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The Molecular Basis for Water Taste in Drosophila

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The Molecular Basis for Water Taste in *Drosophila*

By

Peter Sean Cameron

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in

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in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

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ABSTRACT

The Molecular Basis for Water Taste in *Drosophila*

By

Peter Sean Cameron

Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Kristin Scott, Chair

The sense of taste allows animals to detect and assess potentially nutritive and toxic substances prior to ingestion. Animals have evolved to detect taste substances that are present in their environment. In *Drosophila melanogaster*, these include (but may not be limited to), sugars, salts, toxic or noxious bitter compounds, CO_2, and water. How do flies detect diverse taste substances? The first part of this thesis describes the results of a microarray-based screen performed in order to identify novel taste detection components. More specifically, a screen comparing RNA from proboscises with and without gustatory neurons enriched for known taste sensillum associated transcripts (gustatory receptors and odorant binding proteins) as well as transcripts with no known gustatory ascribed function. This latter group included transcripts with homology to ion channels and transporters, cytochromes, transcription factors, and proteases. A secondary screen with transgenic flies identified genes whose putative cis-regulatory sequence directed reporter expression in specific subsets of taste neurons, including epithelial sodium channel/degenerin (ENaC/Deg) family members, ionotropic glutamate receptors (iGluRs), an orphan G-protein coupled receptor, and a carbonic anhydrase.

The second part of this thesis focuses on the molecular basis for water taste. Here, I identify a member of the ENaC/Deg family, ppk28, as an osmosensitive ion channel that mediates the cellular and behavioral response to water. I use molecular, cellular, calcium imaging and electrophysiological approaches to show that *ppk28* is expressed in water-sensing neurons and loss of *ppk28* abolishes water sensitivity. Moreover, ectopic expression of *ppk28* confers water sensitivity to bitter-sensing gustatory neurons in the fly and sensitivity to hypo-osmotic solutions when expressed in heterologous cells. These studies link an osmosensitive ion channel to water taste detection and drinking behavior, providing the framework for examining the molecular basis for water detection in other animals.

The third part of this thesis describes ongoing work with two ENaC/Deg family members termed ppk23 and CG13568. These molecules are largely co-expressed in a subset of taste neurons on the proboscis. Double labeling experiments strongly suggest that these molecules label a novel class of taste neurons. Mutant analysis suggests that

these molecules are not involved in salt detection. Here I describe ongoing efforts to identify ligands and chemosensory functions for these two molecules.

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1) Routinely engaging in enthusiastic scientific discussions (along with Walter and Sunanda) the first year or two after joining the lab. This often exposed me to the bigger questions in the field (and other fields) and gave me a broader perspective on science that graduate students are not necessarily exposed to. Even though I was somewhat naïve and just starting out, there was no sharp division between "PI" and "graduate student," and I really benefitted from that.
2) Providing tremendous help and working very hard to help get the ppk28 paper out with maximum expedience.
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Growing up, I definitely admired my father's breadth of scientific knowledge and ability to think clearly and logically about problems. This definitely inspired me and is partly why I have pursued a career in science.

CHAPTER 1:

INTRODUCTION

Organisms have evolved sensory systems in order to detect and respond appropriately to stimuli in the external environment. These stimuli include diverse chemicals, light, mechanical force, osmolarity fluctuations, and changes in temperature. In terrestrial animals, including mammals and insects, these sensory systems can be generally categorized into several primary senses: gustation, olfaction, vision, touch, audition, and balance. These senses are moreover specifically tuned to meet an animal's unique ecological needs. For example, human-feeding mosquitoes have evolved an olfactory system that is highly sensitive to human emitted odors (Carey et al., 2010), honeybees can visualize ultra violet light (Townson et al., 1998), and snakes even possess a unique sensory system for detecting infrared radiation (Gracheva et al., 2010). Nonetheless, sensory systems across diverse organisms maintain many common and fundamental features.

Of the above stated senses, vision, touch, audition, and balance can be generally classified as detecting stimuli than can be categorized by continuous properties. Indeed, the visual system detects light, while the tactile, auditory, and vestibular (balance) systems detect changes in force. Alternatively, the gustatory and olfactory systems detect chemical substances in the environment that cannot be readily categorized by any particular continuous property.

How do sensory systems detect "continuous" non-chemical stimuli in the environment? In the visual system, light sensitive cells (rod and cone cells in the mammalian retina or photoreceptor neurons in the Drosophila compound eye, for example) express G-protein coupled receptors termed rhodopsins that are tuned to detect light at differing wavelengths. More specifically, rhodopsin is covalently attached to the light absorbing pigment 11-*cis*-retinal. This pigment absorbs light, undergoes a photoisomerization event, and triggers the catalytic activity of the rhodopsin protein and ultimately a signal transduction cascade that causes subsequent changes in neural activity (Lodish et al., 1999; Zuker, 1996). The amplifying nature of the system allows for exquisite sensitivity in detecting visual stimuli.

Mechanosensation, or force detection, underlies the tactile, auditory, and vestibular systems. In contrast to vision, less is known of the precise molecular mechanisms whereby sensory systems detect mechanical stimulation. In vertebrate audition and balance, hair cells in the inner ear with specialized actin-rich stereociliar protrusions termed "hair-bundles" relay sound wave and gravity induced mechanical stimuli. Hair-bundles are linked together by cadherin "tip-links" which aid in bundle stiffness. Indeed, a transduction channel at the tip of the stereocilia is ultimately gated by bundle deflection (Gillespie and Mueller, 2009). While much is known of the localization and biophysical properties of the stereociliar transduction channel, its precise molecular nature and definitive gating mechanism remain elusive (Gillespie et. al., 2009). In *Drosophila*, hearing and gravity sensing is mediated by chordotonal neurons within the Johnston's organ of the 2nd antennal segment. Various transient receptor potential (trp) channels, including NAN, IAV, *nompC*, *painless,* and *pyrexia* have been shown to have roles in either or both of these processes. To what extent these channels are directly detecting force awaits further confirmation (Kung, 2005; Sun et al., 2009).

Similar to hearing and balance, there is still much to learn of the molecules and gating mechanisms involved in touch sensation. In mammals, investigation into the molecular basis of touch-sensitivity has produced a long list of candidate mechanotransduction channels, such as trp channels, ENaC/Degenerins, and KCNK channels. The complexity and diversity of mechanosensitive cell types tuned to detect different aspects of tactile stimuli in the mammalian somatosensory system makes the problem both daunting and exceptionally interesting (Gerhold and Bautista, 2009). Excitingly, much headway has been made in identifying candidate mechanotransducers in invertebrate systems. In *C. elegans*, genetic screens have identified both ENaC/Deg family members (mec-4 and mec-10) as well as trp family members involved in touch sensation. Similarly, genetic analyses of *Drosophila* touch insensitive mutants identified nompC, a trp channel, as playing an essential role in mediating mechanosensitive currents in epithelial bristles (Walker et. al., 2000). *Drosophila* larval mutants of the ENaC/Deg channel ppk1 are defective in normal crawling behavior and harsh touch sensation (Zhong et. al., 2010). Future work will surely aim to clarify how these different candidate mechanosensitive channels mediate tactile force detection and whether there are additional mechanotransduction channels that have been previously unidentified.

Olfaction

How do animals detect volatile chemical substances? In mammals, odorants are detected by members of a large family (~800-1500) of G-protein coupled receptors (GPCRs) known as odorant receptors (Buck and Axel, 1991; Touhara and Vosshall, 2009). These receptors are expressed in the nasal epithelium, where each olfactory sensory neuron, using a mechanism that is currently not understood, expresses one allele of a given odorant receptor. This most likely sharpens the odorant specificity of a given OSN, which in turn can communicate specific information to downstream olfactory processing brain centers.

How do ORs detect the enormous diversity of potential odorant molecules? Individual odorants are usually recognized by a subset of ORs and individual ORs can be highly tuned for a specific odorant, more generally tuned to many odorants, or even inhibited by odorants (Oka et al., 2004). Efforts to de-orphan ORs on a large scale via heterologous systems have proven non-trivial due to variability in OR plasma membrane translocation efficiency (Touhara K., 2007). Nonetheless, heterologous experiments have demonstrated that ORs can recognize individual features of a given odorant, including functional group, size, and shape, by hydrophobic interactions in the odor-binding pocket of the transmembrane domains as well as hydrogen bonding (Katada et. al., 2005).

The sites of odor recognition in flies are the antennae and maxillary palps. These organs are studded with bristles that harbor olfactory sensory neurons, or OSNs, that generally express 2 (out of 62) odorant receptor proteins: an OR unique to the given OSN and a co-receptor termed Or83b. Thus, similar to mammals, this ensures that a given OSN has unique odorant response properties that can be conveyed directly to olfactory processing circuits in the brain (Touhara and Vosshall, 2009). Interestingly, insects ORs do not show significant homology to mammalian ORs. In fact, recent studies have shown that ORs retain a novel membrane topology and may even function as ion channels (opposed to their previously assumed roles as GPCRs) (Benton et. al., 2006; Sato et. al., 2008). Moreover, the Vosshall laboratory has recently identified a family of ionotropic glutamate receptors (iGluRs) that are expressed in OSNs that are odor responsive but devoid of ORs or GRs (Benton et. al., 2009). This exciting finding lends powerful credence to the idea that ion channels have evolved for smell detection in insects. Future studies will likely aim to further elucidate OR and iGluR structure-function relationships and evolutionary histories.

Mammalian gustation

The gustatory system is employed to detect and assess substances upon contact prior to ingestion. In mammals, these include sugars, salts, chemically diverse toxic or noxious bitter compounds, amino acids (also known as the taste of *umami*), and CO_2. Taste substances are detected by taste receptor cells (TRCs), which are modified epithelial cells located in taste buds (~50-100 TRCs/bud) throughout the surface of the tongue. These cells fire action potentials and send information to afferent nerves that project to the nucleus of the solitary tract (NST) in the brain stem (Yarmolinsky et. al., 2009).

Sugars and amino acids are detected by two heteromers of a family of GPCRs known as the T1Rs (Taste Receptor 1 family). The T1R2+T1R3 heteromer is tuned to detect a broad panel of sugars, artificial sweeteners, D-amino acids, and particular sweet proteins. The T1R1+T1R3 heteromer is tuned to detect a variety of L-amino acids in mice and is highly sensitive to L-glutamate in humans. These receptors are expressed in distinct cell types whereby their activation relays specific taste information to the brain. Bitter substances are detected via a large family of highly sensitive GPCRs known as the T2Rs (Taste Receptor 2 family). In mice, there are 35 different T2Rs that are expressed in a common cell type and mediate detection of chemically diverse bitter compounds. Activation of sugar, bitter, or amino acid taste receptors ultimately impinges on a common G-protein/phospholipase2B (PLC2B) mediated signal transduction cascade that activates the trpm5 cationic channel and subsequently depolarizes the taste cell (Scott, 2006; Yarmolinsky et. al., 2009).

How are salt and sour stimuli detected by the peripheral gustatory system? Previous work showed that mice lacking PLC2B or TRPM5 are deficient in detecting sweet, bitter, or amino acids, whereas their sensitivity to salt and sour stimuli remained unimpaired. This strongly suggested that mice employ a molecular mechanism for detection of acids and salts that is distinct from that used for sweet, bitter, and *umami*. Several molecules have been proposed as sour taste receptors, including the trp channels PKD2L1 and PKD1L3, HCN1, and HCN4. Ablation of cells expressing PKD2L1 abolished acid sensitivity, suggesting that PKD2L1 may be involved in sour detection (Chandrashekar et. al., 2006; Huang et. al., 2006). Future mutant analysis experiments should further clarify the role of these putative sour receptors in acid detection.

Salt, unlike sugar, bitter, and amino acids, possesses the unique quality of being appetitive or aversive depending on the concentration (animals seek out low concentrations of salt and are repulsed by extremely high concentrations). Previous pharmacological and electrophysiological experiments had long suggested that an amiloride-sensitive channel belonging to the ENaC/Deg family mediates some component of mammalian salt sensitivity (Scott, 2005). Indeed, Chandreshekar and colleagues recently confirmed this hypothesis through genetic and functional imaging experiments. They demonstrated that ENaCα is expressed in a cell type dedicated to low salt sensitivity and that ENaCα mutants are deficient in low salt taste cell sensitivity and behavioral attraction. Interestingly, lingual specific ENaCα mutants are still repulsed by highly concentrated saline solutions (Chandrashekar et. al., 2010). Future work will most likely identify other receptors that are involved in high salt detection.

Drosophila gustation

Despite the clear evolutionary divergence of mammals and flies, there is a striking degree of similarity in their taste systems. Firstly, flies and humans taste many of the same compounds, including sugars, salts, CO_2, and toxic or noxious bitter compounds (Scott, 2005). Flies also specifically taste water and cuticular hydrocarbon pheromones (Inoshita and Tanimura, 2006; Lacaille et. al., 2007). Unlike mammals, however, which only have one dedicated taste organ (the tongue), *Drosophila* in fact has multiple sites of taste recognition. Indeed, *Drosophila* has taste bristles located on the legs, wing margins, internal mouthparts, and ovipositor. Each taste bristle contains two to four gustatory receptor neurons (GRNs) and a mechanosensory neuron. These neurons send their dendrites into the shaft of the bristle tip where they come in contact with soluble taste substances. The GRNs send axons directly to the subesophageal ganglion (SOG) where they transmit taste information (Wang et. al., 2004).

How is the peripheral gustatory system organized in *Drosophila*? Previous receptor expression, calcium imaging, and electrophysiology experiments have demonstrated that, like mammals, there are several unique functional classes of taste cells, or GRNs. One class is labeled by the gustatory receptor Gr5a and mediates detection of sugar compounds, low salt, and acceptance behaviors. A second class, labeled by the receptor Gr66a, mediates detection of a wide range of bitter compounds, high salt, and avoidance behaviors (Marella et. al., 2006). A third class of taste neurons responds specifically to water stimulation and is inhibited by increasing taste solute concentration across a broad range of compounds (Inoshita and Tanimura, 2006). There exists additionally a fourth class of neurons, harbored in taste peg sensilla, that detect soluble CO_2 (Fischler et al., 2007). Finally, there is least one additional class of taste neurons for which there is currently no known ligand. These GRNs may serve as pheromone detectors and play a role during courtship, aggression, or other social behaviors. Alternatively, they may have another still undefined chemosensory function.

How are different taste substances detected in the environment by GRNs? A major breakthrough in the *Drosophila* taste field came via the identification of the Gustatory Receptors, or GRs (Clyne et. al., 2000; Scott et. al., 2001). Sugar and bitter sensing neurons both express a complement of distinct GRs that mediate taste recognition. Recent receptor “knock-out” and gene expression studies have suggested that both bitter and sugar detection may be mediated by GR heteromers (Montell, 2009). To what extent different GRs directly bind taste ligands and the potential heteromeric configurations of these receptors remains to be determined. . Unfortunately, efforts to reconstitute GRs in heterologous systems have largely failed (with one notable exception that showed that Gr5a is activated by trehalose), suggesting that there may be other currently unknown components in the taste transduction machinery (Chyb et. al., 2003; Montell, 2009). Excitingly, whether GRs function as GPCRs or potential ion channels is brought into question by recent controversial studies of OR family members (Sato et. al., 2008). Future work will most likely clarify this very interesting and important matter.

