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A genetic tool kit for cellular and behavioral analyses of insect sugar receptors

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Arthropods employ a large family of up to 100 putative taste or gustatory receptors (Grs) for the recognition of a wide range of non-volatile chemicals. In *Drosophila melanogaster*, a small subfamily of 8 *Gr* genes is thought to mediate the detection of sugars, the fly's major nutritional source. However, the specific roles for most *sugar Gr* genes are not known. Here, we report the generation of a series of mutant *sugar Gr* knock-in alleles and several composite *sugar Gr* mutant strains, including a sugar blind strain, which will facilitate the characterization of this gene family. Using Ca^{2+} imaging experiments, we show that most gustatory receptor neurons (GRNs) of sugar blind flies (lacking all 8 *sugar Gr* genes) fail to respond to any sugar tested. Moreover, expression of single *sugar Gr* genes in most sweet GRNs of sugar-blind flies does not restore sugar responses. However, when pair-wise combinations of *sugar Gr* genes are introduced to sweet GRNs, responses to select sugars are restored. We also examined the cellular phenotype of flies homozygous mutant for *Gr64a*, a *Gr* gene previously reported to be a major contributor for the detection of many sugars. In contrast to these claims, we find that sweet GRNs of *Gr64a* homozygous mutant flies show normal responses to most sugars, and only modestly reduced responses to maltose and maltotriose. Thus, the precisely engineered genetic mutations of single *Gr* genes and construction of a sugar-blind strain provide powerful analytical tools for examining the roles of *Drosophila* and other insect *sugar Gr* genes in sweet taste.

Introduction

Detection of sugars and other calorie-containing compounds and their discrimination from other chemicals are critical behavioral tasks that enable animals to feed from nutritious food sources. These processes are embedded in the gustatory system, a hallmark of which is the cellular segregation of receptor proteins that detect different groups of chemicals, such as sugars, proteins, and bitter-tasting compounds. In all characterized animal model systems, food chemicals stimulate different types of taste receptor cells from those stimulated by chemicals with no nutritional value or harmful and toxic compounds by virtue of cell-specific expression of cognate receptors.[1]

In *Drosophila*, taste sensilla constitute the sensory structures for the detection of all soluble chemicals. Taste sensilla, which are the functional equivalents of mammalian taste buds, are found in several major body parts, especially the labellum and the legs. Most taste sensilla contain 4 gustatory neurons (GRNs; some sensilla contain only 2 GRNs), as well as a mechanosensory neuron[2]; the four neurons are thought to be dedicated to different taste modalities, which have been associated with 3 appetitive promoting (sweet, modestly salty, and water) and one aversive (bitter/highly salty) modalities. Additionally, flies are also known to respond with acceptance behavior when provided with amino acids or fatty acids,[3-5] the cellular mechanism of which is not well understood.

Most *Drosophila* species are frugivores, and taste plays a central role in flies' feeding behavior. Sweet sensation is mediated by the sweet GRNs present in most if not all sensilla of the 2 main taste organs, the labial palps and the distal most segments of the tarsi. Each of these sweet GRNs is thought to express members of a *Gr* gene subfamily composed of 8 sugar *Gr* genes (*Gr5a, Gr61a*, and *Gr64a* to *Gr64f*).[6-10] A ninth *Gr* gene, *Gr43a*, was recently shown to be critical for sensing internal (brain hemolymph) fructose.[11] In the taste system, *Gr43a* is expressed in only a pair of tarsal taste sensilla, and its contribution to sucrose and fructose sensing is secondary to receptors formed by sugar Gr proteins.[11]

While *Gr5a* and *Gr61a* have been characterized in some detail and shown to play a critical role in trehalose and glucose sensing, respectively,[8,12-14] the specific functions of each of the *Gr64* genes are less defined, other than the fact that as a whole, this gene cluster is essential for sugar responses both at the behavioral and cellular level.[6,15] This paucity is due to lack of specific mutations in single *Gr64* genes, a consequence of the densely clustered organization of the *Gr64* locus (see **Fig. 1**). Regardless, specific roles have been assigned for some of these genes based on phenotypes of

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deletion and insertion mutations. However, these types of mutations are likely to alter expression of structurally unaffected genes within the locus, which is exemplified by the more severe phenotypes ascribed to the *Gr64ab* mutation (deleting *Gr64a, and Gr64b*) than the more subtle phenotype observed in *Gr64a*[2] (deleting *Gr64a, Gr64b, and Gr64c*).[7,8] Thus, lacking defined mutations and comprehensive expression profiles, it is not possible to determine the specific roles

