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# Calcium taste avoidance in *Drosophila*

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## SUMMARY

Many animals, ranging from vinegar flies to humans discriminate a wide range of tastants, including sugars, bitter compounds, NaCl and sour. However, the taste of $Ca^{2+}$ is poorly understood, and it is unclear if animals such as *Drosophila melanogaster* are endowed with this sense. Here, we examined $Ca^{2+}$ taste in *Drosophila* and showed that high levels of $Ca^{2+}$ are aversive. The repulsion was mediated by two mechanisms—activation of a specific class of gustatory receptor neurons (GRNs) that suppresses feeding, and inhibition of sugar-activated GRNs, which normally stimulates feeding. The distaste for $Ca^{2+}$, and $Ca^{2+}$-activated action potentials required several members of the variant ionotropic receptor (IR) family (IR25a, IR62a, and IR76b). Consistent with the $Ca^{2+}$ rejection, we found that high concentrations of $Ca^{2+}$ decreased survival. We conclude that gustatory detection of $Ca^{2+}$ represents an additional sense of taste in *Drosophila*, and is required for avoiding toxic levels of this mineral.

## INTRODUCTION

The ability to discriminate the chemical composition of food is used by most animals to identify calorie-rich foods, and to consume the appropriate concentrations of minerals. Many animals ranging from flies to humans, perceive sugars as attractive, and bitter compounds as aversive (Liman et al., 2014). This differs from the behavioral responses to $Na^{+}$, which reverse depending on the concentration (Liman et al., 2014). $Na^{+}$ is required for survival, and low levels are attractive, whereas high concentrations can be toxic and are repulsive to the taste. Similar to $Na^{+}$, low or modest levels of $Ca^{2+}$ promote survival, and high

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**CONTRIBUTIONS**

Y.L. S.P., Y.K., and D.T. conducted the experiments, Y.L. and C.M. designed the experiments and wrote the paper.

SUPPLEMENTAL INFORMATION

The supplementary Information includes four figures.

concentrations can lead to adverse effects. In humans, hypercalcemia is associated with many common diseases, and can be life-threatening (reviewed in Žofková, 2016).

In contrast to the extensive behavioral and mechanistic studies focusing on other forms of taste, especially sweet and bitter (Liman et al., 2014), far less is known about $Ca^{2+}$ taste. While some studies support the concept that humans perceive $Ca^{2+}$ in food (Tordoff, 2001), the sensitivity of humans to $Ca^{2+}$ is unresolved. One study suggests that a human taste receptor contributes to sensing $Ca^{2+}$ (T1R3) (Tordoff et al., 2012). However, the mechanism is unclear because this receptor also functions in the gustatory detection of sweet and umami in mammals (Max et al., 2001; Nelson et al., 2002; Nelson et al., 2001). Nevertheless, due to the positive and negative effects of $Ca^{2+}$ on health, in principle, detection of this mineral could be attractive or repulsive depending on its concentration.

To address the fundamental questions concerning the behavioral, molecular and cellular mechanisms through which an animal tastes $Ca^{2+}$, we focused on the vinegar fly. We found that flies are indifferent to modest levels of $Ca^{2+}$ in food. However, they show strong repulsion to high $Ca^{2+}$, indicating that they possess the sense of $Ca^{2+}$ taste. $Ca^{2+}$ avoidance was mediated by neurons distinct from those that respond to bitter compounds. A diet containing high levels of $Ca^{2+}$ diminished viability. We found that three members of family of variant ionotropic receptors (Benton et al., 2009) (IR25a, IR62a, and IR76b) are required for the behavioral and electrophysiological responses to $Ca^{2+}$, indicating that they are critical molecular components that enable flies to avoid ingesting excessive levels of $Ca^{2+}$.

## RESULTS

### Flies taste $Ca^{2+}$ in food, and high levels are aversive

To determine the behavioral response of *Drosophila* to $Ca^{2+}$, we employed a binary food choice assay (Meunier et al., 2003; Moon et al., 2006). We mixed 2 mM sucrose, or 2 mM sucrose plus various concentrations of $CaCl_2$ with either blue or red food dye, and determined the feeding preferences by inspection of the colors of the abdomens. Complete preference for 2 mM sucrose or 2 mM sucrose and $CaCl_2$ results in preference indexes (P.I.) of 1.0 and −1.0, respectively, whereas no taste bias results in a P.I. of 0. In the absence of $CaCl_2$, the flies show no discrimination (Figure 1A), demonstrating that the red and blue dyes do not affect this feeding behavior. 0.1 mM $CaCl_2$ had no impact on their taste preference, demonstrating that it was neither attractive nor aversive (Figure 1A). However, higher concentrations of $Ca^{2+}$ mixed with sucrose (0.5—10 mM $CaCl_2$) induced progressively stronger avoidance (Figure 1A). The $Cl_2$ was not responsible for this repulsion since flies show no distaste for 10—100 mM $MgCl_2$ (Figure S1A), while 1 mM and 10 mM levels of other $Ca^{2+}$-containing compounds were also aversive (Figure S1B). Thus, flies have a sense of $Ca^{2+}$ taste.

We tested a range of $CaCl_2$ levels to determine the concentration necessary to eliminate the strong preference for 5 mM sucrose over 1 mM sucrose (Figure 1B). Addition of 10 mM $CaCl_2$ neutralized this bias, while inclusion of ≥15 mM $CaCl_2$ to the 5 mM sucrose caused the animals to prefer the lower concentration of sugar (Figure 1B). We also applied different concentration of $CaCl_2$ mixed with sucrose directly to the labellum, and performed the

proboscis extension response (PER). 2% sucrose alone stimulated a PER in all flies, while addition of $CaCl_2$ decreased this response in a concentration-dependent manner (Figure S1C). These data indicate that $Ca^{2+}$ that is sensed by the labellum causes repulsion.

## *ppk23* positive GRNs are essential to avoid $Ca^{2+}$

To identify the GRNs required for $Ca^{2+}$ avoidance, we used the *GAL4/UAS* system to selectively express a pro-cell death gene (*UAS-hid*) under the control of *GAL4*s that mark different classes of GRNs. These include the *Gr33a-GAL4* and the *Gr5a-GAL4*, which are expressed in all bitter- and sweet-sensing GRNs, respectively (Moon et al., 2009; Thorne et al., 2004). We also used the *ppk28-GAL4* which is expressed in GRNs that elicit attractive responses to water (Cameron et al., 2010) and the *ppk23-GAL4,* which is reported to function in courtship but not feeding behavior (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). Surprisingly, expression of *UAS-hid* under control of the *ppk23-GAL4/+* background greatly reduced the gustatory aversion to $CaCl_2$ in a dose dependent manner (Figures 1C, 1D and S1D). In contrast, there were no significant effects from combining *UAS-hid* with the other *GAL4*s (Figures 1C and S1D). Thus, *ppk23*-positive GRNs are essential for tasting $Ca^{2+}$.