How do GRNs detect water? Previous electrophysiological experiments in *Drosophila,* as well as a host of other insects, have long demonstrated that insect taste cells are tuned to detect water and inhibited broadly by increasing taste solute concentration (Evans and Mellon, 1962; Werner-Reiss et. al., 1999; Lindemann, 1996; Gilbertson et. al., 2002). This inhibition by chemically diverse substances has led to the hypothesis that water taste detection is mediated by an osmosensor (Meunier et. al., 2009). In this thesis, I address this question and show that an osmosensitive ion channel belonging to the ENaC/Degenerin superfamily, termed ppk28, in fact directly mediates water taste detection in *Drosophila*.

How do GRNs detect salt? This is still a major outstanding question. Previous calcium imaging experiments have shown that Gr5a-expressing GRNs are sensitive to low and high salt, while Gr66a-expressing GRNs are sensitive only to high salt (Marella et. al., 2006). Therefore, similar to mammals, salt taste detection requires at least two functionally distinct cell types. Due to the role of ENaCs in mammalian salt taste, ENaC/Degenerins were also examined for their involvement in *Drosophila* salt taste sensitivity. Expression studies showed that two ENaC/Degenerins, termed *ppk11* and *ppk19*, were expressed in larval taste neurons. Moreover, RNAi and dominant negative experiments suggested a role for these molecules in salt taste sensitivity (Liu et. al., 2003). Further specific genetic mutant studies should further clarify to what extent these molecules mediate salt recognition.

Finally, as stated above, it is also known that flies possess additional unique gustatory neurons, some of which respond specifically to CO_2 as well as some of which that are thought to respond to another undefined stimuli, such as cuticular hydrocarbons or salt (Fischler et. al., 2007; Boll and Noll, 2002). How are these substances detected by the gustatory system? In this thesis, I describe the results of a microarray experiment that identified genes that are expressed in taste neurons. Several of these genes serve as ideal candidates for novel taste detection components. Future gene expression, calcium imaging, and mutant analysis should further elucidate the role, if any, of the microarray-identified molecules in *Drosophila* gustation, including CO_2, pheromone, or even salt detection.

CHAPTER 2:

A microarray-based screen to identify novel taste detection components

Summary

Fruit flies possess taste neurons that detect sugars, bitter compounds, salts, water, and CO_2. Opposed to sugar and bitter detection, there is currently very little known about the molecular mechanisms of salt, water, or CO_2 detection. To address this, I performed a microarray-based screen in order to identify novel taste detection components. A screen comparing RNA from proboscises with and without gustatory neurons enriched for known taste sensillum expressing transcripts (gustatory receptors and odorant binding proteins) as well as transcripts with no known gustatory ascribed function. This latter group included transcripts with homology to ion channels and transporters, cytochromes, transcription factors, and proteases. A secondary screen with transgenic flies identified genes whose putative cis-regulatory sequence directed reporter expression in specific subsets of taste neurons, including ENaC/Deg family members, ionotropic glutamate receptors (iGluRs), an orphan G-protein coupled receptor, and a carbonic anhydrase. Additionally, an *in situ* hybridization screen for 29 EnaC/Deg related *ppk* transcripts in proboscis tissue confirmed taste neuron expression of 3 *ppk* genes originally identified from the microarray. The results of this study provide a broad list of genes that may play fundamental roles in *Drosophila* taste detection, taste neuron development, or homeostasis.

Introduction

How do taste cells detect chemically diverse taste substances? The *Drosophila* gustatory system provides an excellent model system to understand how tastants are detected in the environment by gustatory neurons, as *Drosophila* is amenable to genetic manipulation and GRN activity monitoring, has a wealth of genetic and genomic resources, and exhibits robust taste-driven behaviors to an array of taste compounds. Indeed, *Drosophila* tastes sugars, chemically diverse bitter compounds, salts, water, soluble CO_2, and cuticular hydrocarbons.

Despite steady progress in understanding how sugar and bitter compounds are detected by GRs, there is very little known of how other taste substances are recognized. Why is this the case? Initial pharmacological and subsequent genetic and gene expression experiments in mammalian gustation demonstrated that sweet and bitter taste transduction was mediated by GPCRs (Striem et. al., 1989; Wong et. al., 1996, Hoon et. al., 1999). These observations, taken together with the precedent for GPCRs to mediate sensory detection in general, fueled the search and eventual identification of seven transmembrane domain receptors in the *Drosophila* gustatory system (Clyne et. al., 2000; Scott et. al., 2001). Thus far, all of the individual GRs examined via expression or genetic experiments have been implicated in either bitter or sugar detection (Montell C., 2009).

The precedent for GRs to mediate detection of sugar and bitter compounds as well as the paucity of GRN pharmacological investigation has made the search for molecules involved in water, salt, and CO_2 taste rather daunting. In principal, there are multiple approaches that one could undertake in order to identify molecules involved in these modalities. A forward genetic EMS or transposon insertion screen, for example, could provide an unbiased approach to identify novel taste detection components. This tactic, however, would be significantly hampered by the labor-intensiveness of scoring mutants. One could alternatively examine the pharmacological sensitivity of these taste modalities and subsequently use that information to search for receptors. This technique, while potentially fruitful, may suffer from nonspecific effects of pharmacological agents or of course the possible failure to identify pharmacological sensitivities of a given taste modality. Lastly, one could examine the expression of genes in the taste system to identify molecules that have restricted (or largely restricted) expression in gustatory neurons. This approach was shown to be effective in the mammalian gustatory system and also has the added benefit of being unbiased towards any particular class of molecules (Hoone et. al., 1999). Here, I perform a microarray-based screen to identify candidate novel taste receptors and several secondary GRN expression screens to further characterize various molecules of interest.

Results

A screen to identify genes enriched in taste neurons

To uncover novel molecules involved in taste detection, I performed a microarray-based screen for genes expressed in taste neurons. Proboscis RNA was compared from flies heterozygous versus homozygous for a recessive *poxn* null mutation, as these flies contain or lack taste neurons, respectively (Awasaki and Kimura, 1997; Boll and Noll, 2002). Whole genome microarray comparisons revealed that 256 of ~18,500 transcripts were enriched in heterozygous controls relative to *poxn* mutants (>2 fold enrichment in controls, $p<0.05$, moderated t-test). These include 18 gustatory receptor genes (representing a 21-fold enrichment in the gene set relative to their representation in the genome) and 8 odorant binding protein genes (13-fold enrichment) (Figure 1). In addition, genes belonging to various other classes, including ion channels/transporters (27 genes), cytochromes (13), proteases (12), and transcription factors (19), were decreased in *poxn* mutants (Figure 1; accession number GSE19984 at ncbi GEO).

Are any of these genes novel taste receptors or taste detection components? To further investigate this, I used the Gal4/UAS system to drive expression of a reporter from a given gene of interest's putative cis-regulatory sequence, and subsequently examined the gustatory system for reporter expression. I initially restricted the secondary screen to genes belonging to ion channel classes (as ion channels have been shown to mediate salt detection in the mammalian gustatory system) as well as several other classes of molecules previously implicated in chemosensory or neural circuit functions. More specifically, I focused on 7/11 ion channel related genes and 4 additional miscellaneous genes. In total, 5/7 examined ion channel related genes showed expression in GRNs as well as 2/4 miscellaneous genes (data not shown; I have yet to make transgenic flies to examine Ir11a, Ir76b, CG12344, and CG3078). Of these five, three were ENaC/Degenerin family members (ppk28, ppk23, and CG13568) and two were iGluRs (Ir25a and Ir21a), while the two miscellaneous genes included a putative orphan GPCR (CG31720) and a membrane-tethered carbonic anhydrase (CG3940).

This thesis largely focuses on the function of the ENaC/Deg family members. ENaCs are interesting candidates for novel taste receptors as they have been shown to mediate diverse sensory functions in a wide range of animals, including salt taste and acid sensing in mammals, touch sensation in *C. elegans* and *Drosophila*, peptide signaling, and pheromone signaling (Bianchi and Driscoll, 2002; Lin et. al., 2005). ENaCs have been shown to function as both heteromers and homomers (Bianchi and Driscoll, 2002; Jasti J, Furukawa H et. al., 2007). In *Drosophila*, there are ~29 ENaC/Deg family members, termed *pickpockets* (hereafter referred to as *ppks).* Therefore, to explore the full repertoire of *ppk* expression in the gustatory system, I performed an *in situ* hybridization screen for 28 *ppk* genes in proboscis GRNs. The 29th gene, *ppk1*, was expressed in proboscis neurons that are not GRNs, by Gal4/UAS transgenic studies (Ainsley et. al., 2003)). In total, 3/28 *ppk* genes (*ppk23, ppk28,* and *CG13568*) showed expression in GRNs (table 1). Excitingly, these genes were the only 3 also identified in the primary microarray-based screen and secondary Gal4/UAS-based screen, suggesting that the microarray was potentially thorough in identifying labellar GRN expressing *ppk* genes.

Conclusions

This study sought to identify GRN expressing transcripts in an effort to uncover novel taste detection components. By microarray analyses, I discovered 256 genes as significantly increased in control flies relative to flies lacking GRNs. How many of the identified molecules might have specific involvement in gustation? A large proportion of the genes examined in the secondary screen (7/11) showed GRN expression. Moreover, a genome wide proboscis *in situ* screen for *ppk* genes further reinforced the notion that the microarray-screen was efficient in identifying taste-enriched molecules. These observations taken together with the dramatic enrichment of known gustatory related transcripts (ie GRs and OBPs) strongly suggests that many of the microarray-identified transcripts will indeed have gustatory functions. The dataset may be useful to inform future studies of taste cell differentiation, axon guidance and taste cell signaling, as well as taste recpetor identification, the focus of this thesis.

Materials and Methods

Microarray

A sample of 164-280 proboscises of *poxn70/poxn70* and *poxn70/Cyo* males (8-18 days post eclosure) were dissected (3 samples per genotype) and total RNA was harvested in Trizol according to the manufacturer's instructions (Invitrogen). Twice amplified biotinylated cRNA was prepared from about 50ng total RNA starting material and then hybridized to Drosophila Genome Array 2.0 (Affymetrix). Data was processed and normalized using GC-RMA. Statistical significance was assessed with a moderated t-test. Raw and processed data from the microarray are deposited on the GEO website (accession number GSE19984).

Transgenic flies

Promoter-Gal4 transgenic flies were generated by cloning upstream DNA fragments into pCasper-Gal4. The following primers were used:
CG8546, F:AGTTCGCGGGGAGGGTCAAC,
R: GCGTGGGTAGGTGGCGTTTT;
CG31720, F: TGACCGATTTGCGAGCTTGTG, R:
CTAGTAAATCGGGTAATTGCCAATGGT;
CG17664, F: GGACTGCACTCCAGACCAAG,
R: GTGTGAATTGAATCTTATCGAATAAAC;
5-HT1a [*CG16720*], F: TGCAATATAATCCTTCGGGAATGC,
R: AACGAAAACTTTTTATCAGCAGCAAGC;
Ir21a, F: TGTTACAAAAACGTATCCCTATTAAGC,
R: TCAACATTGGAATATTTCTAAATACAG;
Ir25a: F: CGTTTGTTTGTTTGCCCTAAA,
R: TGTTGCTTGCTTGCCTAATG;
ppk28, see chapter 3 methods; *ppk23*, see chapter 4 methods; *CG13568*, see chapter 4 methods).

Immunohistochemistry and *in situ* hybridization

Double label immunohistochemistry *in situ* hybridization experiments were performed as previously described (Fishilevich and Vosshall, 2005) on flies containing *poxn-Gal4* (Boll and Noll, 2002) and *UAS-Gcamp* (Wang et. al., 2004) to visualize taste neurons in proboscis sections. All probes were labeled with Digoxigenin (Roche) and were between 0.4-1.4-kb. *ppk* genes were tallied as being expressed in GRNs if >1 *poxn*-expressing cells showed *ppk* expression. ~23- 180 *poxn*-expressing cells were counted/gene.

Experimental Animals

Drosophila stocks were maintained on standard cornmeal/agar/molasses medium at 25C. w^{1118} strains were used for transgene injections. P element-mediated germline transformations were performed using standard techniques (Genetic Services Inc). The following lines were used: *poxn70* (Boll et. al., 2002).

Figure Legends

Figure 1: Summary of microarray screen for genes expressed in taste tissue
Distribution of gene categories decreased in poxn homozygous versus heterozygous taste tissue (proboscis). Known chemosensory genes (blue) were decreased in the mutant, as were 11 ion channels (red). In total, 256 of ~18,500 transcripts were significantly decreased in poxn mutants (>2 fold enrichment in control relative to poxn, $P<0.05$, moderated t-test).

Table 1
Results from the *ppk* proboscis *in situ* hybridization screen. The last column lists genes that were significantly enriched in *poxn* heterozygotes relative to *poxn* mutants (>2 fold enrichment, $p<0.05$, moderated t-test). *ppk28* was expressed in taste neurons, though the number of expressing cells/*poxn* expressing cells were not tallied. NA, not available.

Figure 1

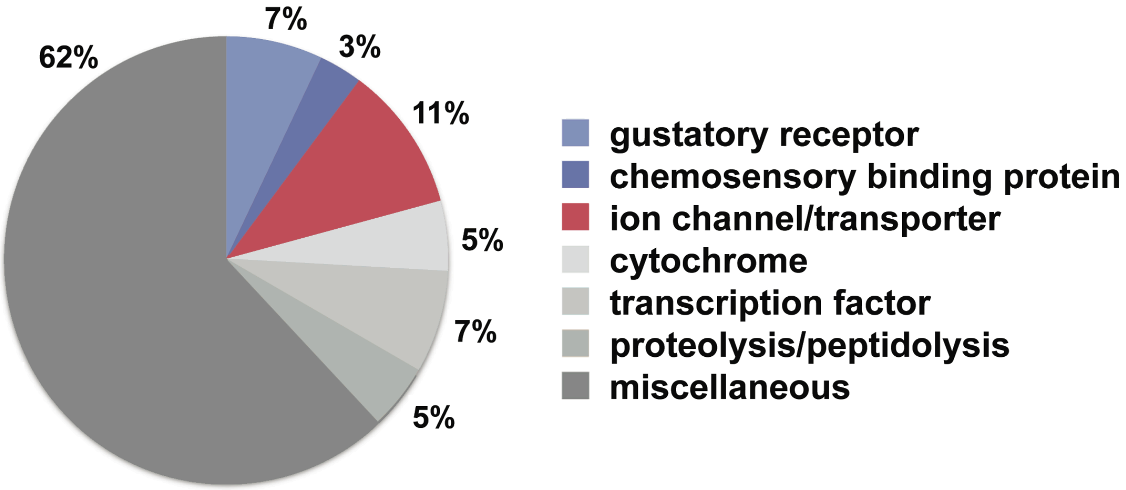
7%
3%
62%
11%
5%
7%
5%
gustatory receptor
chemosensory binding protein
ion channel/transporter
cytochrome
transcription factor
proteolysis/peptidolysis
miscellaneous

Table 1

Gene	GRN expression?	Positive cells/*poxn* GFP cells counted	Significantly enriched in *poxn* heterozygotes?
Ppk23	Yes	60/181	Yes
Ppk28	Yes	NA	Yes
CG13568	Yes	7/25	Yes
CG8546	No	0/126	Yes
CG13490	No	0/75	No
CG30181	No	0/96	No
CG32792	No	0/119	No
CG33289	No	0/91	No
CG18110	No	0/50	No
CG31105	No	0/24	No
CG13120	No	0/29	No
CG31065	No	0/44	No
CG15555	No	0/46	No
CG14239	No	0/27	No
CG10858	No	0/49	No
Ppk4/Nach	No	0/43	No
Ppk6	No	0/53	No
Ppk7	No	0/40	No
Ppk10	No	0/28	No
Ppk11	No	0/30	No
Ppk12	No	0/28	No
Ppk13	No	0/38	No
Ppk14	No	0/77	No
Ppk16	No	0/23	No
Ppk19	No	0/35	No
Ppk20	No	0/23	No
Ppk21	No	0/23	No
Ppk25	No	0/35	No

CHAPTER 3:

The Molecular Basis for Water Taste in *Drosophila*

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Summary

The detection of water and the regulation of water intake are essential for animals to maintain proper osmotic homeostasis. Drosophila and other insects have gustatory sensory neurons that mediate the recognition of external water sources, but little is known about the underlying molecular mechanism for water taste detection. Here, we identify a member of the Epithelial Sodium Channel/Degenerin family, ppk28*,* as an osmosensitive ion channel that mediates the cellular and behavioral response to water. We use molecular, cellular, calcium imaging and electrophysiological approaches to show that *ppk28* is expressed in water-sensing neurons and loss of *ppk28* abolishes water sensitivity. Moreover, ectopic expression of *ppk28* confers water sensitivity to bitter-sensing gustatory neurons in the fly and sensititivy to hypo-osmotic solutions when expressed in heterologous cells. These studies link an osmosensitive ion channel to water taste detection and drinking behavior, providing the framework for examining the molecular basis for water detection in other animals.