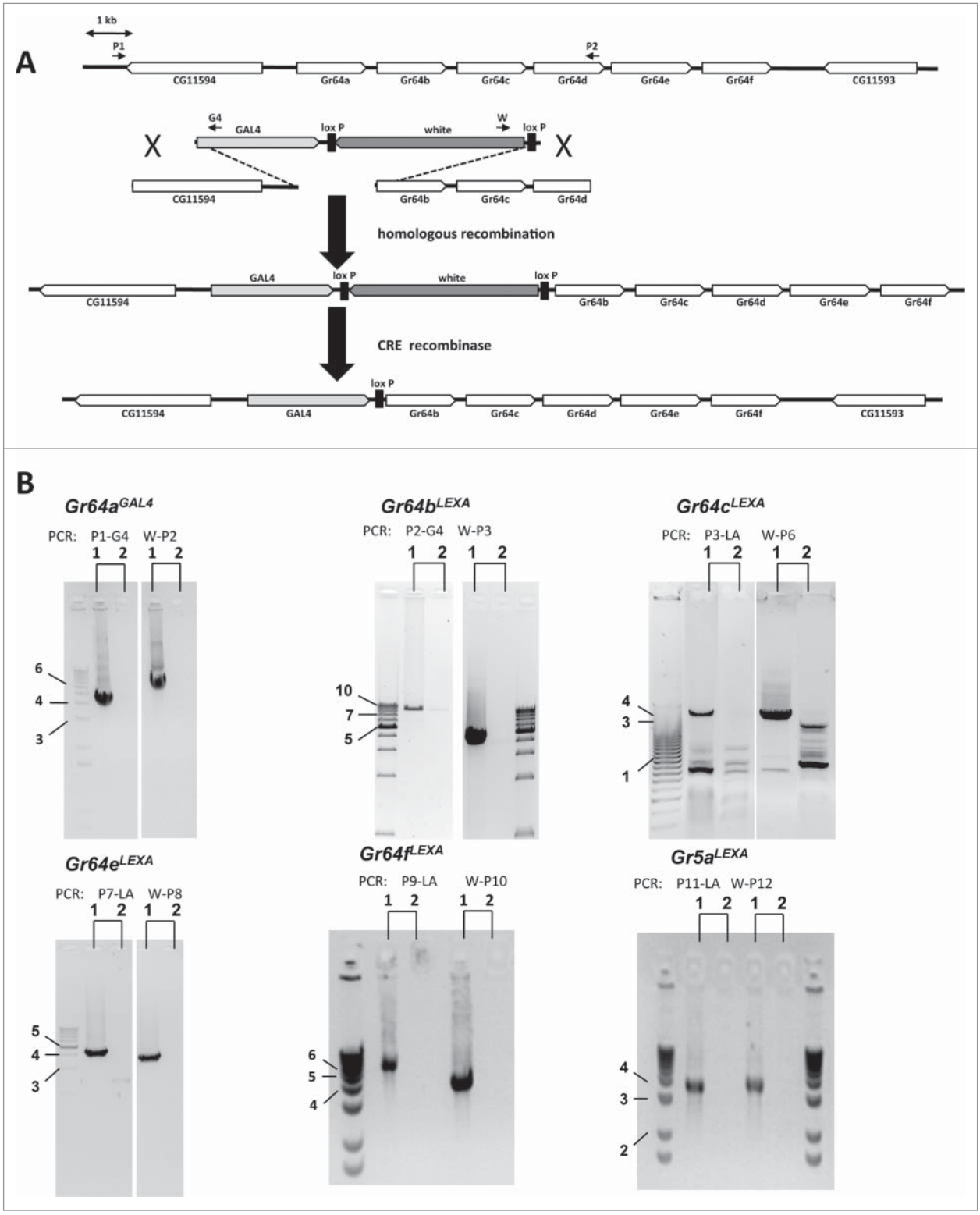

Figure 1. For figure legend, see page 191.

of the 6 *Gr64* genes in sweet taste or the composition and tuning profiles of receptor complexes to specific sugars.

Here, we report the generation of powerful genetic tools that allow us to address these and several additional questions about insect sugar receptors. We constructed a number of *Gr* mutations via homologous recombination that revealed detailed expression of 5 of the 6 *Gr64a* genes.[16] Moreover, we created a sugar-blind strain in which all 8 *sugar Gr* genes were deleted. We use this strain to show, contrary to a recent report,[17] that functional sugar receptors are composed of at least 2 sugar Gr protein subunit. Lastly, we identify 2 functional receptor complexes for recognition of the sugars maltose and sucrose, as well as glycerol.

Results

The genetic tools presented in this paper will overcome 3 major impediments that have slowed progress in our understanding of sweet taste in *Drosophila*. First, there is a lack of precise and useful mutations for the 6 densely clustered genes in the *Gr64* locus. Second, expression for many sugar *Gr* genes has not been established and, hence, the role of such genes in sweet taste remains speculative. Third, we currently lack a tool that unequivocally associates specific sugar chemicals with Gr proteins. The tools presented here will help overcome these obstacles, and they not only provide a path to a clear understanding of the role of each *sugar Gr* gene in sweet taste, but also will aid in elucidating the composition of specific insect sugar taste receptors.

Gene targeting of *Gr5a* and *Gr64a-f* loci

The *GAL4/UAS* expression system has been successfully employed in many studies for the analysis of many *Gr* genes.[9,10,18,19] However, the success rate for *Gr64-GAL4* transgenes has been poor, and for half of the genes, no cellular expression profile has ever been reported with this system. Therefore, we generated a series of sugar *Gr* knock-in alleles through homologous recombination.[20,21] We generated 7 transgenic fly strains containing a *LEXA* or *GAL4* targeting construct on the second chromosome, consisting of 5′ and 3′ non-translated sequences of all 6 *Gr64* genes and *Gr5a* (**Fig. 1**, **Fig. S1**). While null alleles for both *Gr5a* and *Gr61a* are available,[8,13,14] the former, but not the latter, was included in this study because 2 independently generated *Gr5a* transgenes were found to be expressed not only in sweet GRNs, but also in additional taste neurons.[16,19] With the exception of the *Gr64d* construct, all transgenes were successfully recombined into their target site, replacing the *Gr* coding sequence with *LEXA* or *GAL4* and producing 6 new knock-in/null alleles: $Gr5a^{LEXA}$, $Gr64a^{GAL4}$, $Gr64b^{LEXA}$, $Gr64c^{LEXA}$, $Gr64e^{LEXA}$, and $Gr64f^{LEXA}$ (for details, see Experimental Procedures). When these alleles are combined in a fly with specific reporter genes containing transcription factor binding sites for *GAL4* or *LEXA* (*UAS-RFP* or *lexAop-GFP*), they should replicate endogenous *Gr* gene activity. Indeed, all new knock-in alleles revealed expression either only in GRNs or in GRNs and additional chemosensory cells of the olfactory system or nutrient sensing brain neurons. A detailed expression analysis of these alleles is described in a separate study.[16]

