to nicotine of *Gr10a*^{1} flies (Fig. S2 A and B). In conclusion, *Gr10a* is expressed in the labellum (Fig. 2B) and indispensable for sensing nicotine in taste. In addition, the defective nicotine responses of Δ*Gr32a* and *Gr33a*^{1} flies were completely recovered by expression of their own wild-type cDNA driven by their own *GAL4* driver lines (Fig. 2E and F), suggesting these receptors are also required for sensing nicotine. Furthermore, since TRPM5 has critical role in taste transduction of nicotine in mammals (Ren et al., 2015), we tested whether the *Drosophila* TRPM or any other TRP family members play significant roles in nicotine taste sensation. However, all TRP family members tested were dispensable for sensing nicotine in behavioral as well as electrophysiological analyses (Fig. S1 B and C).

3.3. Nicotine induced action potentials are dependent on Gr10a, Gr32a, and Gr33a

To evaluate neural activity to nicotine, we tested the peripheral nerve responses to nicotine of all 21 *Gr* candidates by performing tip recordings from the S6 and S10 sensilla. We elicited reduced levels of action potentials from *Gr10a*^{1}, *ΔGr32a,* and *Gr33a*^{1} compared to *w*^{1118} control flies, consistent with our previous findings (Fig. 3 A and B, and Fig. S3A). Furthermore, we analyzed the responses to nicotine from all sensilla housed in the labellum from these three *Gr* mutants (Fig. 3C). We found that the S1, S3, S5, S6, S7, and S10 sensilla showed the