Introduction

Terrestrial animals must remain appropriately hydrated in order to function properly and survive. Precise regulation of water and electrolyte ingestion and excretion is essential to achieve osmotic homeostasis, critical for maintaining cell volume and intracellular ionic concentrations (Bourque, 2008). Despite the vital role of water consumption in osmotic regulation, surprisingly little is known about how animals detect water in their environment.

The gustatory system is the main sensory modality used to assess the content of fluid prior to ingestion, and is therefore of central importance in regulating water intake. Although taste cells that respond to hypo-osmotic solutions have been described in the mammalian gustatory system, their specificity and contribution to water ingestion remain unclear (Gilbertson, 2002). In fruit flies and other insects, electrophysiological studies have revealed the existence of a unique class of gustatory neurons that responds to water. In *Drosophila*, water-sensing gustatory neurons are activated by hypo-osmotic stimuli, inhibited by increasing concentrations of common taste substances and mediate water detection (Inoshita and Tanimura, 2006).

How do cells detect differences in osmolarity? One hypothesis is that osmosensation is a mechanical process whereby channels detect changes in membrane tension resulting from osmotic fluctuations. In *C. elegans*, *Drosophila* and mammals, members of the transient receptor potential (trp) family of non-selective cation channels have been implicated in osmosensation. *C. elegans osm-9* is expressed in sensory neurons and is necessary for the aversive response to hypertonic environmental conditions (Colbert et al., 1997). In *Drosophila*, two trp channels, water witch and nanchung, have been implicated in humidity detection (Liu et al., 2007). However, evidence that osm9, water witch or nanchung is directly activated by osmolarity is lacking. Two mammalian trp channels, trpv4 and trpv2, have been shown to confer responsiveness to hypotonic stimulation when expressed in heterologous cells, arguing that they can function as osmosensors although their *in vivo* role is less clear (Liedtke et al., 2000; Muraki et al., 2003). Although evidence is accumulating that members of the trp family function as peripheral or central osmosensors, the function of other genes in osmosensation is unknown.

The Drosophila gustatory system provides a unique opportunity to investigate the molecular mechanism of water detection, as Drosophila have well-described water-sensitive taste neurons accessible to electrophysiology and calcium imaging, exhibit robust thirst-driven behaviors and are amenable to genetic manipulation (Inoshita and Tanimura, 2006; Marella et al., 2006; Meunier et al., 2009). In Drosophila, specialized chemosensory bristles located on the proboscis, tarsi, wings and ovipositor detect taste substances. Each chemosensory bristle contains two to four gustatory neurons and a mechanosensory neuron (Falk et al., 1976). Gustatory neurons extend dendrites to the shaft of the bristle tip, where they come in direct contact with soluble taste substances, such as sugars, bitter compounds, salts and water. There are 68 Gustatory Receptors (GRs) in the Drosophila genome, many of which are expressed in gustatory neurons and mediate detection of sugars and bitter compounds (Hallem et al., 2006; Ebbs and Amrein, 2007). Whereas much is known about the molecular mechanism of sugar and bitter detection in Drosophila, there are currently no molecular candidates for water detection.

Here, we examine the molecular basis for water taste detection in *Drosophila* and identify an ion channel belonging to the Epithelial Sodium Channel/Degenerin family, pickpocket 28 (ppk28), as the water gustatory receptor. These studies demonstrate that an ion channel responding to low osmolarity mediates the cellular and behavioral response to water, providing insight into taste detection, drinking behavior and osmosensation.

Results

ppk28 is not expressed in sugar-sensing or bitter-sensing taste neurons

In the mammalian gustatory system, ion channels are thought to mediate the detection of sour and salt tastes (Yarmolinsky et al., 2009), suggesting that ion channel genes may also participate in Drosophila taste detection. We therefore examined the expression pattern of candidate microarray identified taste-enriched ion channels (chapter 2), and found that the putative promoter of one gene, *pickpocket 28* (*ppk28*), directed robust reporter expression in taste neurons on the proboscis (Figure 3.1A). ppk28 is a member of the Epithelial sodium channel family/Degenerin (ENaC/Deg), and these channels have been shown to have roles in detection of diverse stimuli, including mechanosensory stimuli, acids and sodium ions (Kellenberger and Schild, 2002). In the brain, *ppk28-Gal4* drives expression of GFP in gustatory sensory axons that project to the primary taste region, the subesophageal ganglion, arguing that the channel is taste-cell specific (Figure 3.1B). *In situ* hybridization experiments confirmed that transgenic expression recapitulates that of the endogenous gene, as 48/52 of *ppk28-Gal4* neurons expressed endogenous *ppk28*.

Previous studies have identified different taste cell populations in the proboscis, including cells marked by the gustatory receptor Gr5a that respond to sugars (Chyb et al., 2003; Thorne et al., 2004; Wang et al., 2004; Marella et al., 2006) and cells marked by Gr66a that respond to bitter compounds (Thorne et al., 2004; Wang et al., 2004; Marella et al., 2006; Moon et al., 2006). To determine whether these taste neurons express *ppk28-Gal4*, we performed co-labeling experiments with reporters for Gr5a and Gr66a. These experiments revealed that ppk28 did not co-label Gr5a cells or Gr66a cells, and is thus unlikely to participate in sweet or bitter taste detection (Figure 3.1CD, Figure 3.2). An enhancer-trap Gal4 line, *NP1017-Gal4,* marks water-responsive cells in taste bristles on the proboscis (Inoshita and Tanimura, 2006) and carbonation-sensing cells in the taste pegs (Fischler et al., 2007) (Figure 3.3). ppk28 is expressed in taste bristles but not in taste pegs. Interestingly, *ppk28* shows partial co-expression with *NP1017-Gal4* (Figure 3.3CDE), with the majority of ppk28-positive cells also containing *NP1017-Gal4* (22/30). This correlation suggested the intriguing possibility that ppk28 participates in water taste detection.

ppk28-expressing neurons respond to water and are inhibited by high osmolarity

To directly investigate the response specificity of *ppk28*-expressing neurons, we expressed the genetically encoded calcium sensor G-CaMP in *ppk28-Gal4* cells, simulated the proboscis with taste substances and monitored activation of *ppk28-Gal4* projections in the living fly by confocal microscopy (Marella et al., 2006). We tested *ppk28-Gal4* neurons with a panel of taste solutions, including sugars, bitter compounds, salts, acids and water. *ppk28-Gal4* neurons showed robust activity to water stimulation (Figure 3.4), comparable to that observed in Gr5a- and Gr66a-containing neurons when stimulated with their cognate ligands (Marella et al., 2006). In addition, ppk28-positive cells responded to other aqueous solutions even in the presence of a wide range of chemically distinct compounds, and this response diminished with solute concentration. Taste compounds such as NaCl, sucrose and citric acid significantly decreased the response (Figure 3.4, 3.5). In addition, compounds unlikely to elicit taste cell activity such as ribose, a sugar that does not activate Gr5a cells, N-methyl-D-glucamine (NMDG), an impermeant organic cation and the non-ionic high molecular weight polymer polyethylene glycol (PEG, 3350 average molecular weight), all blunted the response in a concentration-dependent manner (Figure 3.4, 3.5). This data demonstrates that *ppk28*-expressing neurons respond to hypo-osmotic solutions and is consistent with previous electrophysiological studies that identified a class of labellar taste neurons activated by water and inhibited by salts, sugars, and amino acids (Inoshita and Tanimura, 2006; Meunier et al., 2009).

ppk28 is necessary for water-invoked taste neuron activity

To determine the function of ppk28 in the water response, we generated a *ppk28* null mutant by piggybac transposon mediated gene deletion, removing 1.769kb surrounding the *ppk28* gene (Parks et al., 2004). We examined the water responses of *ppk28* control, mutant and rescue flies by extracellular bristle recordings of labellar taste sensilla. These recordings monitor the responses of the four gustatory neurons in a bristle, including water cells and sugar cells (Meunier et al., 2000). Control flies showed 12.0±0.9 spikes/sec when stimulated with water (Figure 3.6AB). Remarkably, *ppk28* mutant cells show a complete loss of the response to water (spikes/sec=0.8±0.1), and this response was partially rescued by reintroduction of *ppk28* into the mutant background (spikes/sec=6.4±1.0) (Figure 3.6AB). Responses to sucrose were not significantly different among the three genotypes (58.9±3.3 spikes/sec, 46.9±2.6 spikes/sec and 49.0±1.8 spikes/sec, for control, mutant and rescue flies, respectively) (Figure 3.6AB), arguing that the loss of *ppk28* specifically eliminates the water response. These results were confirmed by G-CaMP imaging experiments, which revealed that *ppk28-Gal4* neurons in the mutant did not show fluorescent increases to water and transgenic re-introduction of *ppk28* rescued the water response (Figure 3.6CDE).

Water consumption is reduced in *ppk28* mutants

The detection of water in the environment and the internal state of the animal may both contribute to water consumption. To examine the degree to which water taste detection contributes to consumption, we examined the behavioral responses of *ppk28* control, mutant and rescue flies to water. Drinking time rather than drinking volume was used to monitor consumption due to difficulty in reliably detecting small volume changes. When presented with a water stimulus, control flies drank on average 10.3±1.1 seconds, mutants drank 3.0±0.5 seconds and rescue flies drank 11.5±1.5 seconds (Figure 3.7). Additionally, control and mutant flies ingested sucrose (at a concentration that produces little or no water cell activity) for the same amount of time, demonstrating that *ppk28* mutants do not have any general drinking defects. Although *ppk28* mutants lack water taste cell responses and drink less, they still do consume water, arguing that additional mechanisms must exist to ensure water uptake. These experiments reveal that water taste neurons are necessary for normal water consumption and establish a link between water taste detection in the periphery and the drive to drink water.

Ectopic expression of ppk28 confers sensitivity to low osmolarity

The loss-of-function studies strongly suggest that ppk28 may function as the water receptor. If ppk28 is indeed the water receptor, then its expression in non-water sensing cells should bestow responsiveness to water. To test this, we used the Gal4/UAS system to ectopically express *ppk28* in *Gr66a*-expressing, bitter-sensing neurons and monitored taste-induced responses by extracelluar bristle recordings and G-CaMP imaging experiments. For extracellular bristle recordings, responses were recorded from i-type sensilla that contain bitter-sensing, Gr66a-positive neurons but not water cells (Hiroi et al., 2004). Expression of *ppk28* in *Gr66a-Gal4* neurons did not significantly affect the response to denatonium (control max%ΔF/F=11.9±1.2; misexpression max%ΔF/F=13.8±0.7) or caffeine (Figure 3.9AB), endogenous ligands for *Gr66a-Gal4* neurons (Marella et al., 2006). In response to water stimulation, *Gr66a-Gal4* neurons showed no significant activity, consistent with previous studies (Marella et al., 2006) (Figure 2.9). Remarkably, misexpression of *ppk28* in *Gr66a-Gal4* neurons conferred sensitivity to water stimulation, as seen by extracellular bristle recordings (Figure 3.9AB) and G-CaMP imaging (Figure 3.9CDE). Moreover, the response was blunted as solute concentration was increased. Both sucrose and NMDG (substances that do not activate *Gr66a-Gal4* neurons at concentrations tested) resulted in dose-sensitive response decreases, similar to that seen in endogenous *ppk28-Gal4* neurons. The finding that both activation by water and inhibition by other compounds are conferred by ppk28 strongly suggests that ppk28 is directly gated by low osmolarity, with the channel activated by hypo-osmotic solutions and inhibited by solute concentration.

To determine if ppk28 requires a taste cell environment to function or confers responsiveness to other cell-types, *ppk28* was expressed in HEK293 heterologous cells. A FLAG-tagged ppk28 (inserted after amino acid 222 in the extracellular domain) was expressed in HEK293 cells, confirming that protein was made and trafficked to the cell surface (Figure 3.8). For physiology experiments, an untagged version of ppk28 was co-transfected with dsRed. Cells expressing mammalian trpv4 osmo-sensitive ion channel were used as a positive control (Liedtke et al., 2000) and cells transfected with vector alone were used as a negative control. Cells were grown in a modified Ringers solution at 303 mmol/kg, loaded with Fluo-4 to visualize calcium changes and challenged with Ringers solution of different osmolalities (236, 216 and 174 mmol/kg; 80%, 70% and 60% osmotic strength to the isotonic solution, respectively). Under these experimental conditions, cells transfected with vector alone showed a modest response at 60% osmotic strength, whereas cells transfected with mammalian *trpv4* showed fluorescence increases to all hypo-osmotic solutions, as expected (Figure 3.10). (Liedtke et al., 2000). Importantly, cells transfected with *ppk28* significantly responded to decreased osmolality, with dose-sensitive responses elicited by osmolalities of 216 and 174 mmol/kg (Figure 3.10). These experiments reveal that *ppk28* confers sensitivity to hypo-osmotic solutions in a variety of non-native environments and strongly argue that the channel itself directly responds to osmolarity. This work provides a foundation for future studies of the biophysical properties of channel activation. Moreover, the ability to express ppk28 in heterologous cells and study its function creates the opportunity to compare its mechanism of gating with other ENaC/Deg family members involved in mechanosensation or sodium sensing.