Effects of individual knock-in mutations on cellular response

The utility of the single gene mutations in assessing the effect on gustatory receptor neuron responses was tested for the $Gr64a^{GAL4}$ mutation. *Gr64a* was chosen because it has been proposed to be essential for proper sensing of many sugars, including fructose, maltose, maltotriose, stachyose, raffinose, and others[7,8] by labellar taste neurons. Yet, lack of expression of $Gr64a^{GAL4}$ in labellar neurons, as well as absence of a PER phenotype,[16] is not consistent with a major role for this *Gr* gene in sugar sensing. Thus, we determined the cellular responses of homozygous and heterozygous $Gr64a^{GAL4}$ mutant flies in tarsal neurons, where $Gr64a^{GAL4}$ is expressed, using Ca^{2+} imaging (**Fig. 2**). We focused these imaging experiments on GRNs of the 5b sensilla, as opposed to the 5v sensilla (albeit both produced similar responses; see **Fig. S2**), because the neurons of the latter also express the *Gr43* fructose receptor,[16] which alone is sufficient to mediate response to sucrose and fructose.[11] Heterozygous control flies showed robust neuronal responses to all sugars tested (**Fig. 2B**). Consistent with the relatively mild behavioral deficits of $Gr64a^{GAL4}$ mutant flies,[16] GRNs of such flies produced robust responses upon stimulation with most sugars, and reduction, but not a complete loss, of Ca^{2+} responses to maltose and maltotriose only (**Fig. 2B**). Thus, the precise gene knock-in mutations are likely to provide a more accurate assessment for the contribution of individual sugar *Gr* genes than gene deletions used in previous studies, many of which also included regulatory sequences.[7,8]

Generating a sugar-blind *Drosophila* strain

Null alleles (i.e., lack of function alleles), such as $Gr64a^{GAL4}$, are useful when determining the contribution of a single gene to a particular taste trait. However, for delineating sufficiency (i.e., which genes together may encode for functional sugar receptors) null alleles are of limited value. Sufficiency is best assessed with the help of heterologous expression systems, which,

Figure 1 (See previous page). GAL4/LEXA knock-in strategy for *sugar Gr* genes using homologous recombination. (**A**) Genomic region of the *Gr64* locus and the targeting construct for $Gr64a^{GAL4}$ are shown in the 2 diagrams at the top. Homologous recombination replaces *Gr64a* with *GAL4* and the w^+ minigene, which is removed via CRE mediated recombination (bottom diagrams). Positions of primers used for PCR analysis are indicated by short arrows. Note that the coding sequences of all other sugar *Gr* genes were replaced by LEXA. (**B**) PCR analysis of genomic DNA isolated from successfully targeted homozygous lines (1) and respective donor lines (2). Location of primes P1, P9 and P2, P10 (see **A**) anneal to genomic DNA upstream and downstream of, but not within, the donor construct; Expected DNA fragment sizes for the 5′ and 3′ products are 4.2 kb and 5.9 kb for $Gr64a^{GAL4}$ (*Gr64a*), 4.6 kb and 4.6 kb (*Gr64b*), 6.3 kb and 4.6 kb (*Gr64c*) and 4.6 kb and 4.6 kb (*Gr64e*), 5.7 kb and 4.6 kb ($Gr64f^{LEXA}$) and 3.7 kb and 3.8 kb (*Gr5a*). Primers G4, LA and w (see Experimental Procedures) are specific for GAL4, LEXA and *white* gene. All PCR fragments had the expected size, except for the 5′ fragment of *Gr64b*. Additional PCR analysis revealed a ~4kb DNA insertion upstream of the *Gr64a* gene. Numbers refer to location of molecular weight marker bands and indicate fragment size (in kb). Primer sequences are shown in **Figure S1**.