To test whether activation of *ppk23* neurons causes aversive behavior, we activated these neurons by expressing the capsaicin receptor, TRPV1. We allowed the flies to choose between 2 mM sucrose versus 2 mM sucrose plus 100 μM capsaicin. Capsaicin alone does not cause repulsion in control flies, or in animals expressing just the *ppk23-GAL4* or *UAS-trpV1* (Figure S1E) (Marella et al., 2006). However, flies expressing *UAS-trpV1* under the control of the *ppk23-GAL4* avoided capsaicin (Figure S1E), indicating that activation of *ppk23* GRNs causes gustatory avoidance.

To address whether $Ca^{2+}$ induces action potentials in GRNs, we performed tip recordings. There are three size classes of taste sensilla. S- and I-type sensilla house both attractive and aversive GRNs, whereas L-type sensilla contain GRNs that respond to attractive but not aversive tastants (Freeman and Dahanukar, 2015; Weiss et al., 2011). We surveyed the taste sensilla using 50 mM $CaCl_2$ and found that six S-type, but no I- or L-type produced robust spikes (Figure 1E). S5 bristles responded with the highest action potential frequency. Therefore, we determined the dose-dependent responses of S5, and found that the action potential frequencies saturated at 50 mM $CaCl_2$ (Figures 1F and 1G). The frequencies of $Ca^{2+}$-induced action potentials were dramatically reduced in *ppk23-GAL4/UAS-hid* flies (Figure 1E), demonstrating that *ppk23*-positive GRNs mediate $Ca^{2+}$-induced action potentials.

## $Ca^{2+}$ suppresses sugar-induced action potentials

Bitter organic tastants such as caffeine and DEET suppress feeding through two mechanisms. They activate bitter-responsive GRNs, and they suppress sugar-activated GRNs (Jeong et al., 2013; Lee et al., 2010; Meunier et al., 2003; Moon et al., 2006; Weiss et al., 2011). To investigate whether $Ca^{2+}$ inhibits sugar-responsive GRNs, we tested 10 mM and 50 mM $CaCl_2$, and recorded from L4 and L6 sensilla. We found that $CaCl_2$ reduced the neuronal firing activated by 50 mM sucrose (Figure 1H). In contrast, neither $MgCl_2$ nor

NaCl diminished sugar-induced action potentials (Figures 1I and 1J). Thus, high levels of $Ca^{2+}$ suppress feeding through dual mechanisms: activation of *ppk23*-positive GRNs and inhibition of sugar-activated GRNs.

## $Ca^{2+}$ taste requires Ionotropic Receptors

To identify candidate receptors/cation channels that are required for $Ca^{2+}$ taste, we performed two-way choice assays using 1 mM sucrose alone versus 5 mM sucrose plus 25 mM $CaCl_2$. We screened mutations disrupting two GRs (GR33a and GR66a), which are broadly required for the aversive responses to most bitter compounds and two other GRs (GR8a and GR98b), which are required for sensing the toxic amino acid, L-canavanine (Lee et al., 2012; Lee et al., 2010; Moon et al., 2009; Shim et al., 2015). None of these *Gr* mutations significantly impaired $Ca^{2+}$ avoidance (Figure 2A), consistent with the absence of a significant impairment of $Ca^{2+}$ repulsion after killing *Gr33a* GRNs with *UAS-hid* (Figure 1C). We also considered PPK23 because it is a member of the family of Degenerin/Epithelial $Na^+$ channels, and ablation of *ppk23*-positive GRNs reduces $Ca^{2+}$-avoidance. Although the *ppk23* null mutant showed a reduced P.I., the difference from the control was not statistically significant (Figure 2A $p$=0.45).

Ionotropic receptors (IRs) (Benton et al., 2009) are candidates for functioning in $Ca^{2+}$ taste since multiple IRs are expressed in gustatory organs (Croset et al., 2010; Hussain et al., 2016; Koh et al., 2014; Zhang et al., 2013), and one member of this family of cation channels (IR76b) is required for tasting low levels of another mineral—$Na^+$ (Zhang et al., 2013). To interrogate potential roles for IRs, we tested requirements for family members that are expressed in the labellum, as well as in GRNs in taste sensilla decorating the legs and wing margins. We examined the effects of mutations and RNAi lines affecting 20 *Ir* genes and found that the $Ir25a^2$, $Ir62a^1$ and $Ir76b^1$ mutations strongly impaired $Ca^{2+}$ avoidance (Figure 2A), and did so over the range of $CaCl_2$ concentrations tested (10—25 mM; Figure 2B). In contrast, we observed normal $Ca^{2+}$ responses elicited by other existing *Ir* mutations ($Ir21a^1$ and $Ir56b^1$), by flies with mutations in *Ir7a*, *Ir47a*, *Ir52a*, *Ir56a*, and *Ir94h*, which we generated using homologous recombination as part of a separate study, and by the RNAi lines tested (Figure 2A). The lack of phenotype using RNAi was not due to ineffectiveness of *elav-GAL4;UAS-Dicer2*, since we replicated the $Ir25a^2$ phenotype by knocking down *Ir25a* (Figure 2A). To provide additional evidence that the deficits in $Ca^{2+}$ avoidance were due to losses of *Ir25a*, *Ir62a*, or *Ir76b*, we conducted genetic rescue experiments. We found that the behavioral impairments exhibited by $Ir25a^2$, $Ir62a^1$ and $Ir76b^1$ flies were suppressed by expression of the corresponding wild-type cDNAs using the *Ir25a-GAL4*, *Ir76b-GAL4* or the *ppk23-GAL4* (Figures 2C—2E). The deficit in $Ir25a^2$ was also reversed using a *genomic Ir25a* transgene (Figure 2C). Furthermore, the profound deficits in $Ca^{2+}$-activated action potentials in $Ir25a^2$, $Ir62a^1$, and $Ir76b^1$ animals were significantly reversed by the wild-type transgenes (Figure 3). We conclude that these IRs are required for sensing $Ca^{2+}$ in GRNs. However, they were not sufficient to confer $Ca^{2+}$ sensitivity since misexpression of all three in sugar-responsive GRNs using the *Gr5a-GAL4* did not confer attraction to $Ca^{2+}$-containing food, or induce $Ca^{2+}$-activated action potentials (Figure S2).