Conclusion

These studies examined the molecular basis for water taste detection in *Drosophila* and identified an ion channel belonging to the ENaC/Deg family, pickpocket 28 (ppk28), as the water gustatory receptor. We showed via calcium imaging experiments that *ppk28* expressing neurons are sensitive to water and inhibited by increasing taste solute concentration across a broad range of chemically diverse compounds. Additionally, calcium imaging and electrophysiology experiments demonstrated that ppk28 is necessary for the cellular response to water, as removal of *ppk28* abolished water sensitivity. Misexpression experiments confirmed that ppk28 is sufficient to confer water sensitivity in *Drosophila* taste neurons and osmosensitivity in heterologous cells, strongly suggesting that ppk28 functions as a homomeric osmosensitive ion channel to mediate water taste detection. It will be very interesting to further examine the biophysical mechanism of ppk28 function, and to compare and contrast its mode of action with other ENaC/Deg family members, osmosensors, and mechanosensitive proteins.

Excitingly, *ppk28* mutants also have defects in drinking behavior, linking water taste detection in the periphery to water uptake. These experiments demonstrate that activation of *ppk28* expressing taste neurons are necessary for normal water ingestion and suggest that their activation may be sufficient to stimulate ingestion behavior. It is notable, however, that *ppk28* mutants still consume water, albeit less. This argues that there are additional mechanisms involved in mediating water consumption, such as activation of touch sensitive neurons located on gustatory sensing appendages during tastant stimulation. It will be intriguing to further examine the integration of water taste with mechanosensation and other taste modalities, as well as to identify additional neurons that are involved in coordinating water-drinking behaviors in the central nervous system.

Materials and Methods

Experimental Animals

Drosophila stocks were maintained on standard cornmeal/agar/molasses medium at 25C. w^{1118} strains were used for transgene injections. P element-mediated germline transformations were performed using standard techniques (Genetic Services Inc). The following lines were used: *NP1017-Gal4* (Inoshita and Tanimura, 2006).

Transgenic flies and *ppk28* mutants

The *ppk28 promoter-Gal4* construct was generated by cloning a 1.004kb genomic DNA fragment upstream of *ppk28* (16699333-16700336, Genbank accession number NC_004354.3) and transgenic flies were generated using standard procedures. Full-length ppk28 (transcript variant a, corresponding to NM_132941) was amplified from whole fly cDNA and was subcloned into pUAST. *ppk28* mutants were generated by gene deletion through FLP-FRT mediated recombination between flanking Piggybac transposons f05788 and e02329, confirmed by sequencing.

Immunohistochemistry and *in situ* hybridization

Labeling of the proboscis and brain was performed as previously described (Wang et al., 2004). In Figure 3.1B, the brain is counterstained with nc82 antisera (Hummel et al., 2000). In Figure 3.1CD, CD2 (magenta) and GFP (green) reporters were detected by immunohistochemistry on flies containing *ppk28-Gal4, UAS-CD2, Gr66a-GFP-IRES-GFP-IRES-GFP* or *Gr5a-GFP-IRES-GFP-IRES-GFP* transgenes (Wang et al., 2004; Marella et al., 2006).

G-CaMP imaging experiments

Imaging studies were performed as described (Marella et al., 2006). For all *ppk28-Gal4* imaging, flies were aged ~2-5 weeks to enhance G-CaMP1.3 levels. For Figure 2, flies were of genotype *UAS-G-CaMP; ppk28-Gal4; UAS-G-CaMP*. For NaCl, sucrose and ribose, flies were given 2-3 stimulations of differing concentrations, with the last stimulation being a positive control (>8% DF/F). For NMDG (adjusted to pH 7.4 with HCl) and PEG (average molecular weight 3,350), flies were given stimulations of various concentrations in random order with the last stimulation being a positive control (>7% DF/F). For Figure 3.4, genotypes were as follows. Control: *UAS-G-CaMP;ppk28-Gal4;UAS-G-CaMP*. Mutant: *Dppk28, UAS-G-CaMP;ppk28-Gal4;UAS-G-CaMP*. Rescue: *Dppk28, UAS-G-CaMP;ppk28-Gal4;UAS-G-CaMP, UAS-ppk28*. Flies were stimulated with taste substances in random order and experiments were performed blind to genotype. For Figure 3.9, genotypes were as follows. Gr66a: *UAS-G-CaMP;Gr66a-Gal4;TM2/TM6b*. Gr66a + *ppk28*: *UAS-G-CaMP;Gr66a-Gal4;UAS-ppk28*. Flies were stimulated with taste substances in random order followed by a positive control of 10mM denatonium (>8% DF/F).

Electrophysiology

2-3 day old flies were transferred on fresh medium one day prior to the experiment. For recording activity from taste neurons, a reference glass electrode filled with AHL solution (Marella et al., 2006) was placed in the head and a recording electrode filled with testing taste solution covered the tip of a single taste bristle. All test solutions contain 1 mM KCl as an electrolyte. The signal was amplified (100X total), filtered (<2800 Hz) by amplifiers (DTP-2, Syntech, Kirchzarten, Germany; CyberAmp 320, Molecular Devices, Sunnyvale, CA) and stored on a PC. Action potentials were counted for the first 1 second.

HEK293 calcium imaging experiments

Measurements in cells were made by using calcium indicator Fluo-4 (Invitrogen) and a confocal laser scanning microscope (Zeiss LSM510, Carl Zeiss, Jena, Germany). Cells were seeded on poly-D lysine coated glass one day prior to transfection (lipofectamine 2000, invitrogen), then incubated for 24-48 hours prior to imaging. Cells were then loaded with 10μM Fluo-4 for 45 min at 37ºC in isotonic calcium imaging buffer (76mM NaCl, 5mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 10mM Glucose, 10mM HEPES, 138mM Mannitol, pH 7.4). Solutions of varying osmolalities (236, 216 and 174 mmol/kg) were prepared by adjusting the mannitol concentration. Osmolality of test solutions was measured using a vapor pressure osmometer (Vapro 5520, Wescor Inc., Logan, UT).
Cells were set in a perfusion chamber with isotonic solution for 3 min prior to stimulating with osmotic test solutions. Solution flow was kept constant at 3.3 mL/min. Fluorescence emission at 480 nm was filtered by 505-530 bandpass filter. Images were analyzed using automated routines written in Matlab. Responses were averaged from 3-5 independent experiments/stimulation/transfected cell line.

Behavioral Assays

Control flies were isogenic w^{1118} fly strain (Exelixis strain A5001, BL-6326). All transgenes were backcrossed seven times to the control strain to significantly reduce genetic background effects on behavior. 2-5 day old flies were starved 15-22 hours with access to water and subsequently mounted on slides. Flies were kept in a humid chamber for ~ 2-3 hours and then stimulated on the proboscis with a taste substance. Flies were allowed to consume freely until they did not ingest after 5 consecutive stimulations with the taste substance. Ingestion time was recorded with a timer. For water ingestion, flies were stimulated on the proboscis with 1M sucrose afterward and only flies that responded with a proboscis extension were kept for data tally.

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Figure Legends

Figure 3.1. The *ppk28-Gal4* transgene labels taste neurons that do not express markers for sugar- or bitter-sensing neurons

(A) The *ppk28-Gal4* transgene drives expression of GFP in proboscis taste neurons.

(B) In the brain, the *ppk28-Gal4* transgene drives expression of GFP in gustatory sensory projections in the subesophageal ganglion. Brain is counterstained with nc82 in magenta. ppk28 expression has been reported in larval tracheae, suggesting additional roles outside the nervous system (Liu et al., 2003).

(C) ppk28 neurons do not contain markers for sugar neurons. Shown is a proboscis with ppk28 neurons (magenta) and Gr5a neurons (green). The proboscises of *ppk28-Gal4, UAS-CD2, Gr5a-GFP-IRES-GFP* flies were used for immunohistochemistry.

(D) ppk28 neurons (magenta) do not contain markers for bitter neurons (Gr66a, green). The proboscises of *ppk28-Gal4, UAS-CD2, Gr66a-GFP-IRES-GFP* flies were used for immunohistochemistry. See also Figure S1 for background on ppk28 identification and Figures S2 and S3 for supplemental expression data.

Figure 3.2. The *ppk28-Gal4* transgene labels projections in the SOG which partially overlap sugar-sensing projections.

(A) Shown are projections in the SOG of ppk28 (magenta) and Gr5a (sugar, green). There is co-mingling of projections from the proboscis (labellar nerve). In addition, ppk28 labels projections from mouthparts (labial nerve) which are segregated (dorsal magenta projections).

(B) The ppk28 projections (magenta) are segregated from Gr66a projections (bitter, green).

Figure 3.3. ppk28 is partially expressed with the enhancer trap line *NP1017-Gal4* that labels some water-sensitive neurons.

(A) NP1017 is a Gal4 enhancer trap that labels many neurons in the brain.

(B). In the SOG, NP1017 predominantly labels gustatory axons from chemosensory bristles (arrow) and taste peg neurons (arrowhead). Water responses were reported from some NP1017 chemosensory bristles (Inoshito and Tanimura, 2006). We previously noted that the taste peg neurons in NP1017 do not respond to water, but instead respond to CO_2 (Fischler et al, 2007) (G-CaMP imaging NP1017 taste peg projections: water response=0.91 ± 0.73; carbonated water response=7.21 ± 0.96; t-test P=0.002, n=4 flies/compound ± s.e.m.. For carbonation, either calistoga or 100mM $NaHCO_3$ pH6.5 was used.) Thus, NP1017 labels CO_2-sensing neurons in the taste pegs and water-sensing neurons in the proboscis.

(C) In the proboscis, ppk28 and NP1017 are partially co-expressed. *NP1017-Gal4* (green) (c), an *in situ* probe for *ppk28* in magenta (d), overlay (e). 22/30 *ppk28* cells expressed *NP1017-Gal4* and 22/78 *NP1017-Gal4* cells expressed *ppk28*. NP1017 labels a single cell in chemosensory bristles and many taste peg neurons. ppk28 is expressed in a single cell in chemosensory bristles and not in taste peg neurons. This likely accounts for the partial co-expression with NP1017. Dual *in situ* hybridization and immunohistochemistry were performed. Anti-rabbit GFP was used at 1:50 for 1 hr. The ppk28 riboprobe was 506 bp. Scale bar is 50 mm in all panels.

Figure 3.4. Neurons labeled by ppk28 respond to water

Taste-induced G-CaMP fluorescent changes to water, NaCl, sucrose, ribose, n-methyl-d-glucamine (NMDG) and polyethylene glycol (PEG). Responses to both taste compounds (NaCl, sucrose) and non-taste compounds (ribose, NMDG, PEG) decrease as a function of concentration. Responses that are significantly different than water by student's t-test are 0.2M NaCl ($P<0.05$), 0.5M NaCl ($P<0.005$), 1M NaCl ($P<0.005$), 0.5M sucrose ($P<0.005$), 1M sucrose ($P<0.005$), 0.5M ribose ($P<0.005$), 1M ribose ($P<0.005$), 1M NMDG ($P<0.005$), 20% PEG ($P<0.05$). Concentrations and details are described in Methods. (n=4-11 flies/compound ± SEM). See also Figure S4 for responses to denatonium, citric acid and a plot of response versus osmolality.

Figure 3.5. Response properties of ppk28 cells.

(A) Taste-induced G-CaMP fluorescent changes to denatonium and citric acid. Responses to denatonium are not statistically different than those to water, consistent with the low osmolality of denatonium. Responses to citric acid decrease as a function of osmolality, (1M different by student's t-test, $P=0.013$). For denatonium, flies were given 2-3 stimulations of differing concentrations, with the last stimulation a positive control (>8% DF/F). For citric acid, flies were given stimulations of various concentrations in random order, with the last stimulation being a positive control (>4% DF/F). The lower responses for citric acid most likely reflect the damaging effects of stimulating sensillum with high acid concentrations (n=9 flies/compound ± s.e.m.).

(B) The response of ppk28 taste cells decreases as a function of osmolality. Most substances tested inhibited the response within a similar range of osmolalities. Data from figure 3.4 plus additional data points are plotted. It is possible that access to taste cells is occluded at the bristle tip at high concentrations, complicating the interpretation of these results.

Figure 3.6. The *ppk28* gene is necessary for the water response

(A) Extracellular bristle recordings of *ppk28* control, mutant and rescue flies after stimulation with water (left traces) or 100mM sucrose (right), showing action potentials. Stimulation begins at the time of recording.

(B) Scatter plot of responses in the three genotypes to water and sucrose, showing mean ± SEM in red bars and each data point as a dot. The response of the *ppk28* control, mutant and rescue to water are all statistically different (***= $P<0.005$); responses to sucrose are not statistically different by Dunn's multiple comparison test.

(C) Pseudocolor images (SOG, scale bar 50mm) of maximum fluorescence increase in projections of *ppk28* control, mutant and rescue flies after stimulation with water (%DF/F).

(D) Example responses of *ppk28* control, mutant and rescue flies after stimulation with water (applied at arrow).

(E) Fluorescence changes in the three genotypes following stimulation with water, 0.1M NaCl, 1M NaCl, 1M sucrose (n=8-11 trials/concentration ± SEM; t-test, *ppk28* control versus *ppk28* mutant, P<0.05=*, P<0.005=***). The response of *ppk28* control and *ppk28* rescue flies to water or other compounds is not significantly different.

Figure 3.7. Flies lacking ppk28 drink less water
(A) Behavioral assays for *ppk28* control, mutant and rescue flies, measuring time consuming water or 500mM sucrose. *ppk28* mutants, *ppk28* mutants + *ppk28-Gal4*, or *ppk28* mutants + *UAS-ppk28* flies all consume less water than control or rescue flies (t-test, versus control, P<0.05=*, P<0.01=**). Water consumption of control and rescue flies is not statistically different (t-test, P=0.53). *ppk28* control, mutant and rescue flies consume similar amounts of sucrose (t-test, no significant difference). n= 3 trials, 18-25 flies/trial/genotype. See Figure S5 for additional behavioral studies.

Figure 3.8. Heterologous cells express ppk28.
FLAG-tagged ppk28 is localized to the cell surface in HEK293 cells. First panel shows cells co-transfected with GFP (green) and unlabeled ppk28. Second panel shows cells co-transfected with GFP (green) and FLAG-tagged ppk28 (magenta). Immunohistochemistry was done to detect the FLAG-tag under non-permeablized conditions. GFP fluorescence is direct. Scale bar is 50 mm. Cells were incubated with 1:1000 mouse anti-Flag (F1804, Sigma) at 4°C for 1 hour, and 1:200 goat anti-mouse Alexa568 (A11004, Invitrogen molecular probes) secondary for 30 min.

Figure 3.9. Expression of ppk28 in bitter taste cells confers water sensitivity
(A) Extracellular bristle recordings of i-type sensilla (non-water responsive) from Gr66a-Gal4 flies lacking (-) or containing (+) UAS-ppk28 after stimulation with water, 0.5M NMDG, 1M NMDG or 0.01M caffeine. Stimulation begins at time of recording.
(B) Scatter plot of water responses for each genotype (mean ± SEM in bars; data points are dots) and summary plot of all responses (mean ± SEM.). Responses are statistically different to water and 0.5M NMDG by t-test (n=7-27, P<0.05=*, P<0.001=***).
(C) Maximum fluorescence increase in Gr66a bitter-sensing projections (left) and Gr66a projections expressing ppk28 (right), after stimulation with water (%DF/F) (SOG, scale bar 50mm).
(D) Example responses in Gr66a cells (left) and Gr66a cells expressing ppk28 (right) to water (applied at arrow).
(E) Summary of fluorescence changes in Gr66a cells without (grey) or with ppk28 (green) tested with water, 0.5 and 1M NMDG and 0.5, 1 and 2M sucrose. Responses are statistically different (n=4-5 trials/concentration ± SEM; t-test, versus Gr66a control, water: P<0.05=*, P<0.01=**, P<0.001=***).