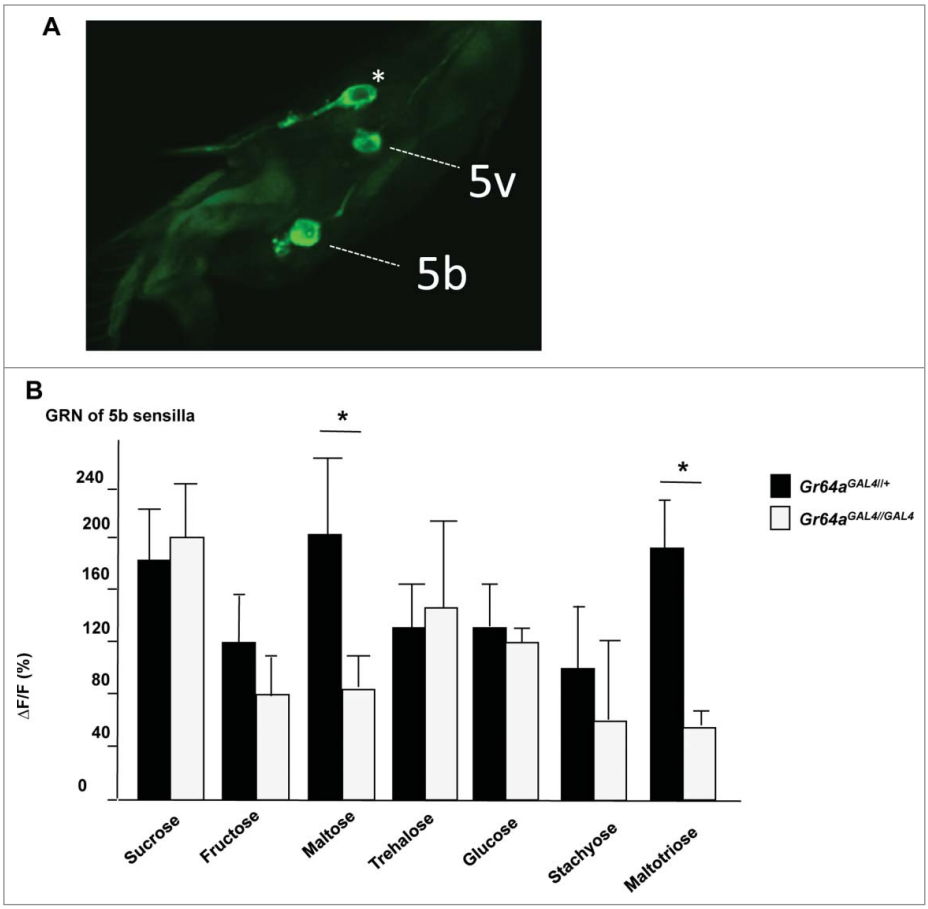

Figure 2. Sweet taste neurons of *Gr64a*^{GAL4} mutant flies respond normally to most sugars. (**A**) Antibody staining of tarsi of *Gr64f-GAL4;UAS-mCD8GFP* shows 3 labeled sweet neurons; the two neurons associated with 5b and 5v sensilla were used for Ca^{2+} imaging. The additional, 5s associated sweet neuron is indicated with an asterisk. Note that *Gr64f-GAL4* and *Gr64a*^{GAL4} are co-expressed in the sweet GNR of these sensilla.[16] (**B**) Ca^{2+} responses of sweet GRNs associated with the 5b sensillum of *Gr64a*$^{GAL4/+}$ (control) and *Gr64a*$^{GAL4/GAL4}$ flies. Responses to maltose and maltotriose, but to none of the other sugars, were significantly reduced in homozygous mutants. All sugars were at 100 mM. Student's t-test: * $P < 0.05$; $4<N<8$.

unfortunately, have had little success in the context of insect taste receptors. An alternative approach to heterologous expression systems is the generation of an "empty neuron" system, whereby deletions/mutations are introduced in every *Gr* gene expressed in a particular neuronal subtype. Such an "empty neuron" system has been powerfully employed in the *Drosophila* olfactory system, where it was used to unambiguously identify the ligands for numerous olfactory receptors.[22] While single GRNs express many more *Gr* genes than olfactory neurons express *Or* genes, we sought to test whether neurons of flies lacking all 8 *sugar Gr* genes could be used as a "*sugar Gr* deficient neuron system." We therefore generated a strain in which all 8 *sugar Gr* genes carried null alleles (octuple mutant). Variations of this octuple mutant strain, also referred to as "sugar-blind" strain (or $\Delta 8Gr^{sugar}/\Delta 8Gr^{sugar}$), were also equipped with a GRN specific GAL4 driver and a transgene for either the calcium indicator GCaMP6.0 (octuple mutant DRIVER strain) or one or more *sugar Gr* transgenes (octuple mutant REPORTER strains; **Table 1**). When octuple mutant flies from these strains are crossed, the effects on the cellular and behavioral responses of single or pair wise combinations of *sugar Gr* genes in

Table 1. Strains generated and used in this study. List of strains used for the examination of phenotypes of (i) mutations in single sugar *Gr* genes (top six lines) and of transgene rescue in octuple mutant (*Gr5a*^{LEXA}; *ΔGr61a ΔGr64a-f*) background (bottom four lines). R1 is an X linked genomic construct that contains two essential non-*Gr* genes missing in the *ΔGr64a-f* deletion.

Genotype	Description	Remarks
***Gr5a*^{LEXA};+;+**	*Gr5a* null allele	Coding Region Replaced by LEXA
+;+; *Gr64a*^{GAL4}	*Gr64a* null allele	Coding Region Replaced by GAL4
+;+; *Gr64b*^{LEXA}	*Gr64b* null allele	Coding Region Replaced by LEXA
+;+; *Gr64c*^{LEXA}	*Gr64c* null allele	Coding Region Replaced by LEXA
+;+; *Gr64e*^{LEXA}	*Gr64e* null allele	Coding Region Replaced by LEXA
+;+; *Gr64f*^{LEXA}	*Gr64f* null allele	Coding Region Replaced by LEXA
R1,Gr5a*^{LexA};+;*Δ61a, Δ64a-f	All sugar Grs deleted	sugar blind strain
R1,Gr5a*^{LexA}; *Gr61a-GAL4:GCamP6m/Cyo; ΔGr61a, ΔGr64a-f	Driver line for the rescue experiment in octuple mutant background	Yields sugar blind flies suitable for Ca^{2+} imaging when crossed to sugar blind strain
R1,Gr5a*^{LEXA}; *UAS-Gr64X/Cyo; ΔGR61a, ΔGR64a-f	UAS lines used for single rescue experiment in octuple mutant background	Yields flies with single sugar Gr suitable for Ca^{2+} imaging when crossed to sugar blind strain
Gr5a*^{LEXA}; *UAS-Gr64X,UAS-Gr64Y/Cyo ; ΔGr61a, ΔGr64a-f/TM6b	Reporter strains used for double Rescue experiment in octupleMutant background	Yields flies with two sugar Gr suitable for Ca^{2+} imaging when crossed to sugar blind strain