### IR co-expression in the labellum

IR25a is expressed in the labellum (Croset et al., 2010) but the specific classes of GRNs expressing IR25a have not been analyzed. We first characterized the *Ir25a-GAL4* reporter using *UAS-mCD8::GFP* to determine whether it reflected the same expression pattern as anti-IR25a. We found that anti-GFP and anti-IR25a staining overlapped extensively (Figure S3A—S3G). We introduced *Ir25a-GAL4;UAS-mCD8::GFP* into the $Ir25a^2$ mutant background and found that the anti-GFP signals were indistinguishable between control and $Ir25a^2$ flies (Figure S3H—S3J), indicating that the $Ir25a^2$ mutation did not result in loss of IR25a-expressing GRNs.

We compared anti-IR25a staining with a variety of reporters and found that IR25a exhibited wide overlap with different classes of GRNs. Anti-IR25a staining overlapped extensively with the *ppk23-GAL4* reporter (Figures 4A—4C, S3K—S3P), and anti-IR25a staining was undetectable in labella expressing *UAS-hid* under control of the *ppk23-GAL4* (Figures S3Q and S3R). We also detected anti-IR25a staining in all bitter-sensing GRNs, which are labeled by the *Gr66a* reporter (Figures 4D—4F), all carbonation buffer-sensing GRNs, which are stained by the *E409 GAL4* enhancer trap reporter (Figure 4G—4I), and a subset of sugar-sensing GRNs, which are marked by the *Gr5a* reporter (Figures 4J—4L). This indicates that IR25a is an exceptionally widely expressed chemoreceptor.

To address whether the three IRs are co-expressed in the same GRNs, we performed double-labeling experiments. We found that IR76b was co-expressed extensively with IR25a (Figures 4M—O and Figures S4A—S4C). The *Ir62a-GAL4* reporter is not detected in the labellum (Koh et al., 2014). However, it is expressed in the legs (Koh et al., 2014), as are the *Ir25a-GAL4* and *Ir76b-GAL4* reporters (Figures S4D—S4L). Therefore, to explore whether *Ir62a* RNA is expressed in the labellum, we performed RT-PCR using dissected labella, and found the predicted 1.8 kb band in wild-type labella (Figure S4M). However, we did not detect the band from labella from flies expressing *UAS-hid* under control of the *Ir25a-GAL4* (Figure S4M). The combination of these data supports the proposal that *Ir25a*, *Ir62a* and *Ir76b* expression overlap extensively in the labellum.

### Toxic effect of $Ca^{2+}$ in food

Because flies avoid high levels of $Ca^{2+}$, we tested whether high $Ca^{2+}$ is toxic to the flies. We compared the survival of flies maintained on 100 mM fructose, or on fructose mixed with different concentrations of $CaCl_2$ (Figure 4P). There was virtually no lethality after 8.5 days in the absence of $Ca^{2+}$. We found that $Ca^{2+}$ decreased viability in a concentration-dependent manner. There was only slight toxicity due to 1 or 10 mM $Ca^{2+}$ (Figure 4P). However, if the fructose was laced with 25 or 50 mM $CaCl_2$, none of the flies survived after 8.5 and 8 days, respectively (Figure 4P). The times in which 50% died ($LT_{50}$) were 163.5 ±9.3 hrs and 121.9 ±10.5 hrs for flies fed 25 and 50 mM $Ca^{2+}$, respectively. 100 mM $CaCl_2$ feeding affected survival rate more significantly (64.1 ±8.5 hrs). If the flies were fed $MgCl_2$ plus fructose, there was minimal lethality even after 204 hours (Figure 4Q).

To clarify if the death is due to $Ca^{2+}$ toxicity or to the detection of an aversive compound which induces stress, we tested $Ca^{2+}$-insensitive mutants using a binary food-choice survival

assay (Figures S4N—S4O). The flies were allowed to choose between 100 mM fructose alone, and 200 mM fructose mixed with either 50 mM $Ca^{2+}$ (Figure S4N) or 100 mM $Ca^{2+}$ (Figure S4O). Nearly all of the control flies survived for 10 days. However, *Ir25a*$^{2}$, *Ir62a*$^{1}$ and *Ir76b*$^{1}$ flies as well as *ppk23-GAL4/UAS-hid* animals showed high levels of concentration-dependent lethality (Figures S4N—S4O). After 10 days on the fructose versus fructose plus 100mM $Ca^{2+}$, <26.5% of the *Ir25a*$^{2}$ or *Ir62a*$^{1}$ flies were alive, while none of the *Ir76b*$^{1}$ flies survived (Figure S4O). These findings support the conclusion that feeding on high $Ca^{2+}$ induces lethality due to $Ca^{2+}$ toxicity.

## DISCUSSION

### Avoiding dangerous environmental $Ca^{2+}$ through dual mechanisms

$Ca^{2+}$ is an essential mineral. However, at high levels it causes toxicity (Žofková, 2016). Thus, consuming the ideal $Ca^{2+}$concentration is critical. Despite the fundamental importance of $Ca^{2+}$ for health, behavioral studies focusing on $Ca^{2+}$ attraction or avoidance have been limited, and are unexplored in *Drosophila*. We found that flies are endowed with the ability to taste $Ca^{2+}$. Surprisingly, rather than displaying attraction and repulsion to low and high concentrations of $Ca^{2+}$, respectively, which would be reminiscent of the $Na^{+}$ response, there is no attraction to low $Ca^{2+}$. The only reaction to $Ca^{2+}$ is repulsion to high levels. The lack of $Ca^{2+}$ attraction suggests that the normal environmental sources of nutrition for vinegar flies have ample $Ca^{2+}$, and there is no need for selecting a taste modality for $Ca^{2+}$ attraction. The avoidance behavior is due to $Ca^{2+}$ rather than $Cl^{-}$, because there is no avoidance to $MgCl_2$, while other $Ca^{2+}$-salts are aversive.

The question arises as to why vinegar flies have the capacity to avoid very high levels of $Ca^{2+}$. Indeed, the $Ca^{2+}$ concentration in the leaves of plants, such as tomatoes, eggplants and others, reaches 100 mM (Watanabe et al., 2016). Therefore, the capacity of flies to sense and avoid $Ca^{2+}$ levels at least as high as 100 mM deters them from consuming high $Ca^{2+}$ in their environment.

Unexpectedly, in addition to activating GRNs in S-type sensilla, $Ca^{2+}$ also suppresses sugar-activated GRNs in L-type sensilla. The influence of $Ca^{2+}$ on two types of taste receptor cells is reminiscent of the simultaneous effects of bitter compounds on activation of avoidance GRNs, and inhibition of sugar-activated GRNs (Jeong et al., 2013; Meunier et al., 2003). We suggest that employment of two strategies to detect high $Ca^{2+}$ levels provides a double safeguard to avoid consuming excessive $Ca^{2+}$, which is deleterious. Although the mechanism through which $Ca^{2+}$ suppresses the sugar response remains to be defined, it might involve an odorant binding protein (OBP), as bitter compounds suppress the sugar response by binding to an OBP, which in turn associates with and inhibits sugar receptors (Jeong et al., 2013).