Figure 3.10. Heterologous cells expressing ppk28 respond to hypo-osmolarity
(A-C) Pseudocolor images of maximum fluorescence increases (maxDF) in response to isotonic (303mmol/kg) and reduced osmolality (174 mmol/kg) for cells expressing ppk28, TRPV4 or vector alone. On the right, plots of fluorescence change per frame over the stimulation period (noted by bar) at 80%, 70% and 60% of isotonic osmolality (236, 216 and 174 mmol/kg). Total fluorescence change for the field was calculated and divided by dsRed-positive cell area to normalize for different cell densities.

(D) Concentration curve of peak responses to different osmolalities for ppk28, TRPV4 and vector. The response of HEK293 cells containing ppk28 is statistically different from vector for 216 mmol/kg and 174 mmol/kg, as is the response of TRPV4 cells. (n=4-5 trials/concentration ± SEM; t-test, versus vector, $P<0.01$=**, $P<0.001$=***).

Figure 3.1

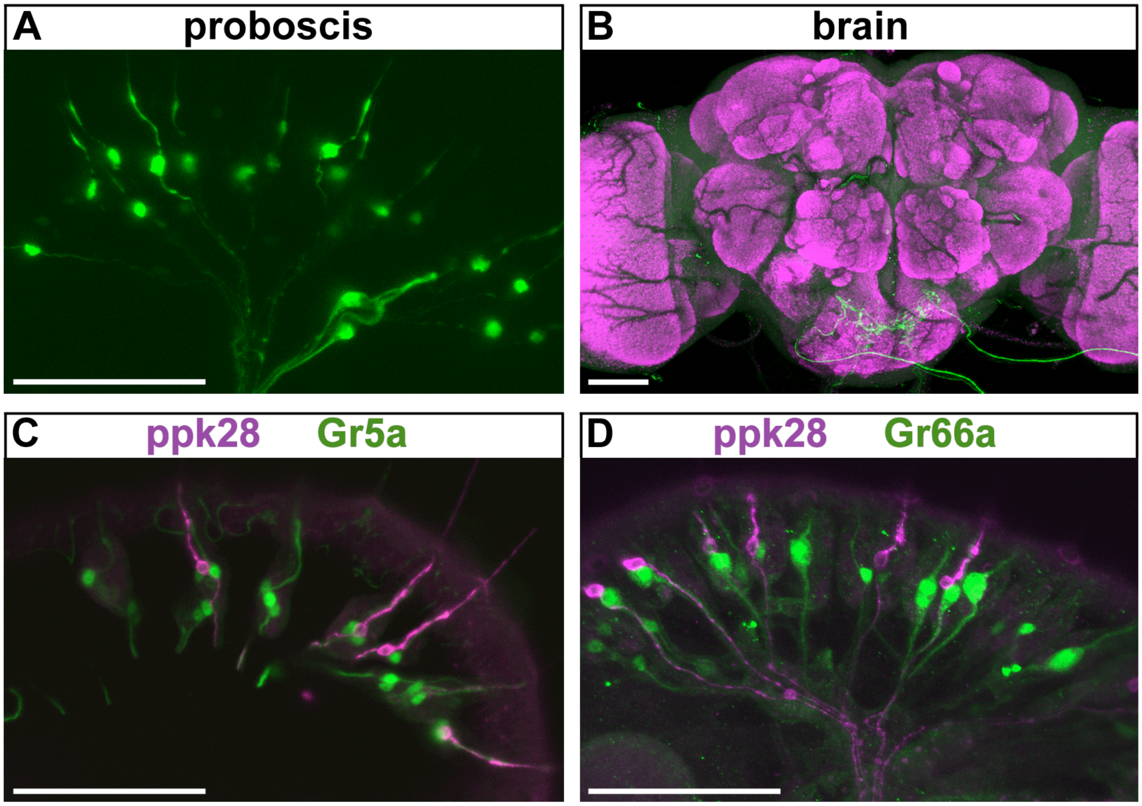


Figure 3.2

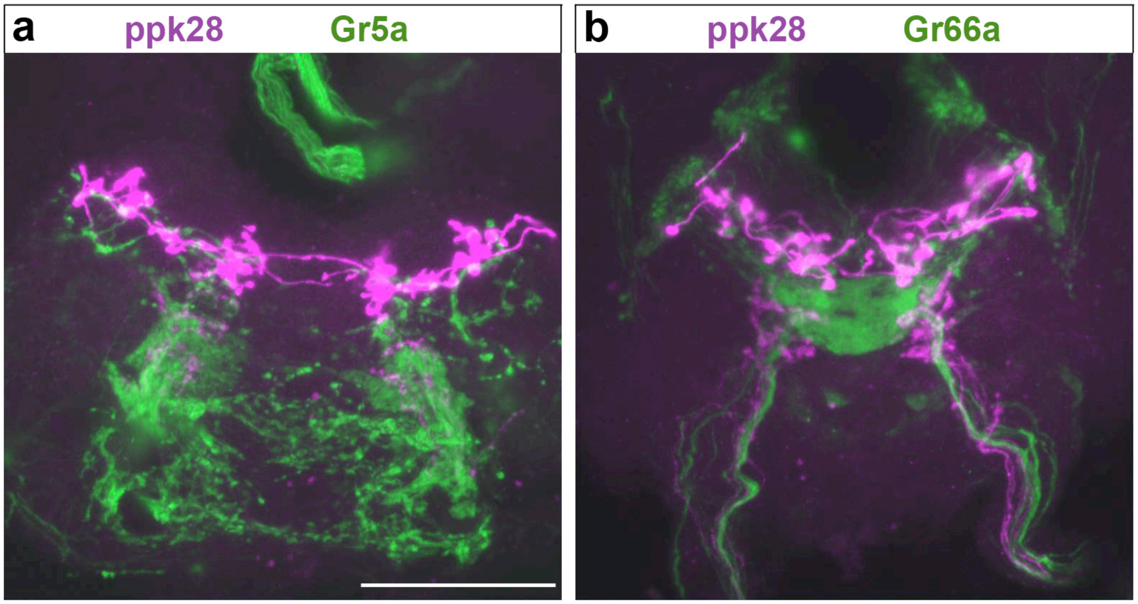
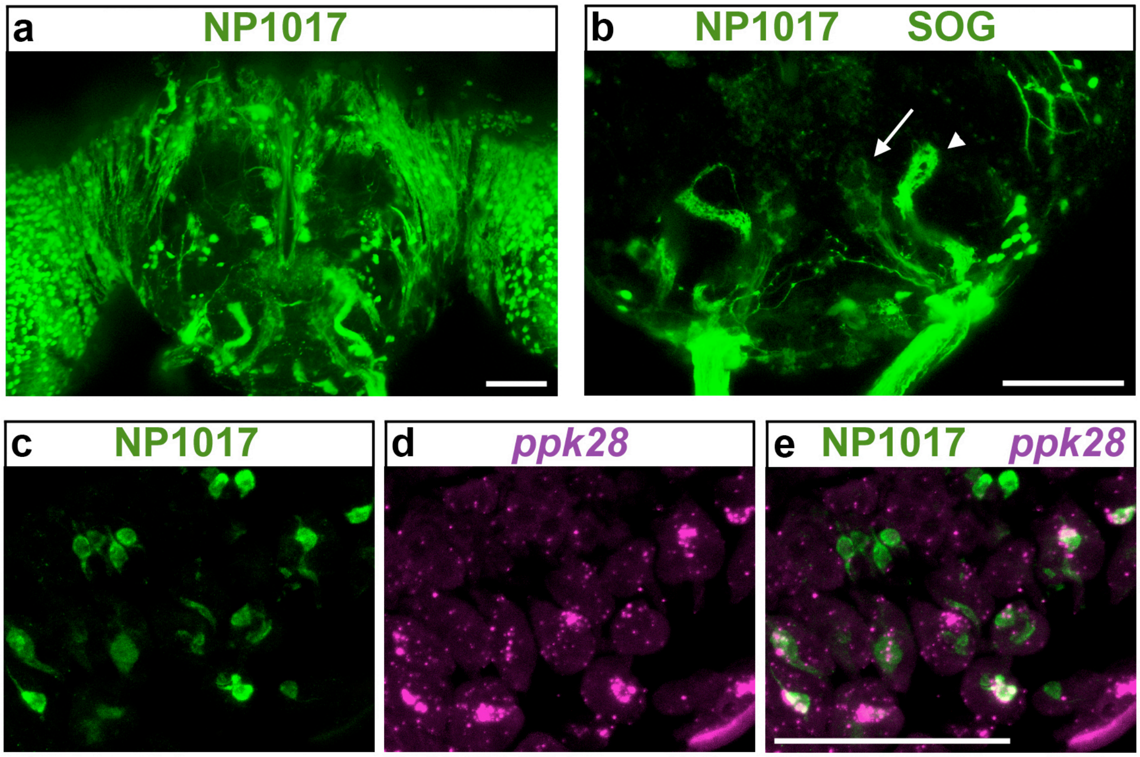


Figure 3.3



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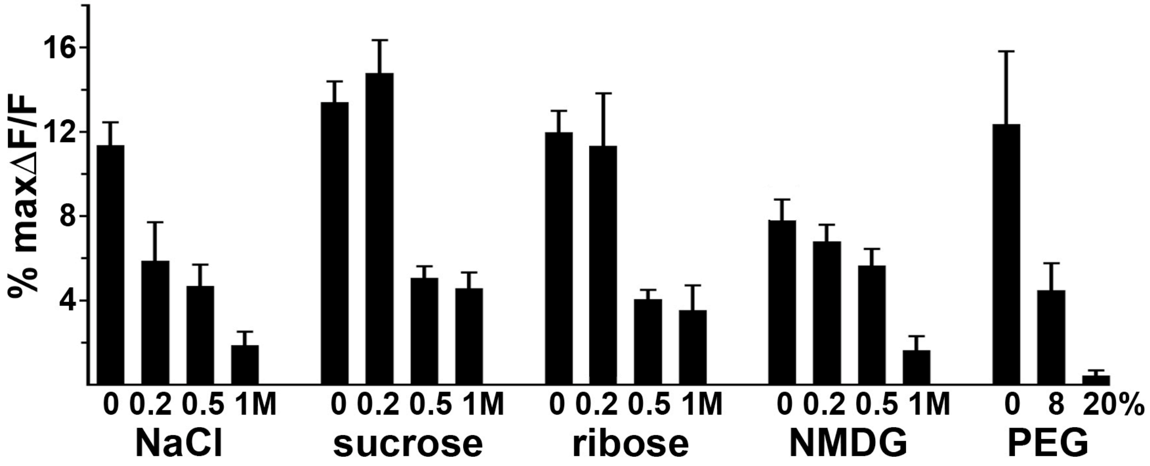

% maxΔF/F
16
12
8
4
0 0.2 0.5 1M
NaCl
0 0.2 0.5 1M
sucrose
0 0.2 0.5 1M
ribose
0 0.2 0.5 1M
NMDG
0 8 20%
PEG

Figure 3.5

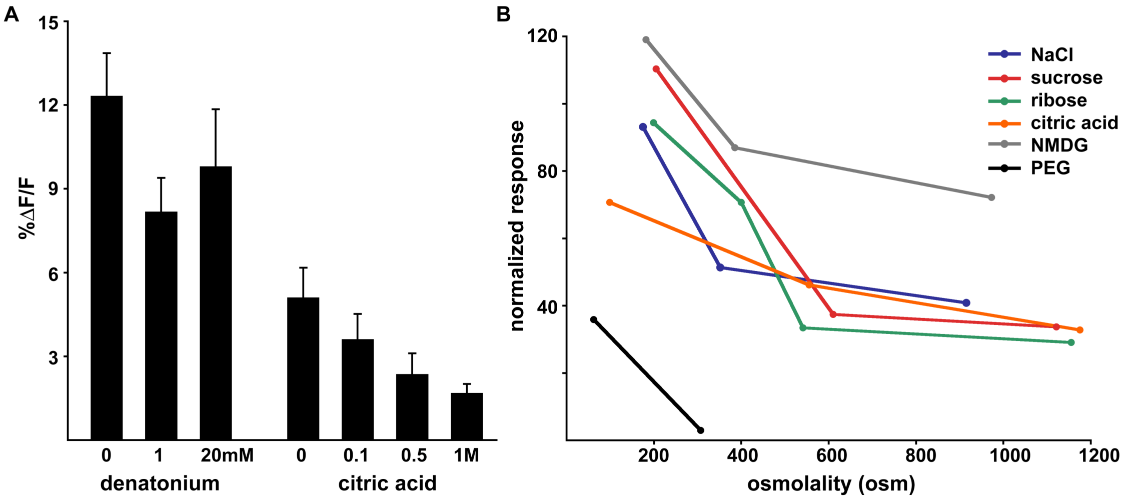

A
15
12
9
6
3
%ΔF/F
0
1
20mM
denatonium
0
0.1
0.5
1M
citric acid
B
120
80
40
normalized response
200
400
600
800
1000
1200
osmolality (osm)
NaCl
sucrose
ribose
citric acid
NMDG
PEG

Figure 3.6

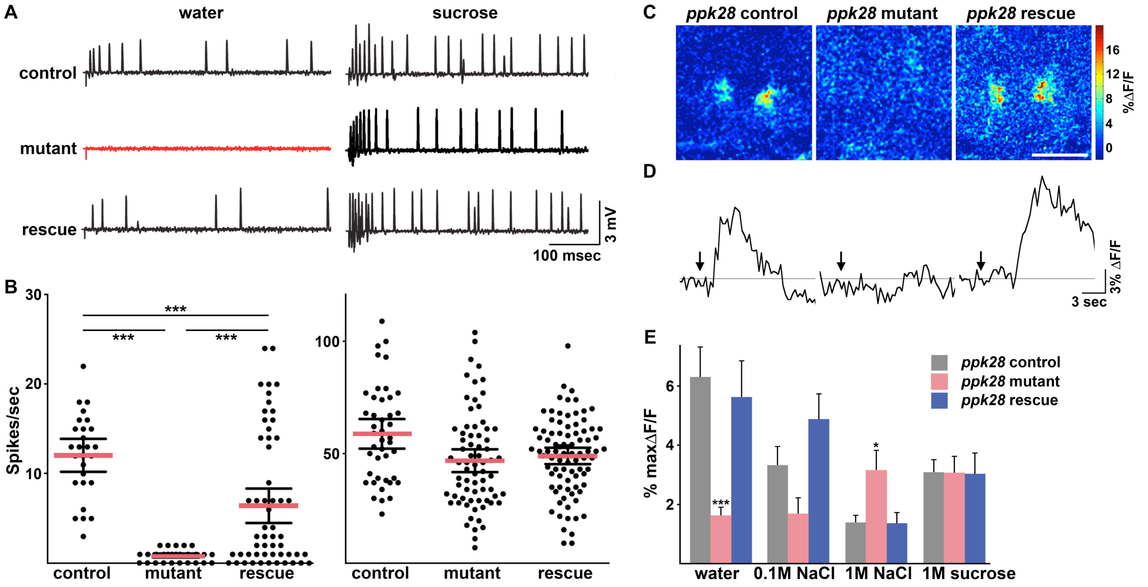

A
water
sucrose
control
mutant
rescue
3 mV
100 msec
B
30
20
10
Spikes/sec
***
***
***
control
mutant
rescue
100
50
control
mutant
rescue
C
ppk28 control
ppk28 mutant
ppk28 rescue
16
12
8
4
0
%ΔF/F
D
3% ΔF/F
3 sec
E
% maxΔF/F
6
4
2
***
*
ppk28 control
ppk28 mutant
ppk28 rescue
water
0.1M NaCl
1M NaCl
1M sucrose