**UAS-Gr64f* construct is on the X chromosome (recombined onto *R1, Gr5a*^{LEXA} chromosome) and *UAS-Gr5a* construct is on third chromosome (recombined onto *ΔGr61a, ΔGr64a-f* chromosome).

otherwise sugar-blind flies can be quantitatively assessed using Ca^{2+} imaging and the proboscis extension reflex (PER) assay, respectively. To verify suitability of the $\triangle 8Gr^{sugar}/\triangle 8Gr^{sugar}$ strain, we first examined the sugar-induced neuronal responses in 2 types of GRNs, one expressing the non-canonical fructose receptor Gr43a (associated with the 5v sensilla) and one lacking expression of that gene (5b sensilla), of octuple mutant flies (as well as heterozygous control flies).[16] No neural activity was observed in 5b associated sweet GRNs in homozygous flies upon stimulation with any sugar solution tested, whereas control flies responded robustly to all sugars (**Fig. 3A**). In contrast, the 5v-associated GRN, which express *Gr43a*, was activated when stimulated with fructose and sucrose, to a level approximating that of heterozygous control flies. These observations are consistent with our previous analysis of the *Gr43a*, which showed that this receptor functions independently of the sugar Gr proteins as a fructose sensor.[11] Moreover, they suggest that the "*sugar Gr* deficient neuron system" is adequate to determine the response profile upon re-introduction of *sugar Gr* genes.

Sugar receptors are encoded by 2 or more *sugar Gr* genes

It was recently reported that olfactory neurons expressing any single *sugar Gr* gene are activated when bathed in a sugar solutions, and the authors suggested that single Grs function in the absence of other sugar Grs. This observation, however, contradicts evidence from numerous studies that strongly argue that functional sugar receptors are composed of 2 (or more) different sugar Gr proteins.[6-8,15] To address whether or not single Gr proteins can mediate sugar responses, we expressed each of the 8 *sugar Gr* genes in sweet GRNs of octuple mutant flies and performed Ca^{2+} imaging experiments on the tarsal 5b sensilla. None of the Gr proteins, when expressed singly, led to