### Composition and potential mechanism of activation of $Ca^{2+}$ sensor

The taste of $Ca^{2+}$ depends on three members of the variant *Ir* family: *Ir25a*, *Ir62a*, and *Ir76b*. Two of these IRs (IR25a and IR76b) are required to detect multiple types of stimuli, and this diversity appears to be defined by unique combinations of IRs, or by the IRs acting as

homomeric ionotropic receptors. For example, IR25a appears to be sufficient for sensing low-amplitude temperature cycles necessary for temperature synchronization of the circadian clock (Chen et al., 2015), but collaborates with IR93a and IR21a for cool sensation (Knecht et al., 2016; Ni et al., 2016). IR25a also acts as a humidity sensor along with IR93a and IR21a. Similarly, IR76b is sufficient for sensing low salt, and acts in concert with IR41a for olfactory attraction to polyamines (Hussain et al., 2016). However, the gustatory attraction to polyamines requires just IR76b (Hussain et al., 2016). IR76b also contributes to the taste of amino acids, and in the adult, this also depends on IR20a (Croset et al., 2016; Ganguly et al., 2017). Both IR76b and IR25a are also required in leg sensilla for sensing acids (Chen and Amrein, 2017).

There are at least two models to account for the requirements for the three IRs for $Ca^{2+}$ sensation. According to one model, one or both of the broadly required IRs (IR25a and IR76b) act as co-receptors and contribute to dendritic localization, in a manner similar to ORCO, which serves as a co-receptor for Odorant Receptors (ORs) (Abuin et al., 2011; Larsson et al., 2004). In this model, IR25a and IR76b only contribute to trafficking. Because no other function has been ascribed to IR62a, the specificity for $Ca^{2+}$ taste would be conferred exclusively by IR62a. A second model is that IR25a and IR76b, in addition to IR62a, contribute to the response to $Ca^{2+}$ independent of any contribution of their roles in trafficking. Nevertheless, as in the first model, IR62a is essential for the specificity for the $Ca^{2+}$ response. We favor the second model, as IR76b is cation channel (Zhang et al., 2013), and therefore does more than contribute to subunit trafficking. However, the repertoire of IRs required to sense $Ca^{2+}$ appears to be greater than *Ir25a*, *Ir62a*, and *Ir76b* since ectopic co-expression of these IRs in sugar-sensing GRNs was insufficient to confer a $Ca^{2+}$ response to these cells. Nevertheless, it is plausible that the IR complex might be directly activated by $Ca^{2+}$. The external $Ca^{2+}$ concentration in the endolymph of insect chemosensory neurons is estimated to be similar to the hemolymph (~1 mM) (Kaissling and Thorson, 1980). This ionic concentration may account for the observation that GRNs are not very sensitive to $Ca^{2+}$, and require mM concentrations of $Ca^{2+}$ to generate action potentials.

## $Ca^{2+}$ sensing through *ppk23* GRNs and a “mineral GRN”

The *ppk23* GRNs in forelegs contribute to sensing pheromones and in courtship behavior. However, the roles of *ppk23* GRNs in the labellum were unknown, but proposed to “detect a novel taste modality” (Toda et al., 2012). We conclude that *ppk23* GRNs in the labellum function in detecting aversive levels of $Ca^{2+}$, since killing these neurons virtually eliminates $Ca^{2+}$ sensitivity. Moreover, their roles in gustatory aversion is supported by our observation that artificial gustatory stimulation of these neurons causes avoidance to a chemical (capsaicin), to which flies are normally indifferent (Marella et al., 2006). However, it seems highly unlikely that these *ppk23* GRNs are tuned specifically to $Ca^{2+}$.

Only six sensilla were robustly activated by high levels of $Ca^{2+}$, and all were S-type. Among these sensilla is S8, which is unresponsive to bitter organic compounds (Weiss et al., 2011), but is also activated by high levels of $Na^{+}$ that cause repulsion (Zhang et al., 2013). This indicates that S8 is tuned to aversive levels of multiple minerals, rather than to bitter-tasting

organic chemicals. We suggest that this *ppk23* GRN in S8 is a broadly tuned "mineral GRN" that senses aversive levels of these elements.

A question arises as why only six sensilla elicit robust responses to $Ca^{2+}$, since *Ir25a* and *Ir76b* are expressed in GRNs in nearly all sensilla in the labellum. The *Ir62a* reporter is not detected in the labellum. Nevertheless, our RT-PCR results demonstrate that *Ir62a* RNA is expressed in the labellum, and specifically in *Ir25a* GRNs. Thus, *Ir62a* expression might be limited to the six S-type sensilla, and define the specificity. However, we suggest that this is not the explanation. *Ir25a* and *Ir76b* are widely expressed in many more than six sensilla, and expression of *Ir62a* in *Ir25a* GRNs, does not greatly extend $Ca^{2+}$ responses to other sensilla, with the exception of endowing modest response to a couple of I-type sensilla. However, there were some minor differences in $Ca^{2+}$-induced action potential between control flies, and mutant animals expressing wild-type transgenes. For example, while S1 sensilla are normally unresponsive to $Ca^{2+}$, some $Ca^{2+}$ response is induced upon expression of a *Ir76b*$^{+}$ rescue transgene using the *GAL4*/*UAS* system, possible due to higher expression of *Ir76b* than in control flies. Nevertheless, our misexpression experiments with *Ir62a* further support the model that the IR heteromultimer required for the $Ca^{2+}$ response is more complex than three subunits. Whether this involves additional IRs, or a β-subunit, remains to be determined.

### Concluding remarks

Our results demonstrate that $Ca^{2+}$ represents a previously unappreciated taste modality in flies, and is mediated through *ppk23* GRNs in the labellum—a previously enigmatic class of GRNs. In contrast to $Na^{+}$, which elicits robust attraction and avoidance at low and high concentrations respectively (Liu et al., 2003; Nakamura et al., 2002; Zhang et al., 2013), flies show $Ca^{2+}$ avoidance only, and exclusively to high concentrations. Moreover, the $Ca^{2+}$ GRNs are distinct from bitter GRNs, and at least one appears to be dedicated to sensing aversive levels of at least two minerals: $Ca^{2+}$ and $Na^{+}$. Repulsion to $Ca^{2+}$ is mediated through the dual activation of avoidance GRNs and suppression of sugar-activated GRNs. The findings from this study raise the possibility that the taste of $Ca^{2+}$ might be sensed exclusively as a deterrent in a host of other animals.

## STAR METHODS

### CONTACT FOR REAGENT AND RESOURCE SHARING

The fly stocks generated in this study will be deposited with the Bloomington Stock Center for public distribution (http://flystocks.bio.indiana.edu/). Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, C.M. (craig.montell@lifesci.ucsb.edu).