Figure 3.7

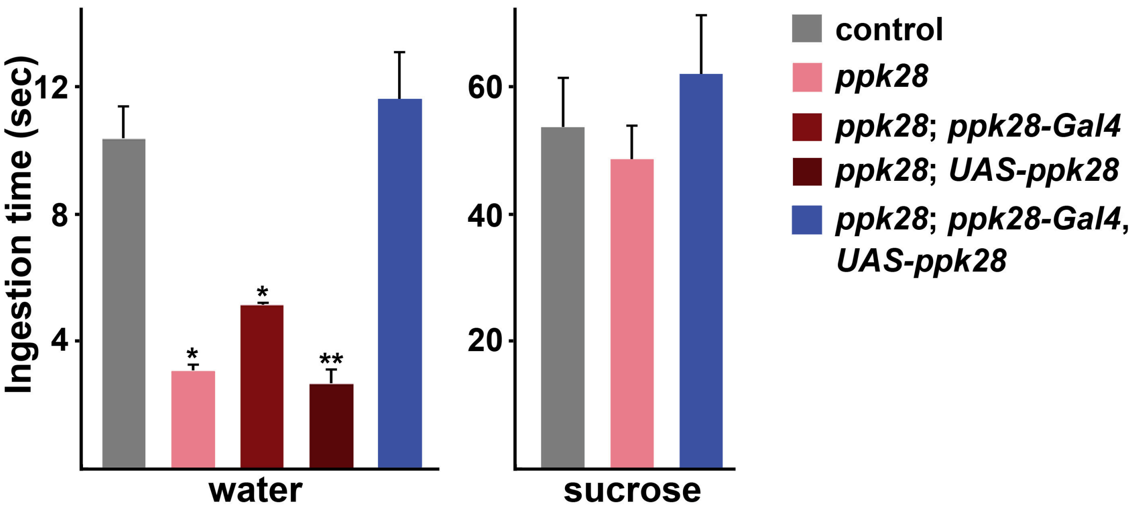

Ingestion time (sec)
12
8
4
*
*
**
water
60
40
20
sucrose
control
ppk28
ppk28; ppk28-Gal4
ppk28; UAS-ppk28
ppk28; ppk28-Gal4, UAS-ppk28

Figure 3.8

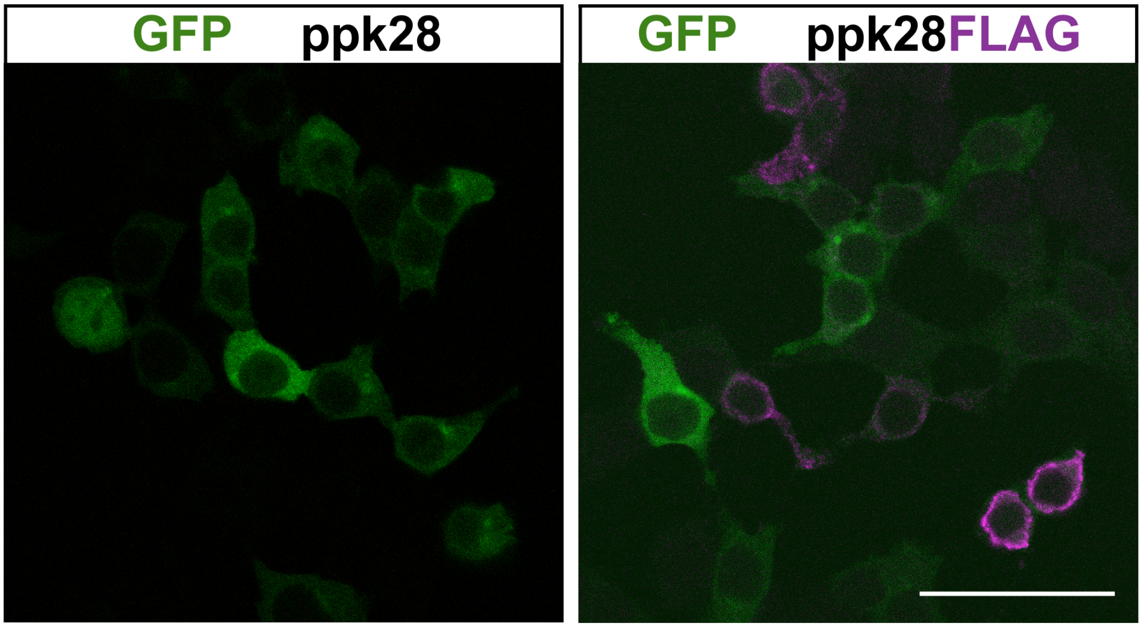


Figure 3.9

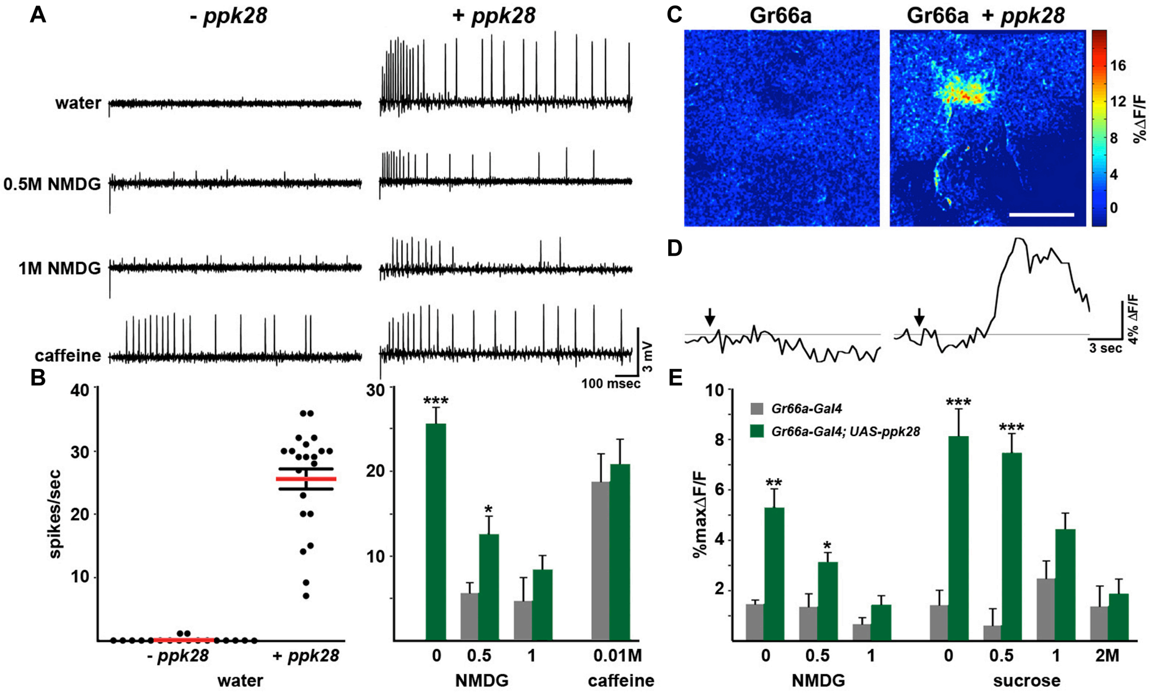
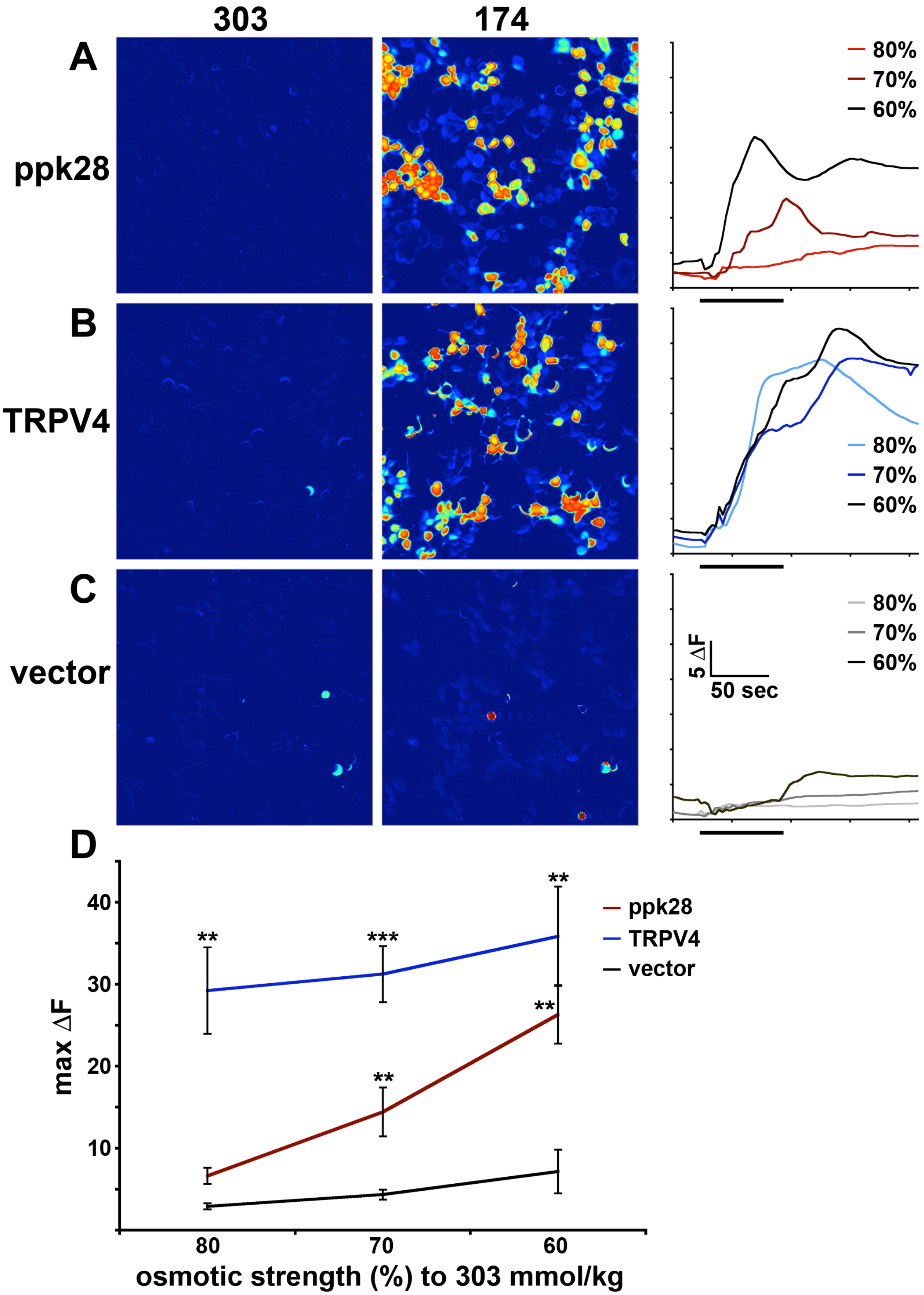

A
- ppk28
+ ppk28
water
0.5M NMDG
1M NMDG
caffeine
3 mV
100 msec
B
spikes/sec
40
30
20
10
- ppk28
+ ppk28
water
30
20
10
***
*
0
0.5
1
0.01M
NMDG
caffeine
C
Gr66a
Gr66a + ppk28
16
12
8
4
0
%ΔF/F
D
3 sec
4% ΔF/F
E
10
8
6
4
2
%maxΔF/F
Gr66a-Gal4
Gr66a-Gal4; UAS-ppk28
**
*
***
***
0
0.5
1
0
0.5
1
2M
NMDG
sucrose

Figure 3.10


303
174
A
ppk28
— 80%
— 70%
— 60%
B
TRPV4
— 80%
— 70%
— 60%
C
vector
— 80%
— 70%
— 60%
5 ΔF
50 sec
D
max ΔF
40
30
20
10
80
70
60
osmotic strength (%) to 303 mmol/kg
**
***
**
**
**
— ppk28
— TRPV4
— vector

CHAPTER 4:

Two ENaC/Deg channels label a putative novel population of taste neurons

Summary

This chapter summarizes ongoing work with two ENaC/Deg family members termed ppk23 and CG13568. *In situ* hybridization and transgenic expression experiments show that *ppk23* and *CG13568* are expressed in subsets of GRNs in the major sites of taste recognition, including the proboscis and tarsi. Moreover, double label *in situ* hybridization experiments show that *CG13568* and *ppk23* are co-expressed in a putative novel population of taste neurons distinct from known sugar, water or bitter sensing GRNs. Interestingly, *ppk23* is additionally expressed in a subpopulation of bitter sensing neurons. Preliminary mutant analysis suggests that *ppk23* and *CG31568* are not involved in NaCl or sucrose detection. Future work will aim to characterize the ligands and chemosensory functions of these two channels.

Introduction

What is the chemosensory function of GRN expressing ENaC/Deg genes? My thesis work has demonstrated that *Drosophila* recognizes water directly via an ENaC/Deg termed ppk28. Interestingly, ENaC/Deg genes have been shown to mediate sensory detection across a broad range of stimuli in many diverse organisms (Bianchi and Driscoll, 2001). Here, I present data from an ongoing investigation into the chemosensory function of two ENaC/Deg molecules, ppk23 and CG13568, which were originally identified from a microarray-screen (chapter 2) and show expression in specific subsets of GRNs.

Results

ppk23 and *CG13568* label a putative novel population of taste neurons

The putative *ppk23* promoter drove taste neuron expression in all the major sites of taste recognition, including the proboscis, wing margins, tarsi, and larval terminal organ (Figure 4.1). The putative *CG13568* promoter drove taste neuron expression in proboscis and the male tarsi (Figure 4.1). Intriguingly, both *ppk23* and *CG13568* promoters drove sexually dimorphic expression in the forelegs, with more neurons showing expressing in males compared to females (Figure 4.1ABE). Importantly, double label immunohistochemistry and *in situ* hybridization experiments showed that the *ppk23-Gal4*$^{2.1}$ driver faithfully recapitulated the *ppk23* endogenous mRNA expression pattern in the proboscis (84/93 [90.3%] *ppk23-Gal4*$^{2.1}$-expressing neurons expressed *ppk23*, 84/84 [100%] *ppk23*-expressing neurons expressed *ppk23-Gal4*$^{2.1}$) (Figure 4.2).