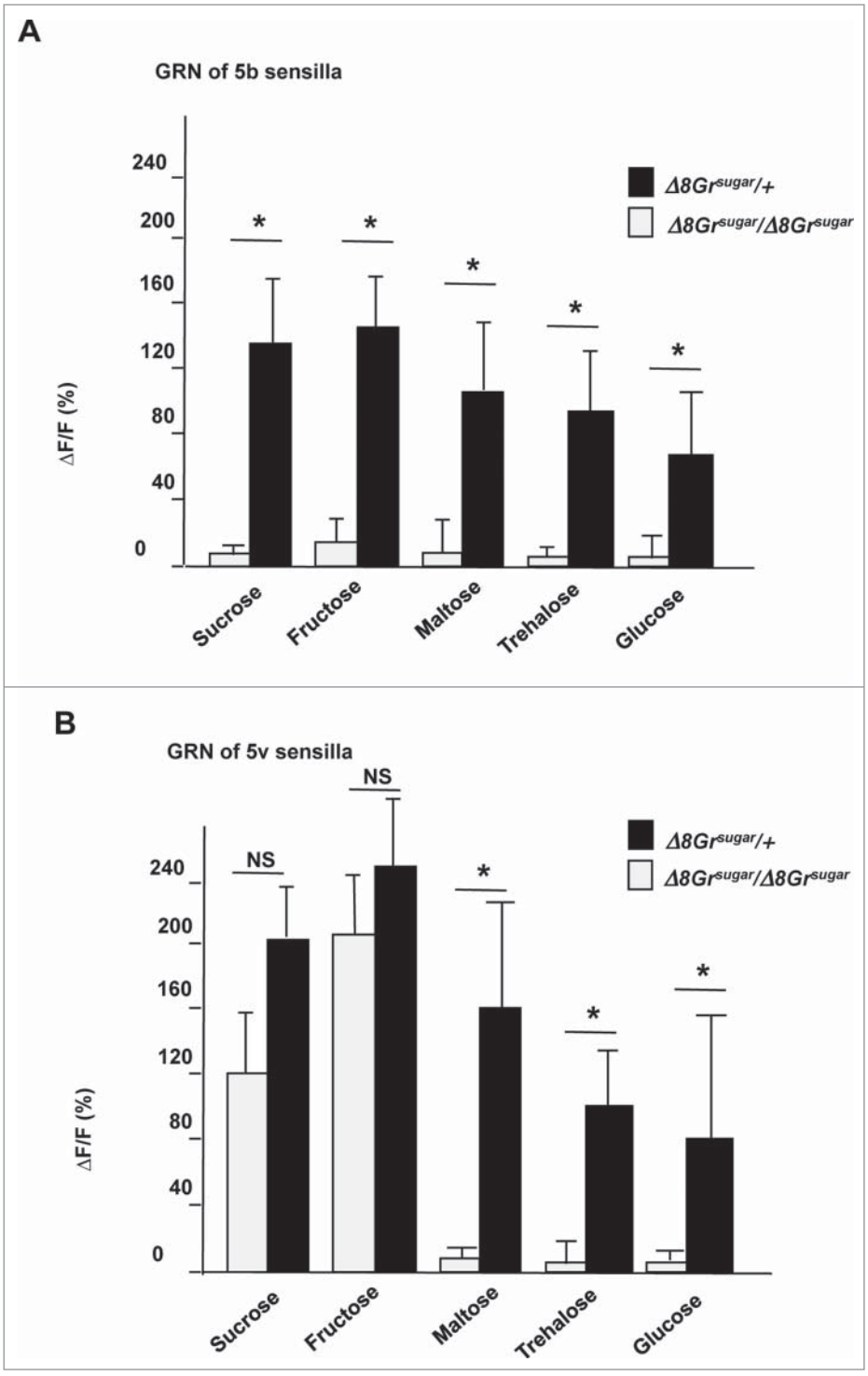

Figure 3. Many sweet taste neurons of *octuple* mutant flies lack sugar responses. (**A**) 5b associated sweet GRNs of $\triangle 8Gr^{sugar}/\triangle 8Gr^{sugar}$ flies (homozygous mutant for all 8 sugar *Gr* genes) lack responses to any sugar tested, while imaging of the same neuron of *sugar* $\triangle 8Gr^{sugar}/+$ flies respond robustly to all sugars tested. (**B**) 5v associated sweet GRNs, which express the atypical fructose receptor Gr43a, of $\triangle 8Gr^{sugar}/\triangle 8Gr^{sugar}$ flies respond to sucrose and fructose, but not to maltose, trehalose, and glucose. Heterozygous flies show somewhat stronger responses to sucrose and fructose, indicating that receptors for these sugars are formed by sugar Gr proteins. All sugar concentrations were at 100 mM. Student's t-test: NS, Not Significant; * $P < 0.05$; $3<N<7$.

Table 2. Summary of single and double rescue Ca^{2+} imaging experiments. Sweet GRN responses using Ca^{2+} imaging observed in the 5b sensilla (lacking expression of *Gr43a*; red), and in the 5v sensilla (expressing *Gr43a*; blue) of sugar blind ($\Delta 8Gr^{sugar}/\Delta 8Gr^{sugar}$) flies expressing a (top) or pairs of select sugar *Gr* genes are shown. Empty fields indicate no statically significant increase compared sugar blind ($\Delta 8Gr^{sugar}/\Delta 8Gr^{sugar}$) flies; +, ++ and +++ indicate statistically significant increase compared to sugar blind flies with cellular response < 33%, 33 to 66% and > 66% compared to control flies ($\Delta 8Gr^{sugar}/+$), respectively. Note that the GRN of the 5v sensilla responds to sucrose and fructose, due to expression of the *Gr43a* gene in that neuron. Also note that expression of single *UAS-Gr* transgene in the 5b-associated GRN fails to restore responses to any sugar, while expression in the 5v-assocaited GRN leads to the recovery of some sugar responses. However, expression of pairs of *UAS-Gr* genes recovers select sugar response in the 5b-associated neuron (red). UAS transgenes were expressed under the control of *Gr61a-GAL4*.

SugarTransgene(s	Maltose	Glucose	Trehalose	Fructose	Arabinose	Melezitose	Glycerol	Sucrose
none				+++				+++
UAS-Gr64a	+++			+++				+++
UAS-Gr64b				+++	++	++	+++	+++
UAS-Gr64c				+++				+++
UAS-Gr64d				+++				+++
UAS-Gr64e				+++	++	++		+++
UAS-Gr64f				+++				+++
UAS-Gr61a		++		+++				+++
UAS-Gr5a			+	+++		+		+++
UAS-Gr64aUAS-Gr64b				+++				+++
UAS-Gr64aUAS-Gr64d				+++				+++
UAS-Gr64aUAS-Gr64e	+++			+++				+++ +++
UAS-Gr64bUAS-Gr64e				+++			++	+++

a fluorescence increase after application of 8 different sugar solutions. Interestingly, when we measured cellular responses of the 5v associated Gr43a expressing neuron, expression of single *sugar Gr* genes was sufficient for activation following application of some sugars (**Table 2**). For example, expression of *Gr64a* alone elicited a maltose response in the *Gr43a* neuron, expression of *Gr64b* alone or *Gr64e* alone elicited responses to arabinose, melezitose, and glycerol, and expression of *Gr61a* alone elicited a glucose response. The interpretation of these results is that Gr43a can form complexes with sugar Gr proteins to form receptors for select sugars.

The experiments described thus far are consistent with the hypothesis that Gr proteins do not function as single receptors or homodimers, but are composed of at least 2 different Gr subunits. To test this idea further, we randomly chose 4 pairwise *UAS-Gr* combinations, expressed them in octuple mutant flies, and monitored activity in the sweet GRN of the 5b sensillum (which does not express *Gr43a*; **Figure 4**). Indeed, 2 combinations lead to strong neural responses to a select group of sugars. Specifically, the Gr64a/64e pair induced strong response to maltose and sucrose, but not to glucose, trehalose, fructose, arabinose, melezitose, and glycerol. In contrast, the Gr64b/64e pair was able to induce glycerol-specific responses, but did not mediate responses to the other 7 sugars we tested. We note that 2 other combinations of Gr proteins—Gr64a/Gr64b and Gr64a/Gr64d—failed to convey cellular responses in 5b associated sweet GRNs of octuple mutant flies when tested with any of the 8 sugars.