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

All experiments were performed with the indicated strains of adult male and female *Drosophila melanogaster*. The flies were 3—6 days-old for the two-way choice assays, 3—7 days-old for the tip recordings, and 3—4 days old for the survival assays. We used *w*$^{1118}$ as the "wild-type" control. *Ir21a*$^{1}$, *Ir56b*$^{1}$, *Ir62a*$^{1}$, *UAS-hid, UAS-DsRed*, *UAS-GFP* and *UAS-*

*mCD8::GFP* were from the Bloomington Stock Center. We previously described *Ir76b*[1], *Ir76b-GAL4*, *UAS- Ir76b* (Zhang et al., 2013), *Gr66a*[ex83] (Moon et al., 2006), *Gr33a*[1], *Gr33a*[GAL4] (Moon et al., 2009), *Gr8a*[1] (Lee et al., 2012), and *Gr98b*[1] (Shim et al., 2015). *Ir7a*[1], *Ir47a*[1], *Ir52a*[1], *Ir56a*[1], and *Ir94h*[1] mutants were generated by ends-out homologous recombination for other studies, and will be described elsewhere. The *Ir* RNAi lines were: *Ir11a* (v100422), *Ir20a* (v109324), *Ir21a* (v2471), *Ir47a* (v11812), *Ir52a* (v37173), *Ir52d* (v8963), *Ir56a* (v109691), *Ir56b* (v105928), *Ir56d* (v6112), *Ir60b* (v106225), *Ir67c* (v107921), *Ir76a* (v101590), *Ir94a* (v107734), *Ir94c* (v100967), *Ir94f* (v109702), and *Ir94h* (v100407) were tested after crossing *elav-GAL4;UAS-Dicer2* (Bloomington Stock Center). K. Scott provided the *ppk23*, *ppk23-GAL4* (Thistle et al., 2012), the *E409-GAL4* (Hussain et al., 2016), and the *ppk28-GAL4* (Cameron et al., 2010). H. Amrein provided the *Gr66a-GAL4* and the *Gr5a-GAL4* (Dunipace et al., 2001; Thorne et al., 2004). R. Benton provided *Ir25a*[2] and the *Ir25a-GAL4* (Abuin et al., 2011).

## METHOD DETAILS

**Generation of Transgenic Flies—**To generate *UAS-Ir25a* animals, we first subcloned the full-length EST clone (IP13516) between the EcoRI/XhoI sites of the pUAST vector. The transformation vector was injected into *w*[1118] embryos (BestGene Inc.). To obtain the *Ir25a* genomic transgene, P[g*Ir25a*], we subcloned the genomic region from P[*acman*] CH321-90F22 (www.pacmanfly.org) into the attP154 insertion site on the 3rd chromosome (BestGene Inc.).

To generate the *UAS-Ir62a* transgene, we prepared RNA from labella, and amplified the full-length *Ir62a* cDNA by RT-PCR using the following primer pair: 5'-CAGAATTCACGAGCGAAAATGT-3' and 5'-TACCTCGAGTGCATTAATCC-3'. We subcloned the cDNA between the EcoRI/XhoI sites of the pUAST vector, verified the cDNA by DNA sequencing, and injected the plasmid into *w*[1118] embryos (KAIST *Drosophila* Research Center).

### Chemicals

Sucrose and sulforhodamine B, $MgCl_2$, KCl, $CaCl_2$ and tricholine citrate were purchased from Sigma-Aldrich Co. Brilliant blue FCF was obtained from Wako Pure Chemical Industry Ltd.

### Behavioral Assays

We performed two-way choice assays using 72-well plates as previously described (Meunier et al., 2003; Moon et al., 2006). We employed two paradigms. First, we used 2 mM sucrose versus 2 mM sucrose and different concentration of $CaCl_2$, which we added to alternating wells. Second, we used 1 mM sucrose versus 5 mM sucrose mixed with different concentration of $CaCl_2$. The two food alternatives used in each assay were added to 1% agarose, mixed with either blue (brilliant blue FCF, 0.125 mg/ml) or red food coloring (sulforhodamine B, 0.1 mg/ml).

To conduct each assay, we starved 50–70 flies (3–6 days old) for 18 hr in a humidified chamber, and introduced the animals into a dish. The dishes were kept in a dark humidified

chamber, and the flies were allowed to feed for 90 min. To determine their food preferences, the flies were frozen at -20°C, the color of their abdomens were analyzed using a stereomicroscope, and the number of flies that were that were blue ($N_B$), red ($N_R$), or purple ($N_P$) were counted. In cases in which there was a mixture of red and blue dyes in the abdomens, we assigned the animals as red and blue if the percentage of the blue and red colors in the abdomens, respectively, was <5%. If the other color crossed the 5% threshold, we designated the flies as purple. The preference indexes (P.I.) were calculated according to the following equation: $PI = (N_{red} + 0.5N_{purple}) - (N_{blue} + 0.5N_{purple})/(N_{red} + N_{blue} + N_{purple})$, or $PI = (N_{blue} + 0.5N_{purple}) - (N_{red} + 0.5N_{purple})/(N_{red} + N_{blue} + N_{purple})$, depending on the dye/tastant combinations. P.I.s of 1.0 and -1.0 indicate complete preferences for one food option or the other. A P.I. of 0 indicates no bias between the two food alternatives.

## Tip Recordings

We performed tip recordings as previously described (Moon et al., 2006). Briefly, we first immobilized 3—7 days old male or female flies on ice. We then inserted a reference glass electrode filled with Ringer's solution into the thorax of the fly, and extended the electrode toward the proboscis. We stimulated the sensilla with tastants dissolved in the buffer solution in the recording pipette (10–20 μm tip diameter). 1 mM KCl was used as the electrolyte for recording from S-type, I-type sensilla and 30 mM tricholine citrate was used for L-type sensilla. The recording electrode was connected to a preamplifier (TastePROBE, Syntech, Germany), and we collected and amplified the signals 10x using a signal connection interface box (Syntech) in conjunction with a 100–3000 Hz band-pass filter. Recordings of action potentials were made using a 12-kHz sampling rate and analyzed using Autospike 3.1 software (Syntech). Spike sorting was used as an indicator of the spike amplitudes that correspond to the action potentials of $CaCl_2$ sensitive neurons, rather than the relatively small amplitudes of water spikes. The number of action potentials was counted from 50—550 msec after application of the $CaCl_2$. The numbering nomenclature for the sensilla was as described (Hiroi et al., 2002).

## Survival Assays

We performed survival assays using male and female control flies as previously described (Lee et al., 2010). The food consisted of 1% agarose and either 100 mM fructose alone, or 100 mM fructose plus different concentrations of $CaCl_2$ or $MgCl_2$ as indicated in Figures 4P, 4Q, S4N and S4O. To perform the assays, we exposed 10 male and 10 female flies (3—4 days old) to the food source at 25°. The number of viable flies were tabulated every 12 hr, and then transferred to new vials containing the same food source. The assays were terminated after 204 hrs. Each condition was tested 4—7 times.