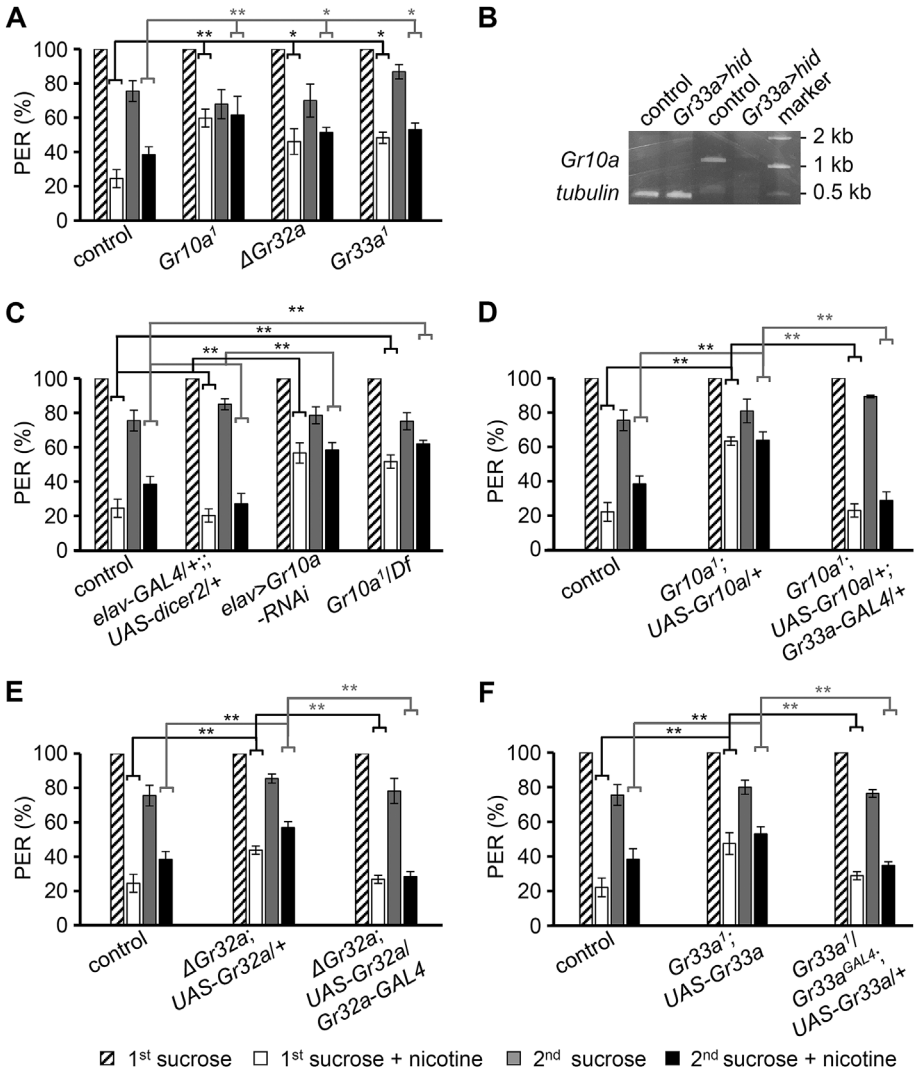

Fig. 2. *Gr10a*, *Gr32a*, and *Gr33a* are indispensable for nicotine sensation.
(A, C–F) PER assay with indicated strains in response to 2% sucrose or 2% sucrose with 0.5% nicotine. n = 4–6. (A) Candidates screening results extracted from Figure S1A. (B) Analyses of *Gr10a* and *tubulin* RT-PCR products from the labellum of control and bitter-sensing GRNs' ablated flies (*Gr33a-GAL4/UAS-hid*). (C) PER assay with 1) control, 2) *elav-GAL4/+;UAS-dicer2/+*, 3) *elav-GAL4/+;UAS-Gr10a^{RNAi}/+;UAS-dicer2/+*, 4) *Gr10a^{1}*/Df flies harboring the *Gr10a^{1}* mutation *in trans* with a deficiency that removes *Gr10a*. (D) PER assay with control, parent strain (*Gr10a^{1};UAS-Gr10a/+*), and rescue of *Gr10a^{1}* (*Gr10a^{1};UAS-Gr10a/+;Gr33a-GAL4/+*). (E) PER assay with control, parent strain (*ΔGr32a;UAS-Gr32a/+*), and rescue of *ΔGr32a* (*ΔGr32a;UAS-Gr32a/Gr32a-GAL4*). (F) PER assay with control, parent strain (*Gr33a^{1};UAS-Gr33a*), and rescue of *Gr33a^{1}* (*Gr33a^{1}/Gr33a^{GAL4};UAS-Gr33a*). *Gr33a^{GAL4}* is knock-in mutant. All error bars represent SEMs. Single factor ANOVA with Scheffe's analysis was used as a *post hoc* test to compare multiple sets of data. Asterisks indicate statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$). Gray asterisks in C–F indicate statistical significance compared with each mutant.

strongest responses to nicotine. We also found that the S2, S11, S12, and some I-type sensilla could be mildly activated by nicotine. However, none of the L-type sensilla, the I1, I2, and I3, and the S4 and S8 sensilla were significantly activated by nicotine (Fig. 3C). In addition, all three *Gr* mutants had significantly suppressed responses to nicotine compared with control flies (Fig. 3C).

To rescue the nicotine sensing defect in *Gr10a*, *Gr32a*, and *Gr33a* mutant flies, we expressed the respective wild type cDNA in bitter neurons using bitter-sensing *GAL4* drivers. We recovered the responses to nicotine in the *Gr* mutant lines with rescued flies and confirmed the other mutants (*Gr10a^{1}/Df* and *elav > Gr10a-RNAi*) for *Gr10a* (Fig. 3D and E, F, and G). These results suggest that *Gr10a, Gr32a*, and *Gr33a* are required for nicotine responses in taste.

The above evidence strongly suggests that nicotine activates fly GRs, which function as ligand-gated ion channels (Sato et al., 2011; Shim et al., 2015). Expression of wild-type cDNAs for *Gr10a*, *Gr32a*, and *Gr33a* successfully rescued defective nicotine responses in mutant flies. However, we found that none of those three GRs is sufficient to confer a response to nicotine when the three cDNAs are expressed in sweet-sensing GRNs driven by *Gr5a-GAL4* (Fig. S3B). These results suggest that there remains at least one more unidentified receptor or subunit that is required to form the multimeric complex necessary to sense nicotine.