Previous experiments have demonstrated that a population of taste neurons labeled by the receptors Gr5a and Gr64f respond to sugars, a population of taste neurons labeled by the receptor Gr66a respond to bitter compounds, and a population of neurons labeled by the receptor *ppk28* respond to water (Dahanukar et. al., 2007; Marella et. al., 2006; chapter 3). To directly investigate gene expression, I performed double label proboscis *in situ* hybridization experiments. Excitingly, double label experiments showed that *CG13568* was expressed in a large subset of *ppk23* expressing neurons (92/99 [93%] *CG13568*-expressing neurons expressed *ppk23,* 92/128 [71.9%] *ppk23*-expressing neurons expressed *CG13568*) (Figure 4.3D). *ppk23* and *CG13568* were not expressed in *ppk28*-expressing neurons (0/51 *ppk23*-expressing neurons expressed *ppk28*, 0/17 *ppk28*-expressing neurons expressed *ppk23*, 0/27 *CG13568*-expressing neurons expressed *ppk28,* 0/20 *ppk28*-expressing neurons expressed *CG13568*)(Figure 4.3C, data not shown). Additionally, *in situ* hybridization experiments confirmed that *ppk23* was expressed in a subset of *Gr66a* expressing neurons (25/84 [29.7%] *ppk23*-expressing neurons expressed *Gr66a*, 25/47 [53.19%] *Gr66a*-expressing neurons expressed *ppk23*) (Figure 4.3A). *In situ* experiments with the *Gr5a* probe failed to give a conclusive signal.

Interestingly, *CG13568* was not expressed in *Gr66a* expressing neurons (0/63 *CG13568*-expressing neurons expressed *Gr66a*, 0/68 *Gr66a*-expressing neurons expressed *CG13568*)(Figure 4.3B). Finally, double label *in situ* hybridization and immunohistochemistry experiments showed that *ppk23* is expressed in a population of neurons largely distinct from those labeled by the *Gr64f-Gal4* promoter construct (2/74 [2.7%] *Gr64f-Gal4*-expressing neurons expressed *ppk23*, 2/54 [3.7%] *ppk23*-expressing neurons expressed *Gr64f-Gal4*)(Figure 4.3E). Therefore, *ppk23* is expressed in a subset of *Gr66a*-expressing, bitter sensing neurons as well as an additional class of neurons that are distinct from *ppk28*-expressing, water sensing neurons and *Gr64f*-expressing, sugar sensing neurons. Additionally, *CG13568* is expressed specifically in a subpopulation of *ppk23* that does not co-label with *Gr66a*-expressing neurons.

***ppk23* and *CG13568* mutants have normal proboscis extension reflex (PER) responses to salt and sugar**

To further investigate the role of *ppk23* and *CG13568* in taste detection, I used FLP-recombination target (FRT) mediated trans-recombination (Parks et. al., 2004) to generate deletion mutants of *ppk23* and *CG13568*. I confirmed the deletions by using genomic polymerase chain reaction (PCR) and DNA sequencing from the trans-recombined chromosomes (Figure 4.4). *ppk23* mutants were definitive null mutants, as the entire open reading frame was deleted. *CG13568* deletion mutants were likely null mutants, as the first putative two exons (including the first putative transmembrane domain) and part of the third are deleted (it is noteworthy, however, that there is an additional downstream exon that begins with a start codon). *ppk23* and *CG13568* mutants were viable and fertile, with no obvious morphological or behavioral defects. Preliminary proboscis extension reflex (PER) experiments strongly suggest that *ppk23* and *CG13568* are not involved in NaCl or sucrose detection, consistent with their expression patterns (Figure 4.5).

Conclusion

Here I present the results of an ongoing investigation into the chemosensory function of two ENaC/Deg members, ppk23 and CG13568. Expression analysis revealed that *ppk23* and *CG13568* are co-expressed in a population of neurons distinct from *Gr64f-Gal4*-expressing (sugar sensing), *ppk28*-expressing (water sensing), and *Gr66a*-expressing (bitter sensing) GRNs (*ppk23* is additionally expressed in a small subset of *Gr66a-Gal4* expressing neurons). What is the ligand specificity of these neurons? Recent GRN labellar *in situ* hybridization experiments from the Carlson lab showed that Gr64 family members are expressed with Gr5a in a single class of neurons that fully account for the vast majority of sugar sensitivity (Dahanukar et. al., 2009). Therefore, *ppk23* and *CG13568* co-expressing neurons are most likely not sensitive to sugar and are therefore likely to mediate another taste modalitiy. Previous electrophysiological experiments have suggested that taste sensillum house four different classes of taste neurons: sugar sensing, water sensing, high salt sensing, and low salt sensing or bitter sensing (Hiroi et. al., 2004). This would potentially suggest a role for ppk23 or CG13568 in salt detection. Intriguingly, preliminary behavioral analysis suggests that these molecules are in fact not involved in salt (NaCl) detection.

What other taste substances does *Drosophila* recognize? It has long been assumed that *Drosophila* taste cuticular hydrocarbons which most likely modulate their social and sexual behaviors to both conspecifics and heterospecifics (Billeter et. al., 2009). Additionally, it is known that the sexually dimorphically spliced transcription factor *fruitless* is expressed in pheromone sensing ORNs as well as candidate pheromone sensing gustatory neurons (stockinger et. al., 2005). Future experiments will aim to determine whether *ppk23* and *CG13568* are expressed in *fruitless* expressing neurons, which would suggest their involvement in pheromone recognition. Indeed, the hypothesis that *ppk23* and *CG13568* may mediate pheromone detection is made even more tantalizing by the observation that both genes are expressed in a sexually dimorphic pattern as well as the fact that another *ppk* member has been previously implicated in courtship behavior (Lin et. al., 2005).

Alternatively, *ppk23* and *CG13568* may still mediate another less examined taste modality, such as fat, amino acid, or even acid detection. Indeed, the mere observation that taste genes are expressed in a sexually dimorphic pattern does not confirm their involvement in pheromone recognition, as electrophysiological recordings of tarsal sensillum showed that there were sex differences in non-pheromonal stimuli (pheromonal stimuli were not tested) (Meunier et. al., 2000). Future calcium imaging, behavioral mutant analysis, and misexpression experiments should uncover the chemosensory roles of both *ppk23* and *CG13568*.

Materials and Methods

Experimental Animals

Drosophila stocks were maintained on standard cornmeal/agar/molasses medium at 25C. w^{1118} strains were used for transgene injections. P element-mediated germline transformations were performed using standard techniques (Genetic Services Inc).

Transgenic flies

The *CG13568* promoter-*Gal4* construct was generated with a 3.612-kb upstream fragment (19942397-19946008, GenBank accession number NT_033778.3). The primers used for making the *CG13568* promoter-*Gal4* construct were F:5'-ttcgtattcatgaaatcctttccacaatttctt-3' (G4CG13568F2) and R: 5'-atctgccgcacaagacacaagatgt-3' (G4CG13568R3). The *ppk23* promoter-*Gal4* construct was generated with 2.695-kb upstream fragment (17463170-17465864, GenBank accession number NC_004354.3). The primers used for making the *ppk23* promoter-*Gal4* construct were F:5'-tccgtttcaggaacattgctcgc-3' (G4ppk23F) and R:5'-cattagttgtatagttcgcagcaaattga-3' (G4ppk23R). Full-length *ppk23* (transcript variant RA, NM_132992 and transcript variant RB, NM_001014749) was cloned into pUAST. Full-length *CG13568* (transcript variant RD, NM_001103972, bp 283-1674, labeled as "uas-*cg13568* short" in the stocks. I also generated a "uas-*CG13568* long" which includes the 5th putative intron, which has a stop codon in frame). *ppk23* mutants were generated by FLP-FRT mediated recombination between *piggybac* transposons d04369 and e03639, removing 8.284-kb surrounding the *ppk23* gene, including a 3' fragment of the closest predicted downstream gene, *CG8465*, which has no known function. *ppk23* deletion was confirmed by genomic PCR (primer pairs:5,F:5'-tcggcacactactctcgctctc-3',R:5'-caaagtgaacacgtcgagatc-3'; G, F:5'-gcgacgaggacataccctgtt-3', R: 5'-tgaggtgctccggtcttaacg-3'; 3, F:5'-tccaagcggcgactgagatg-3', R: 5'-agcgaggacgaggaaaactt-3'). Products of the expected size were sequenced to confirm correct DNA sequence identity. *CG13568* mutants were generated by FLP-FRT mediated recombination between *piggybac* transposons f06838 and f02213, removing the first two putative exons and part of the third exon as well as 331-bp "downstream" of the gene (this deletion also removes part of the gene, *cg13563*, which has no known function). *CG13568* deletion was confirmed by genomic PCR and sequencing for the trans-recombined chromosome (primer pair: F:5'-agtcgcagacttcggttgtt-3', R: 5'-tttggtgcttaaaatgtctctg-3'). Expected size of PCR products were 3.51-kb and 7.57-kb for wild-type and *CG13568* deletion chromosome, respectively. Products of the expected size were sequenced to confirm correct DNA sequence identity.

In situ hybridization

Double label *in situ* hybridization experiments were performed as described (Fishilevich and Vosshall, 2005). Probes were labeled with either FITC or Digoxigenin (Roche). *ppk23* probe corresponded to full length *ppk23* transcript variant RA, NM_132992. *CG13568* probe template was generated with the primers 5'-ACGTCAACAGTCCCGAGGAT-3' (CG13568F) and 5'-AATGAAGTACGAAATGATCTCCA-3' (CG13568R). *ppk28* probe template was generated with the primers 5’-GGTTTCAAGGTTCTTGTGCAC-3’ (ppk28f) and 5’-CTATATGGCCCGTGGAAGA-3’ (ppk28r). *Gr66a* probe corresponded to bp 934-1409 in *Gr66a-RB*, NM_079247

Behavior

Flies were mounted on myristic acid slides and tested for taste sensitivity 2-3 days post eclosure (dpe). Flies were deprived of water for ~25 hours prior to testing. For figure 4A and NaCl on figure 4B: flies were allowed to drink water until satiety (as defined by no PER/drinking elicited upon 5 consecutive proboscis water stimulations), and then a taste substance was applied to the proboscis. For figure 4B (50mM sucrose) and 4C, tastant sensitivity was assayed with tarsal PER, as previously reported (Wang et. al., 2004). Flies were given 3 stimulations/taste substance. N=~25-30 flies/taste substance for A,B, and N=~14 flies/taste substance for C. Flies were kept for data tally if they extended their proboscis to stimulation with 1M sucrose at the end of the trial.

Figure legends

Figure 4.1. *ppk23-Gal4* and *CG13568-Gal4* is expressed in taste neurons.
ppk23-Gal4$^{2.1}$ expression in male (A) and female (B) foreleg tarsi, wing margin (C,D), proboscis (F), and larvae terminal organ (H). *CG13568-Gal4*$^{5.3}$ expression in male foreleg tarsi (E) and *CG13568-Gal4*$^{1.2}$ expression in proboscis (G). Scale bar is 50 μm.

Figure 4.2. *ppk23-Gal4*$^{2.1}$ faithfully recapitulates endogenous *ppk23* mRNA expression.
In the proboscis, *ppk23* and *ppk23-Gal4*$^{2.1}$ are co-expressed. An *in situ* probe for *ppk23* (magenta) (A), *ppk23-Gal4*$^{2.1}$ (green)(B), overlay (C). Scale bar is 50 μm.

Figure 4.3. *ppk23* and *CG31568* are co-expressed in a putative novel population of taste neurons.
In the proboscis, *ppk23* (magenta) is partially co-expressed with *Gr66a* (green) (A), *CG13568* (magenta) is not co-expressed with *Gr66a* (green) (B), *ppk28* (green) is not co-expressed with *ppk23* (magenta) (C), *ppk23* (green) is co-expressed with *CG13568* (magenta) (D), *ppk23* (magenta) is not expressed with *Gr64f-Gal4* (green) (E). Scale bar is 50 μm.

Figure 4.4. FLP-FRT deletion of *ppk23* and *CG31568* genomic DNA.
(A). Genomic structure of *ppk23* and relevant piggybac transposons. Primer pairs used in confirmation of genomic deletion are shown.
(B). Genomic PCR to confirm *ppk23* deletion (prime pairs are written below line; genotype is listed above line). Product size for *ppk23* (G) product: 124-bp.
(C). Genomic PCR to confirm *CG13568* deletion.

Figure 4.5. *ppk23* and *CG13568* mutants have normal NaCl and sucrose sensitivity.
(A). *ppk23* and control flies have similar sensitivity to 50mM NaCl and 50mM sucrose. PER was elicited by proboscis stimulation.
(B). *ppk23/CG13568* double mutants and control flies have similar sensitivity to 50mM NaCl and 100mM sucrose. NaCl PER elicited by proboscis stimulation. Sucrose PER was elicited by tarsi stimulation. N=~25-30 flies/taste substance.
(C). *ppk23/CG13568* double mutants and control flies have similar sensitivity to high salt. PER was elicited by tarsi stimulation. NaCl was added to 100mM sucrose. N=~14 flies/taste substance.