## Immunohistochemistry

The labella of the indicated flies were dissected and fixed using 4% paraformaldehyde (Electron Microscopy Sciences, Cat No 15710) with 0.2% Triton X-100 for 15 min at room temperature (Lee et al., 2012). The labella were washed ≥3 times in PBST (1x PBS and 0.2% Triton X-100), placed in blocking buffer (0.5% goat serum in PBST), cut in half with a razor blade, and incubated with blocking buffer for 30 min at room temperature. The labella

were transferred to new blocking buffer containing the primary antibodies (mouse anti-GFP, Molecular Probe, 1:1000; rabbit anti-DsRed, Clontech, 1:1000; rabbit anti-IR25a, L. Vosshall, 1:1000) and incubated overnight at 4°C. The labella were washed three times with PBST and incubated with secondary antibodies (goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 568) for 4 hr at 4°C. The labella were washed three times with PB ST and mounted in 1.25x PDA solution (37.5% glycerol, 187.5 mM NaCl, 62.5 mM Tris pH 8.8), and viewed by confocal microscopy (Carl Zeiss LSM510).

### RT-PCR Analyses of *Ir62a*

20–25 proboscises from control and *Ir25a-Gal4/UAS-hid* flies were dissected, and RNA was extracted using TRIZOL (Invitrogen). cDNA was synthesized from the extracted RNA using AMP transcriptase (Promega). The *Ir62a* cDNA was amplified using the *Ir62a* specific primer used to generate *UAS-Ir62a* as described above (5'-CAGAATTCACGAGCGAAAATGT-3' and 5'-TACCTCGAGTGCATTAATCC-3'), and the primer pair used to amplify the *tubulin* cDNA was: 5'-TCCTTCTCGCGTGTGAAACA- 3' and 5'-CCGAACGAGTGGAAGATGAG-3'.

### QUANTIFICATION AND STATISTICAL ANALYSIS

All error bars represent standard error of the means (SEMs). The number of times each experiment was repeated (n) is indicated in the figure legends. For the two-way choice assays, each "n" represents a single test performed with 50—70 animals. For the survival assays, each "n" includes 20 flies (10 male and 10 female flies). Each "n" for the tip recordings represents an analysis of a single, independent fly. Single factor analysis of variance (ANOVA) with Scheffe's analysis as a *post hoc* test was used to compare multiple sets of data. Asterisks indicate statistical significance compared with the control (*$P < 0.05$, **$P < 0.01$).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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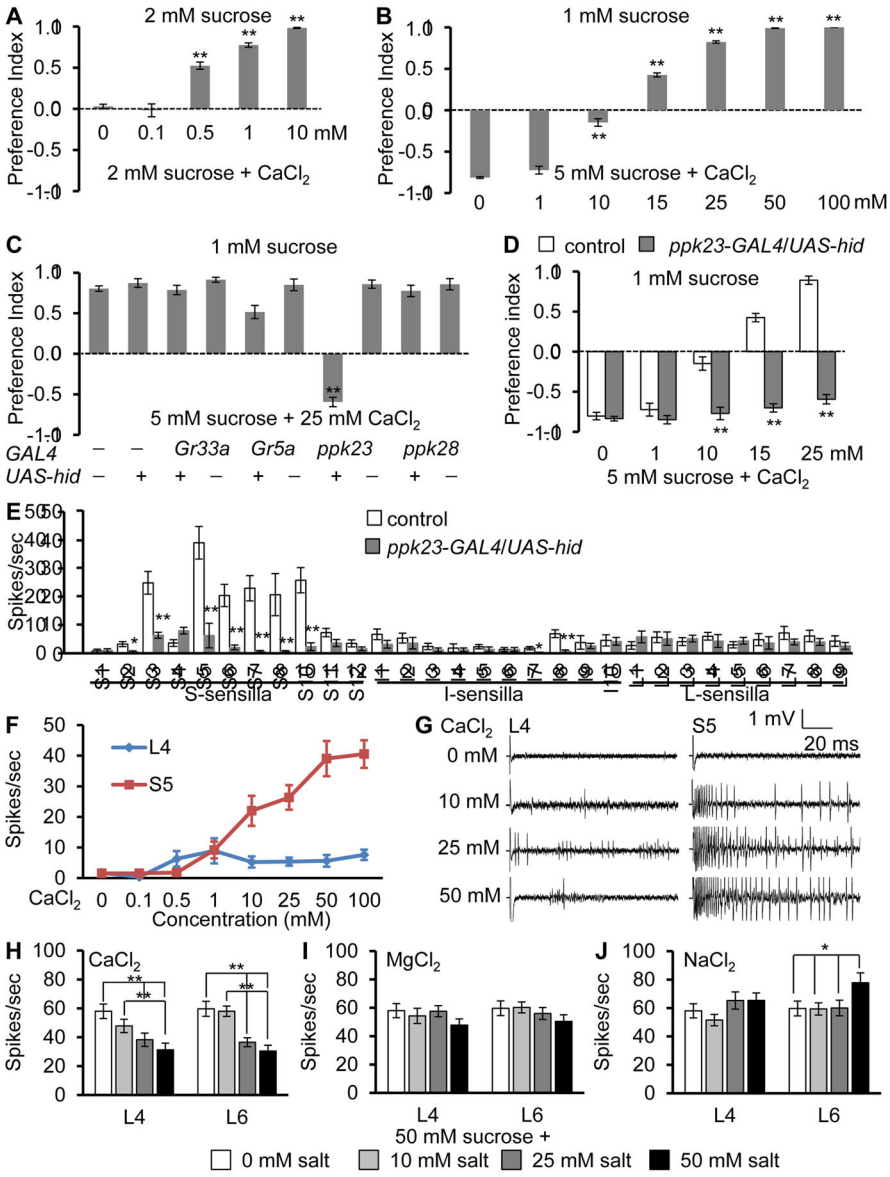


**Figure 1. Flies avoid $Ca^{2+}$ via *ppk23* GRNs**

(A—D) Two-way choice taste assays. 50—70 flies/assay

(A) Preferences of control flies (*w*$^{1118}$) to 2 mM sucrose alone versus 2mM sucrose and the indicated concentrations of $CaCl_2$. n=4.

(B) Preferences of control flies to 1 mM sucrose alone versus 5 mM sucrose and the indicated concentrations of $CaCl_2$. n=4.

(C) Two-way choice assays after ablating different GRNs by *UAS-hid* under control of the indicated *GAL4*s. n=4.