3.4. Gustatory receptors impart the survivability of insects against the toxicity of nicotine

Nicotine has various adverse effects in the body and can cause severe poisoning (Benowitz and Burbank, 2016; Morris et al., 2018). To evaluate the minimal concentration required for nicotine intoxication in insects, we performed survival assays. We compared the viability of control flies with different concentrations of nicotine-laced fly food (Fig. 4A). Survival of flies feeding on 0.01% nicotine was normal up to 6.5 days, suggesting this level of nicotine is relatively mild (Fig. 4A). However, survival of the flies was severely impaired when feeding on 0.2%–1% nicotine, indicating a high level of toxicity at concentrations greater than 0.2% (Fig. 4A). The times when 50% died (LT_{50}) were 13.8 ± 1.8 h, 10.8 ± 0.5 h, 9.0 ± 1.7 h, and 3.0 ± 1.2 h for flies fed 0.2, 0.3, 0.5, 1.0% nicotine, respectively. Consumption of 0.1% nicotine had a relatively mild toxic effect compared with 0.2% nicotine (Fig. 4A). To examine whether *Gr10a, Gr32a, or Gr33a* play a role in nicotine toxicity, we tested mutant and control flies in a binary food survival assay with 100 mM fructose versus 200 mM fructose laced with 0.1% nicotine (Fig. 4B). The advantage in feeding fructose rather than cornmeal food is that flies need to consume greater amounts of fructose to reach satiation. Since fructose alone is preferred over a nicotine-laced food, we used a 2-fold higher concentration of fructose mixed with nicotine to match the valence between the two foods. Although the mortality rates were variable, all three of the *Gr* mutants died earlier than control flies (LT_{50} of *Gr10a^{1}*: 40.0 ± 15.3 h; LT_{50} of *ΔGr32a*:

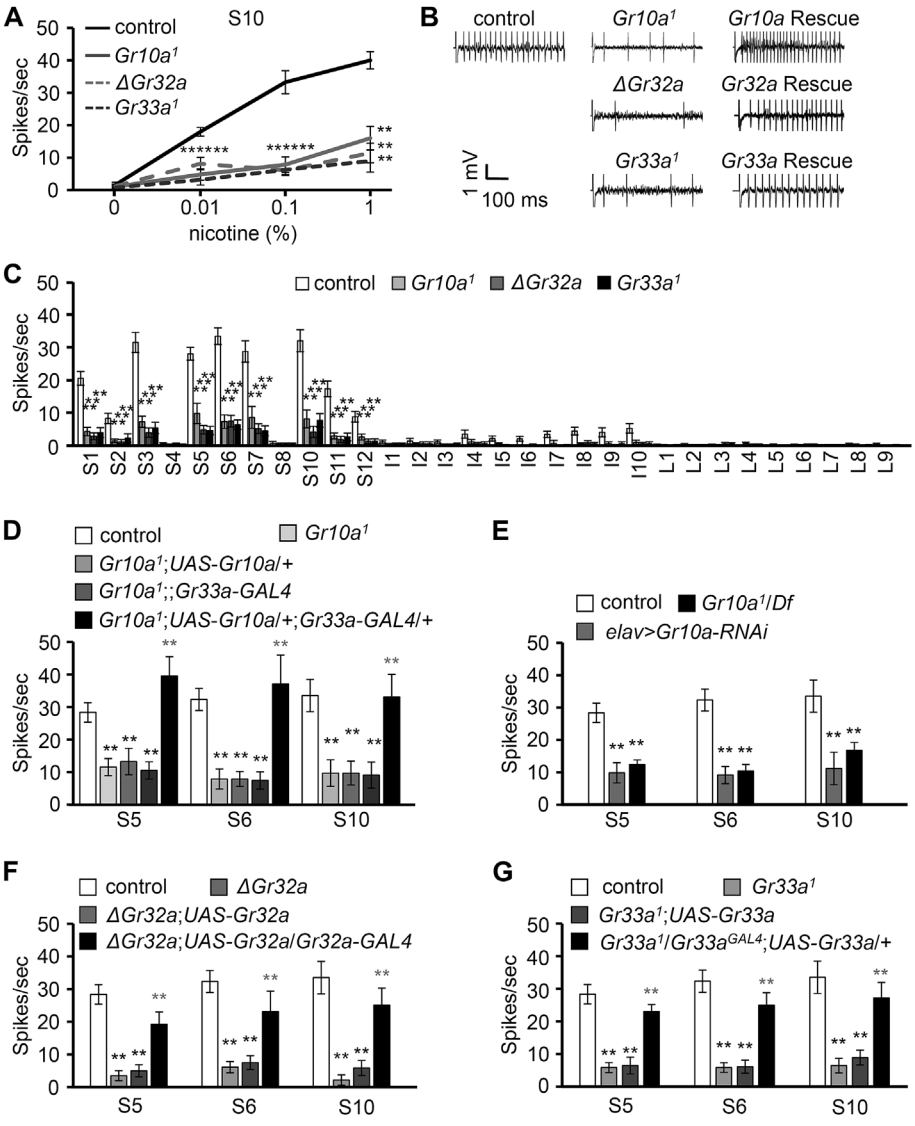

Fig. 3. Nicotine-induced action potentials are dependent on *Gr10a*, *Gr32a*, and *Gr33a*. (A) Frequencies of action potentials elicited from control, $Gr10a^1$, $\Delta Gr32a$, and $Gr33a^1$ by the stimulus of 0, 0.01, 0.1, and 1% nicotine. n = 10–16. (B) Representative traces on S10 obtained from control, $Gr10a^1$, $\Delta Gr32a$, $Gr33a^1$, and their respective rescue strains: $Gr10a^1$;*UAS-Gr10a/+*;*Gr33a-GAL4/+*, $\Delta Gr32a$;*UAS-Gr32a/Gr32a-GAL4*, and $Gr33a^1/Gr33a^{GAL4}$;*UAS-Gr33a/+* flies to 0.1% nicotine. (C) Mapping of all the sensilla on the labellum with 0.1% nicotine from control, $Gr10a^1$, $\Delta Gr32a$, and $Gr33a^1$ flies. n = 18–25. The nomenclature system is used, based on the Tanimura study (Hiroi et al., 2002). (D–G) Frequencies of action potentials elicited on S5, S6 and S10 sensilla with the indicated flies. n = 10–14. All error bars represent SEMs. Single factor ANOVA with Scheffe's analysis was used as a *post hoc* test to compare multiple sets of data. Asterisks indicate statistical significance ($^*P < 0.05$, $^{**}P < 0.01$). Gray asterisks in D, F, and G indicate statistical significance compared with each mutant.

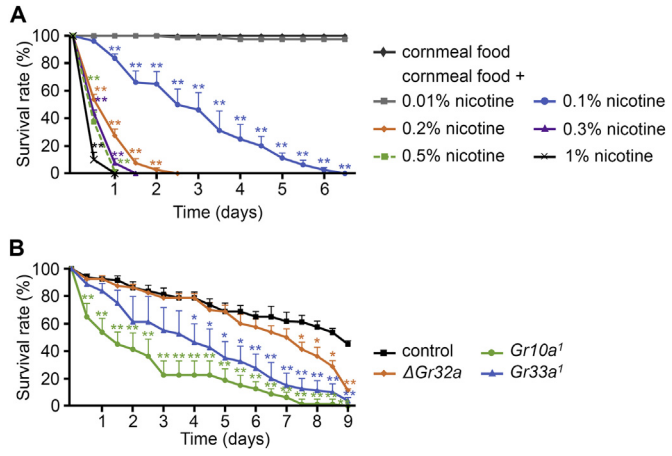

Fig. 4. Toxicity of nicotine. (A) Time-dependent effects on the survival rate of adult control flies fed with cornmeal food, or cornmeal food mixed with the indicated concentrations of nicotine. n = 4–6. (B) Binary food choice survival assay with control, $Gr10a^1$, $\Delta Gr32a$, and $Gr33a^1$ flies fed with 100 mM fructose versus 200 mM fructose mixed with 0.1% nicotine. n = 4–6. All error bars represent SEMs. Single factor ANOVA with Scheffe's analysis was used as a *post hoc* test to compare multiple sets of data. Asterisks indicate statistical significance compared with control ($^*P < 0.05$, $^{**}P < 0.01$).