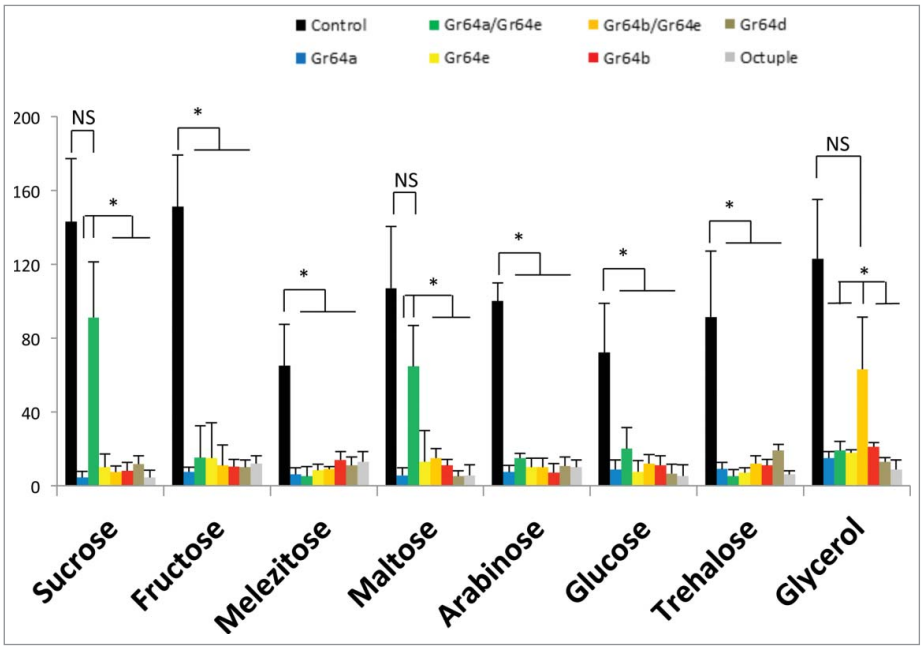

Figure 4. Two sugar Gr proteins are necessary to form functional sugar receptors 5b-associated sweet GRNs of octuple mutant flies (gray) expressing *Gr64a* (blue), *Gr64b* (red), *Gr64d* (tan), or *Gr64e* (yellow) do not respond to any of the 8 sugars we tested. However, when octuple mutant flies were provided with *Gr64a* and *Gr64e* (green), or *Gr64b* and *Gr64e* (orange), responses to maltose and sucrose, or glycerol, was recovered. All sugars were at 100mM. Student's t-test: $P < 0.05$; $3<N<7$.

Conclusion

We have generated a number of precise *sugar Gr* mutations that can be used both as null alleles as well as expression alleles. We also generated a sugar-blind fly strain that lacks measurable sugar responses in sweet taste neurons (except in those expressing Gr43a), and we have explored the utility of the sugar-blind strain to answer some important, pressing questions. Indeed, one of the main findings from our study is that sugar receptors are multimeric complexes composed of 2 or more subunits, rejecting the suggestion derived from pseudo-heterologous expression studies that single *sugar Gr* genes can mediate sugar responses on their own.[22] Using sugar-blind flies, we have determined the necessary components of 2 sugar receptors, one tuned to the disaccharides sucrose and maltose and one to the sugar alcohol glycerol.

Surprisingly, while the Gr43a fructose receptor functions on its own in the brain and probably in other chemosensory organs,[11] as well as in heterologous expression systems,[23] it is capable of forming additional sugar receptors when combined with other Gr proteins (**Table 2**). This observation suggests that some Grs, albeit none of the sugar Grs, function as homomultimers, but in combination with other Gr proteins, they can combine to form receptors with novel ligand properties.

Our imaging analysis of *Gr64a* mutant flies, together with behavioral studies of single *Gr64* mutant flies,[16] demands re-evaluation of the promoted model of sweet taste, which suggested that 2 multimeric receptors composed by only 3 Gr proteins (including Gr64a) function as the major, if not sole, receptors for sweet chemicals. Indeed, the conspicuous expression of *Gr64a* in nutrient sensing neurons in the brain, along with the absence in labial palp neurons,[16] suggests that the main function for this gene is likely the sensing of an internal sugar, rather than a dietary one.

Lastly, whether the GRNs in the octuple mutant strains represent a true empty neuron system remains to be determined. It is impossible to rule out that other *Gr* genes are expressed in sweet GRNs; moreover, a number of *Ionotropic chemoreceptor* genes are expressed in the gustatory system[24-26] and it is not known whether they are expressed in sweet GRNs. Finally, sweet GRNs were recently shown to mediate taste response to fatty acids, suggesting expression of receptors in these neurons that recognize such chemicals.[4] Regardless, the complete lack of sugar mediated responses in GRNs that lack expression of Gr43a should make the octuple mutant strain a powerful tool to analyze not only *Drosophila* sugar *Gr* genes, but also putative sugar *Gr* genes from other insect species.