(D) Effects of ablation of *ppk23* GRNs (*UAS-hid*/*ppk23-GAL4*) on $Ca^{2+}$avoidance. n=4.

(E—G) Assaying $Ca^{2+}$-induced action potentials using tip recordings.

(E) $CaCl_2$ (50 mM)-induced action potential frequencies in control and *UAS-hid*/*ppk23-GAL4* sensilla. n=10–24.

(F) Responses of L4 and S5 sensilla to different $CaCl_2$ concentrations . n=17–22.

(G) Tip recordings from S5 and L4 sensilla using 0—50 mM $CaCl_2$.
(H—J) Effects of different salts on sucrose-induced action potentials in L4 and L6 sensilla. The pipets contained 50 mM sucrose and 0—50 mM salts: (H) $CaCl_2$, (I) $MgCl_2$, (J) NaCl. n≥10. Error bars indicate SEMs. ANOVA tests with Scheffe's post hoc analyses between control and *ppk23*-GRNs ablated sensilla. $*P < 0.05$, $**P < 0.01$.

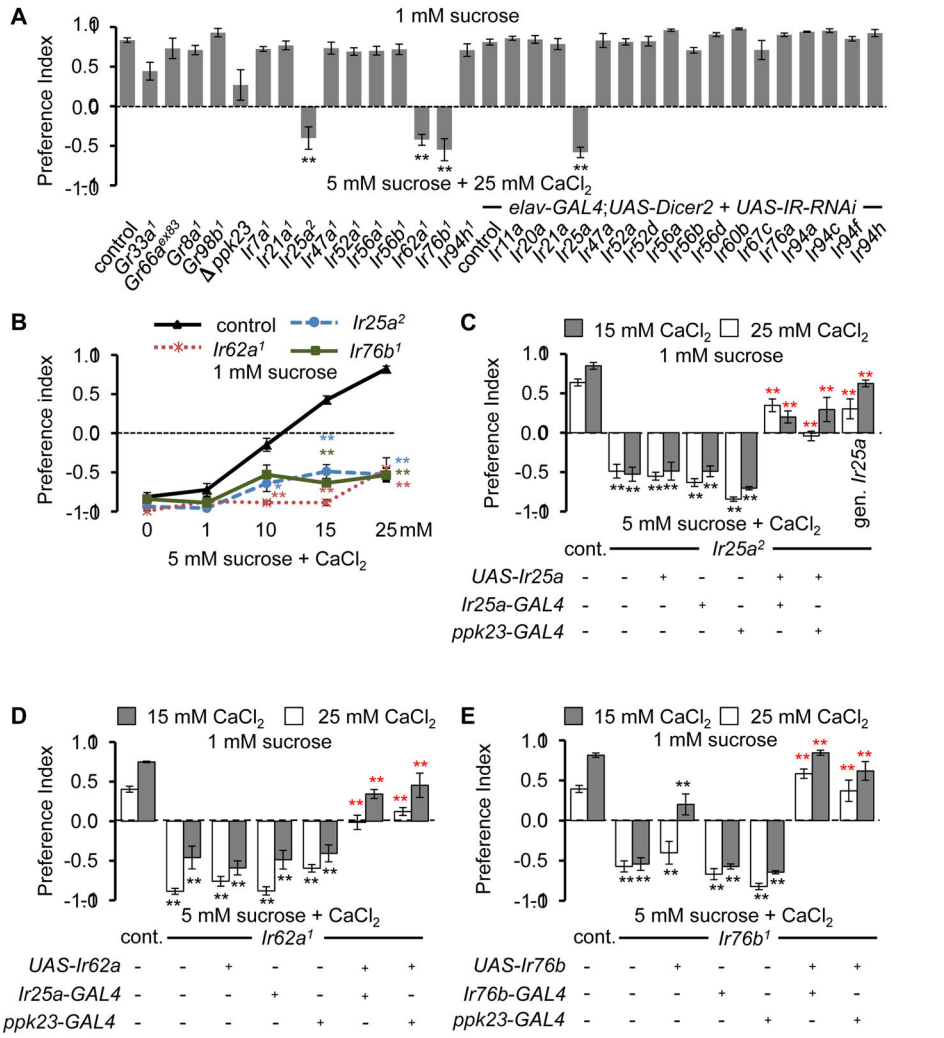


**Figure 2. Requirements for three *Ir*s for rejecting $Ca^{2+}$-containing food**

(A) Screening candidate chemoreceptors for defects in $Ca^{2+}$ aversion. n=4—12.

(B) Dose-dependent avoidance of *Ir* mutants to $Ca^{2+}$. n=4.

(C) Rescue of $Ca^{2+}$ avoidance defects in *Ir25a*$^{2}$ by expressing *UAS-Ir25a* using the indicated *GAL4*, or the *Ir25a*$^{+}$ genomic transgene (gen. *Ir25a*). n=5—12.

(D) Rescue of $Ca^{2+}$ avoidance deficits in *Ir62a*$^{1}$ by expressing *UAS-Ir62a* using the indicated *GAL4*. n=4.

(E) Rescue of $Ca^{2+}$ avoidance impairments in *Ir76b*$^{1}$ by expressing *UAS-Ir76b* using the indicated *GAL4*. n=5—13. Error bars represent SEMs. Asterisks in A and B indicate significant differences from controls (*$P < 0.05$, **$P<0.01$) using ANOVA with Scheffe's *post hoc* test. The black and red asterisks in C—E indicate significant differences from the controls and mutants, respectively (*$P < 0.05$, **$P<0.01$), using ANOVA with Scheffe's *post hoc* test.