162.0 ± 12.7 h; LT_{50} of $Gr33a^1$: 88.5 ± 23.7 h; LT_{50} of control was not measurable). To investigate whether the mutants consumed more nicotine due to inability to detect and avoid nicotine-laced food, we performed a two-way food choice ingestion assay with 0.1% nicotine. All the mutants consumed more nicotine-laced food compared with control flies (Fig. S3C). Furthermore, we repeated a binary food survival assay with normal cornmeal food containing 0.1% nicotine. In this condition, wild-type flies as well as mutants can prefer normal cornmeal food to nicotine-laced food since 0.1% nicotine may inhibit sugar sensation. Nevertheless, all three mutants died earlier than control flies (Fig. S4). These results suggest that inhibiting the function of these GR10a, GR32a, and GR33a could be an effective strategy to maximize the insecticidal effect of nicotine.

3.5. High concentrations of nicotine inhibit the sugar response from sweet GRNs

Animals utilize two mechanisms to sense bitter or toxic chemicals: direct activation of bitter-sensing GRNs and inhibition of the sugar-sensing GRNs, which serves as an indirect mechanism (Charlu et al., 2013; French et al., 2015; Jeong et al., 2013; Meunier et al., 2003). Many toxic compounds employ both of these pathways to maximize avoidance behavior. Since nicotine confers a high level of toxicity, we hypothesized that it would inhibit sugar responses. Previous research

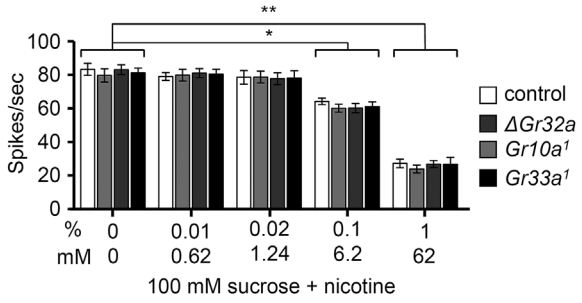

Fig. 5. Nicotine inhibits the sugar response from sweet GRN. Electrophysiological examination showing frequencies of action potentials elicited from 100 mM sucrose or 100 mM sucrose with the indicated concentrations of nicotine on L6 sensilla of control, *Gr10a*^{1}, Δ*Gr32a*, and *Gr33a*^{1} flies. n = 18–27. All error bars represent SEMs. Single factor ANOVA with Scheffe's analysis was used as a *post hoc* test to compare multiple sets of data. Asterisks indicate statistical significance compared with the same genotype ($^*P < 0.05$, $^{**}P < 0.01$).

has shown the dubitable result that nicotine doesn't inhibit sugar responses (French et al., 2015). However, we decided to test a wider range of nicotine concentrations against sucrose responses compared to the previous report. We performed tip recordings from L6 sensilla with stimulation of 100 mM sucrose alone and with different concentrations of nicotine. While the responses from L6 sensilla to 100 mM sucrose +0.01% (0.62 mM), and 0.02% (1.24 mM) nicotine were comparable with sucrose only responses, we elicited significantly decreased responses with the addition of 0.1%, and 1% nicotine mixed with 100 mM sucrose (Fig. 5). We also found that three *Gr* mutants, *Gr10a*^{1}, Δ*Gr32a*, and *Gr33a*^{1} showed similar sugar inhibition with nicotine as control flies (Fig. 5). These results suggest that nicotine inhibits the responses of sugar GRNs at high concentrations ranging from 0.1 to 1%.