Experimental Procedures

Molecular cloning of knock-in constructs

Targeting constructs for ends-out homologous recombination were based on the CMC-loxP-Gal4[11] and CMC-loxP-LexA::VP16 vectors. CMC-loxP-LexA::VP16 was obtained by first adding loxP sites into the *Avr*II the *BstE*II sites of the CMC vector.[27] From the resulting plasmid ("CMC-loxP"), we cloned the LexA::VP16 sequence into the *Spe*I and *Avr*II sites yielding the targeting vector CMC-loxP-LexA::VP16. To generate gene-specific targeting constructs, PCR fragments flanking the gene being targeted were cloned into the TOPO-XL vector (Life Technologies) and then subcloned into the upstream and downstream multiple cloning sites of CMC-loxP-Gal4 (*Gr64a*) or CMC-loxP-LexA::VP16 (*Gr5a, Gr64b, Gr64c, Gr64e*, and *Gr64f*).

In most cases, restriction sites were introduced into the primer sequence used to generate the PCR fragments, with the following exceptions: (1) Gr5a 3′ flank—Internal *Spe*I site in the PCR product and a SpeI site in the TOPO vector were used to ligate fragment to *Nhe*I site in 3′ MCS. (2) Gr64b 3′ flank—Internal *Nhe*I site in the PCR product was used to ligate fragment to *Nhe*I site in 3′ MCS. (3) Gr64c 3′ flank—Internal *Nhe*I site in the PCR product was used to ligate fragment to *Nhe*I site in 3′ MCS (4) Gr64f 3′ flank—Internal *Nhe*I site in the PCR product was used to ligate fragment to *Nhe*I site in 3′ MCS.

Primer Gr5a 5′ Flank Sense—CGTACGCCGCAACTGGAAATGGAAATCTGA

Primer Gr5a 5′ Flank Antisense—ACTAGTTGTGTACAAGCTCTAAATCCTGACTAAACG Primer Gr5a 3′ Flank Sense—GGTGACCCACCCTTCAATCTTGATTAGACGCAC Primer Gr5a 3′ Flank Antisense —GCTAGCGTTTTTACGCCTGCTGTCTGCTG Primer Gr64a 5′ Flank Sense—GGCGCGCCCTGTCGTTGGTTCTCCAGCAGC Primer Gr64a 5′ Flank Antisense—CGTACGGACGCTGGTCCCTTTTGCACTGAC Primer Gr64a 3′ Flank Sense—GCGGCCGCTGGACAACAATAGCCACCAACACC Primer Gr64a 3′ Flank Antisense—GCTAGCCAAGCCGCACTTCCCACATAGG Primer Gr64b 5′ Flank Sense—GGCGCGCCGCAAATGGGGGAAGATCATTACTGGG

Primer Gr64b 5′ Flank Antisense—CGTACGGGCCAAACTAGCACTAACCAAACGAC

Primer Gr64b 3′ Flank Sense—GCGGCCGCATCCTAGAATTTACTACTCGTATCTCCAATTCAAGAACG

Primer Gr64b 3′ Flank Antisense—GCTAGCCTCACTTTTCGAACTGGCATCAAAGC

Primer Gr64c 5′ Flank Sense—GGCGCGCCGTAGCTATAT-TACTACTGCCCTACGTTCACTG

Primer Gr64c 5′ Flank Antisense—ACTAGTGGCTTGACT-GTTGGGTAGCAAATG

Primer Gr64c 3′ Flank Sense—GCGGCCGCTTCTAGTTTGAAATTTGCATTCTGTCGCACCTTC

Primer Gr64c 3′ Flank Antisense—GCTAGCCTTTTCTTCAGCCGCCTCAACTTG

Primer Gr64e 5′ Flank Sense—GGCGCGCCGTGAGTTGAGAAATGACTTTACACAGCTTAG

Primer Gr64e 5′ Flank Antisense—ACTAGTGTTCCGTACTCGACTGACAACCAATC

Primer Gr64e 3′ Flank Sense—GCGGCCGCATTTTGTGGAAGTGGCAGGGGGTTAAG

Primer Gr64e 3′ Flank Antisense—GCTAGCGATGCGGATGTGTCCCAGTACTTG

Primer Gr64f 5′ Flank Sense—GGCGCGCCGTGGAGTGCAAGCTGGATGCGAAC Primer Gr64f 5′ Flank Antisense—ACTAGTCCTAGGACCTGCTGGGGTAAACTG Primer

Gr64f 3′ Flank Sense—GCGGCCGCCCGCTAGAGAGATTCTACGTGTGTCCG Primer Gr64f 3′ Flank Antisense—GCTAGCCTTATGGCGGACACTGCAATCCTGG.

The transgenes were excised and linearized as described by Miyamoto et al.[11] and potential relocation onto the third chromosome for the *Gr64*$^{GAL4/LEXA}$ constructs and the X for the *Gr5a*^{LEXA} construct was evaluated based on segregation from respective chromosome balancers. Between 2 and 10 lines with integration on the respective chromosome were generated and genomic DNA of homozygous flies with putatively recombined alleles was isolated. To determine whether the coding sequence of the respective *Gr* genes was precisely replaced with either that of LEXA or GAL4, we performed PCR using a primer within the targeting construct and a primer complementary to a sequence just upstream of downstream of the targeting construct for each *Gr* gene.

Ca^{2+} imaging of tarsal taste sensilla

Preparation of forelegs and Ca^{2+} imaging of taste sensilla was performed as described by Miyamoto et al.[12]. Concentration of all sugar was 100 mM.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.