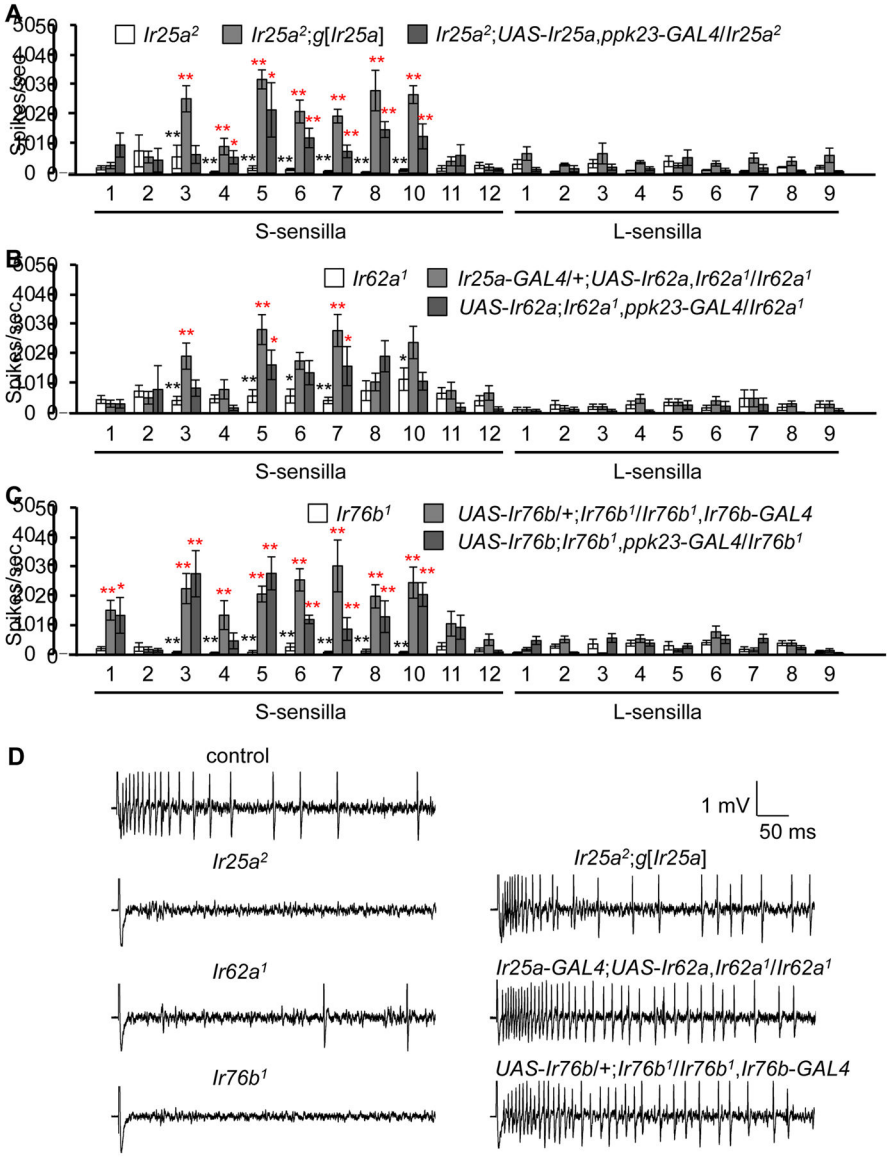


**Figure 3. Dependence on *Ir25a, Ir62a,* and *Ir76b* for $Ca^{2+}$-induced action potentials**

Tip recordings were performed in response to 50 mM $CaCl_2$.

(A—C) Mean responses of sensilla from *Ir25a*$^{2}$, *Ir62a*$^{1}$ or *Ir76b*$^{1}$, or the mutants expressing the genomic rescue transgene (*g*[*Ir25a*]) or a *UAS-cDNA* rescue transgene under control of the indicated *GAL4*s. n=10—26.

(D) Representative traces showing $Ca^{2+}$-induced action potentials.

Error bars represent SEMs. The black and red asterisks indicate significant differences from the controls and the mutants, respectively (**$P$<0.01, *$P$<0.05), using single factor ANOVA with Scheffe's *post hoc* test to compare two sets of data.

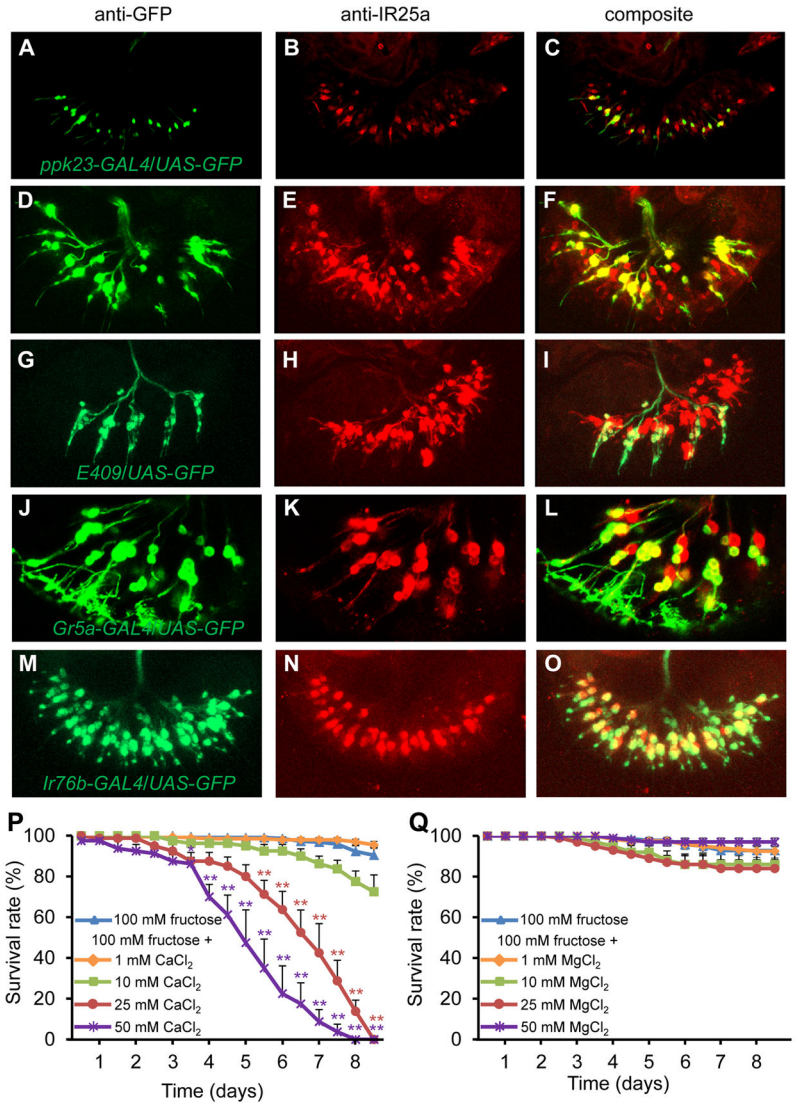


**Figure 4. Labellar expression of IR25a, and toxicity of high $Ca^{2+}$**

(A—O) Staining of labella from controls viewed by confocal microscopy. 3-D reconstructions generated by maximum transparency. Scale bars represent 25 μm.

(A, D, G, J, M) *UAS-GFP* expression driven by the indicated *GAL4*s. The signals were detected by anti-GFP staining (green).

(B, E, H, K, N) Anti-IR25a staining (red).

(C, F, I, L, O) Merged images of anti-GFP and anti-IR25 staining.

(P and Q) Survival of control flies fed 100 mM fructose or 100 mM fructose mixed with: (P) $CaCl_2$, or (Q) $MgCl_2$. n=4—7. Error bars represent SEMs. The asterisks indicate significant differences from the fructose only feeding (**$P$<0.01, *$P$<0.05.) using single factor ANOVA with Scheffe's as a *post hoc* test to compare two sets of data.