4. Discussion

4.1. Nicotine activates bitter-sensing GRNs

It is generally believed that plants and insects are evolutionary symbionts (Yuan et al., 2013). They have a long history of association and advancing fitness for acclimation (Gómez et al., 2010). The phytophagous arthropods have acclimated to their host using their sensory modality, whereas plants evolved a defense system that includes toxins, antifeedants and secondary metabolites to hinder attacks (Anderson and Mitchell-Olds, 2011). So, there is a strong relationship between an insect's niche and plant variability. Depending on where arthropods live, they evolve their GRs and other sensory receptors in order to survive in their environments. To avoid edible toxins, insects have divergently developed their bitter or toxin-sensing GRs.

In *Drosophila*, dedicated bitter-sensing GRNs marked by *Gr33a-GAL4* are responsible for detecting toxic compounds. Naturally toxic compounds such as caffeine, strychnine, saponin, and coumarin, and synthetic compounds including DEET and denatonium are well known to activate bitter-sensing GRNs (Lee and Poudel, 2014; Sang et al., 2019). Here we provide evidence that nicotine is a bitter chemical to insects. First, we show that nicotine activates S-type sensilla and that this response disappears in flies whose bitter-sensing GRNs have been eliminated by inducing apoptosis. Second, at least two GRs involved in sensing nicotine, GR32a and GR33a, belong to the bitter-sensing GRs because their reporters are solely expressed in bitter-sensing GRNs. Furthermore, we provide the evidence that the deficits in nicotine sensation of the newly found *Gr10a* can be rescued by the expression of wild-type *Gr10a* in bitter-sensing GRNs. Finally, all three *Gr* mutants show a significantly reduced survival rate in a nicotine-containing food-choice survival assay. These pieces of evidence strongly suggest that insects perceive nicotine as a bitter chemical.

4.2. Nicotine negatively affects development and adult survival of insects

Prenatal exposure to nicotine is deleterious to fetal health and leads to many abnormalities ranging from developmental defects to loss of dopaminergic neurons and small brain size. Recent findings in flies show that nicotine exposure during the early larval stage leads to small brain size and that exposure during the adult stage causes the loss of dopaminergic neurons in the PPM3 cluster (Morris et al., 2018). This PPM3 cluster is a circuit to control locomotion, sleep and wakefulness, and ethanol-induced behavior (Kong et al., 2010; Liu et al., 2012; Ueno et al., 2012).

Here we show the effects of nicotine in adult flies by evaluating their survival rates during exposure to nicotine. Exposure to just 0.1% nicotine decreases survivability by 50% within 3 days, and exposure to 0.2% and 0.3% nicotine were even more fatal to the insects. Nevertheless, our study did not dissect the mechanism how 0.1% nicotine fed flies survived for 6 days and how the flies were dead. It would be intriguing to determine the nicotine detoxifying ability of flies, since lower concentrations of nicotine enhance dopamine levels and have been proposed to be beneficial for Parkinson's disease (Barreto et al., 2015). We provide a good example for the proper concentrations in adult stage in order to dissect how nicotine regulates dopaminergic neuronal regulation. Furthermore, the *Gr* mutants that can't taste nicotine could be useful tools for dissecting the relationship between nicotine and brain functions since feeding aversions to nicotine can be avoided.

4.3. Nicotine-based insecticide can be developed using GR modulation

Insecticides targeting specific transient receptor potential (TRP) channels in insects have recently been proposed (Nesterov et al., 2015). However, TRP channels are highly conserved from insects to humans. So TRP channel targeted insecticides carry a risk that they will affect humans as well. GRs and IRs are distantly related ionotropic channels that are unique to insects, making them prime potential targets for insecticides (Rimal and Lee, 2018). Targeting the GRs that are specific to harmful insects would also mitigate that detrimental effects that nicotine and its derivatives such as neonicotinoids can have on useful pollinator insects. More studies focusing on GRs involved in sensing nicotine may reveal better GR targets that are not expressed in beneficial pollinators. Our findings would facilitate the development of nicotine-based insecticides that are GR-targeted and ecofriendly by reducing the risk of harming pollinators while still achieving the expected pest control.

Conflicts of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ibmb.2019.103178.

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