Figure 4.1

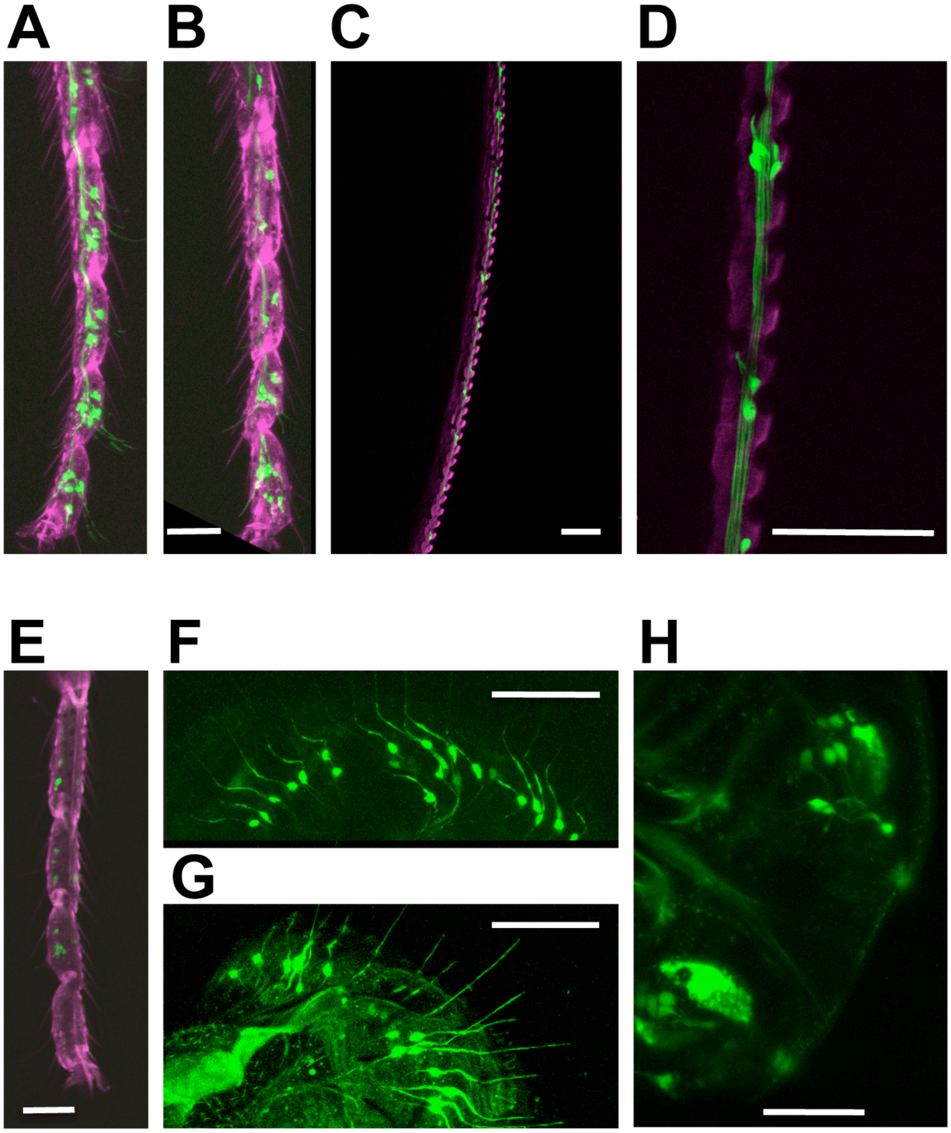


Figure 4.2

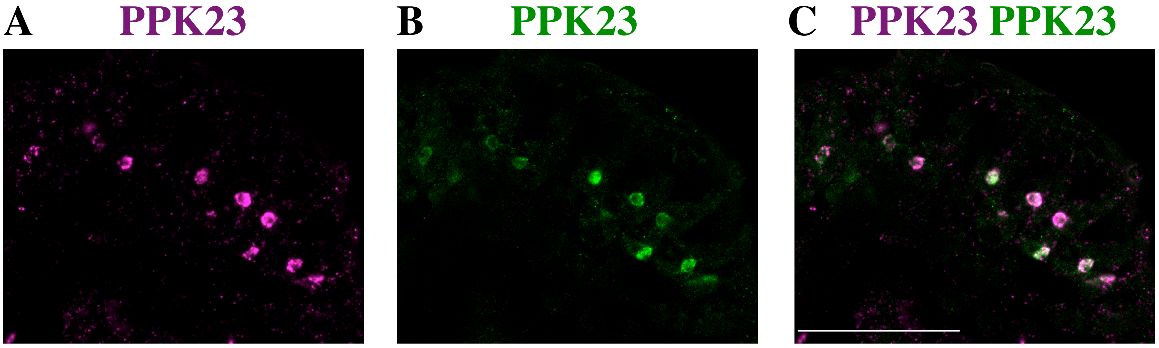


Figure 4.3

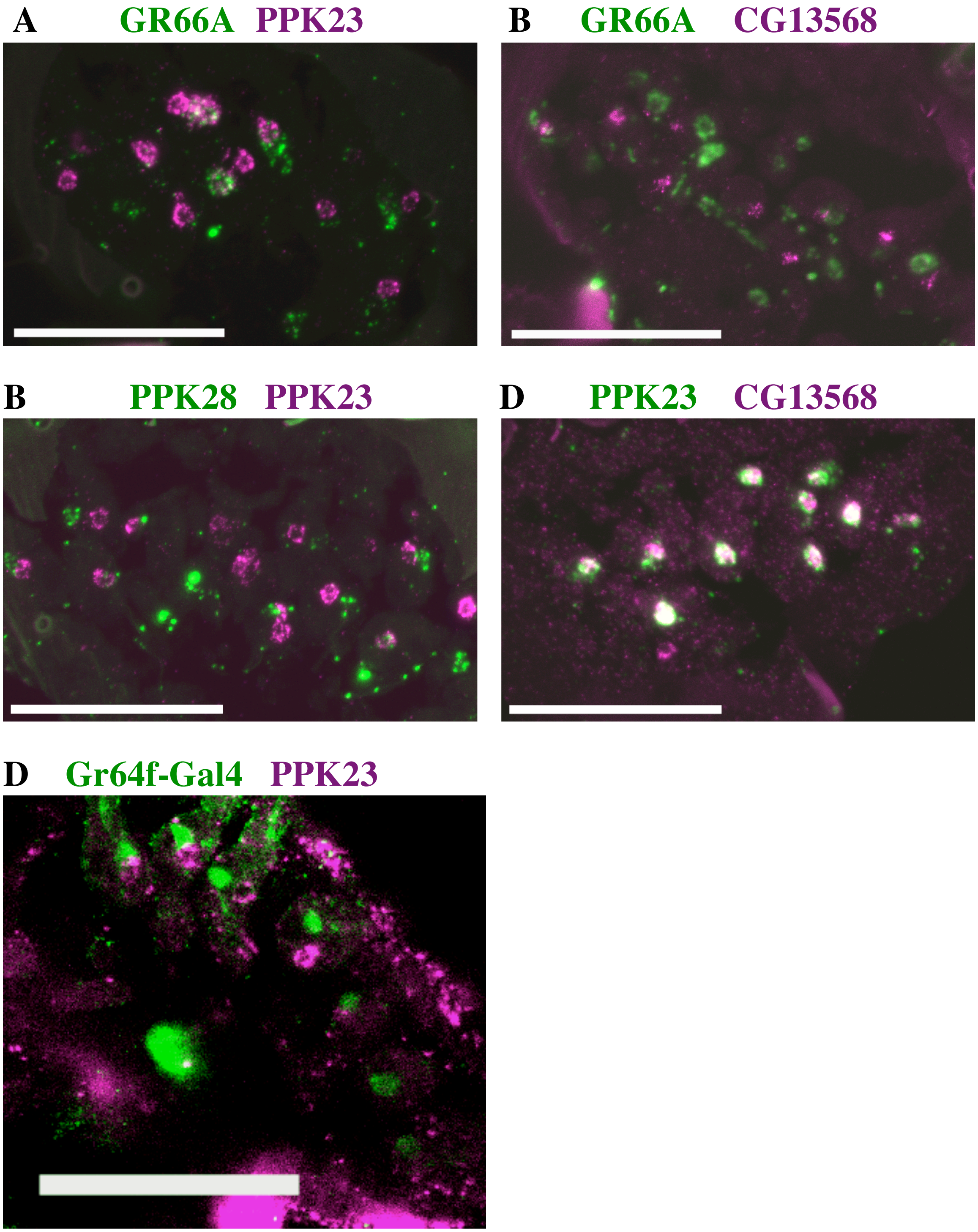

A GR66A PPK23
B GR66A CG13568
B PPK28 PPK23
D PPK23 CG13568
D Gr64f-Gal4 PPK23

Figure 4.4

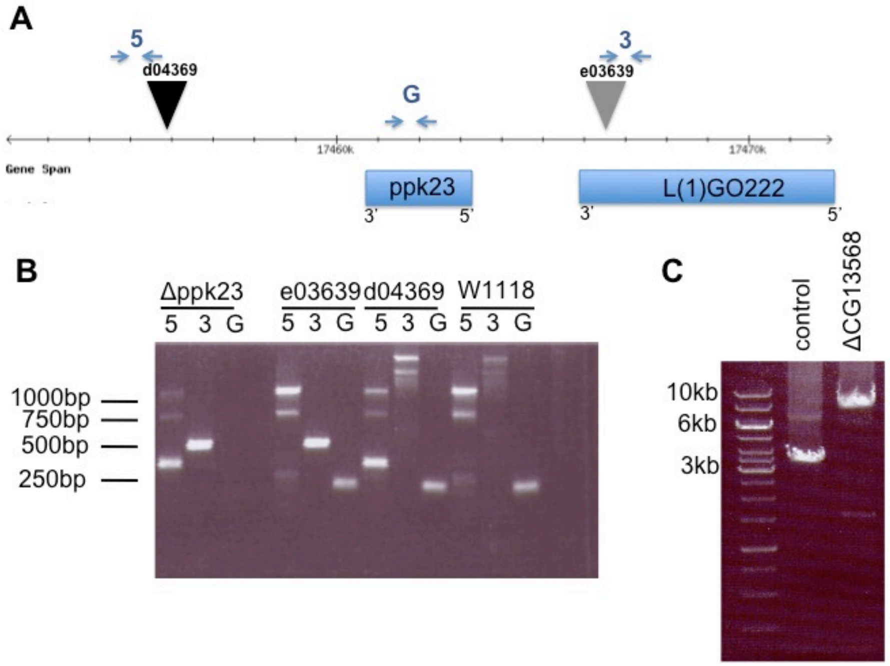
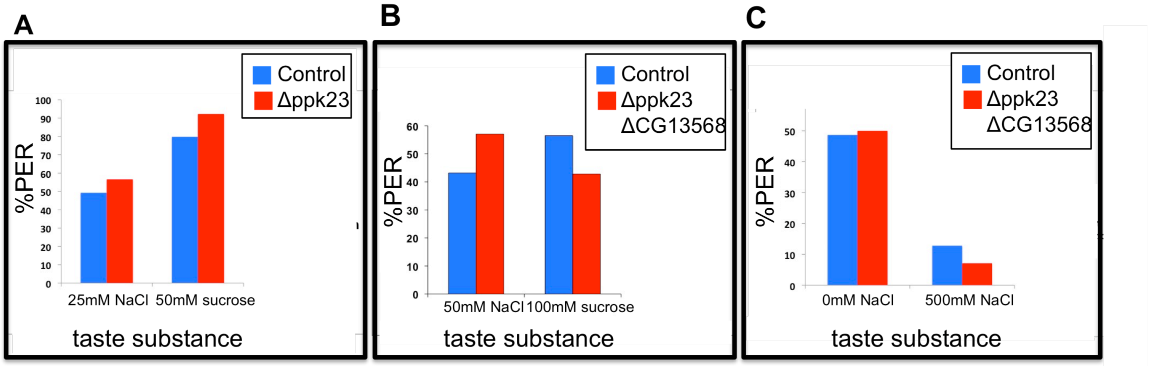

A
5
d04369
G
3
e03639
17460k
17470k
Gene Span
ppk23
3'
5'
L(1)GO222
3'
5'
B
Δppk23
5 3 G
e03639
5 3 G
d04369
5 3 G
W1118
5 3 G
1000bp
750bp
500bp
250bp
C
control
ΔCG13568
10kb
6kb
3kb

Figure 4.5


A
Control
Δppk23
%PER
0
10
20
30
40
50
60
70
80
90
100
25mM NaCl
50mM sucrose
taste substance
B
Control
Δppk23
ΔCG13568
%PER
0
10
20
30
40
50
60
50mM NaCl
100mM sucrose
taste substance
C
Control
Δppk23
ΔCG13568
%PER
0
10
20
30
40
50
0mM NaCl
500mM NaCl
taste substance

CHAPTER 5:

DISCUSSION

A microarray-based screen identified known and novel taste detection components

A microarray screen comparing RNA from heterozygous controls and mutants lacking GRNs enriched for both known and novel taste detection components. 11 of the genes with no previously ascribed gustatory function were putative ion channels. Moreover, 9/11 of these belonged to either the ENaC/Deg family (5) or the recently characterized iGluR family (4) (Benton et. al., 2009). Indeed, one of the ENaC/Deg molecules, ppk28, is the *Drosophila* water sensor. Thus, these gene families may play important roles in insect gustation.

What about other molecules that were recovered from the screen? Since 2005, when the screen was performed, several studies have uncovered genes involved in olfactory pheromone reception, such as *cyp6a20* (a cytochrome P450), and CD-36 related *snmp-1* (Benton et al., 2007; Wang and Anderson, 2009). Interestingly*, cyp6a20* as well as 2 SNMP family members (CG7422 and CG7227) were enriched in heterozygotes, suggesting that they may play roles additionally in gustatory pheromone detection (Nichols and Vogt, 2008).

Of the 256 enriched transcripts, 158 (62%) did not belong to any particularly overrepresented class of molecules (relative to their distribution in the genome). Moreover, ~30% of these transcripts could not be readily identified through literature or BLAST searches as belonging to any specific gene family or homologous to genes of known function. An analysis of these transcripts in GRNs may elucidate novel families of genes or novel physiologies. Lastly, one of the rationales for undertaking the microarray was to explore the molecular mechanism of salt detection, as it remains a major outstanding problem in *Drosophila* gustation. Analyses of the recovered ENaC/Deg molecules have surprisingly confirmed that they are likely not involved in salt detection. It is formally possible that the identified transporter or iGluR genes may play roles in salt detection, and may be interesting candidate molecules for further examination.

A member of the ENaC/Deg family is an osmosensitive ion channel

Drosophila and other insects have gustatory neurons that respond to water, but the molecular mechanism for water sensing has been mysterious. Our calcium imaging and electrophysiological studies of ppk28-containing taste cells and *ppk28* mutants argue that this channel is necessary for water detection. Although ion channels may participate in detection or transduction of sensory signals, the misexpression of ppk28 in ectopic systems provides strong evidence that this ion channel is directly activated by low osmolarity. In non-native cells, ppk28 confers responses that are maximal to water and decrease with solute concentration, demonstrating that these properties are inherent to the channel. This work also suggests that ppk28 functions as a homomer in osmodetection, although it is possible that accessory proteins might modulate its response properties *in vivo*.

Osmosensation is important not only for the detection of external water sources by peripheral neurons but also for monitoring plasma osmolality by central neurons (Bourque, 2008). In mammals, peripheral osmosensors are found in the oropharyngeal cavity, the gastrointestinal tract and blood vessels. Central osmosensors are found in regions of the brain lacking a blood-brain barrier, such as circumventricular organs

(Bourque, 2008). Together, these osmosensors maintain the extracellular fluid osmolality near a stable value by regulating ingestion and excretion of water and ions. Despite the critical nature of osmotic homeostasis, there are very few molecular candidates for osmodetection. Several studies have identified members of the transient receptor potential family as candidate osmosensors (Colbert et al., 1997; Liedtke et al., 2000; Muraki et al., 2003; Liu et al., 2007), but the role of other ion channel families has not been considered. Our finding that ppk28 is an osmosensitive ion channel raises the possibility that members of the ENaC/Deg family may participate more broadly in peripheral and central osmosensation than previously appreciated.

ENaC/Deg members provide a molecular connection between osmosensation and mechanosensation

Members of the ENaC/Deg family have been shown to be involved in the detection of mechanosensory stimuli, acids, sodium ions and small peptides (Kellenberger and Schild, 2002). The basis for channel gating is not well understood. In *C. elegans*, a mechanosensory channel involved in touch detection is composed of two ENaC/Deg members, mec-4 and mec-10, and accessory proteins (Goodman and Schwarz, 2003). The observation that ENaC/Deg members participate in mechanosensation and osmosensation may reflect fundamental similarities between these senses, with changes in membrane tension driving channel activation. However, an important distinction exists: mechanotransduction in *C. elegans* involves proteins that form a specialized extracellular matrix and cytoskeleton, suggesting that the mec-4/mec-10 channel may be tethered to the extracellular matrix and cytoskeleton and detect relative displacements (Goodman and Schwarz, 2003). By contrast, our data suggests that ppk28 does not require additional subunits or accessory proteins to detect osmolarity changes, indicating that it may be directly activated by membrane swelling. An interesting avenue for future studies will be to examine whether the gating properties of ppk28 and mec-4/mec-10 reveal basic differences or commonalities between osmosensation and mechanosensation.

The water taste modality may provide insight into taste integration and modulation by internal states

In *Drosophila*, electrophysiological experiments have supported the notion that the four gustatory neurons in a chemosensory bristle respond to different taste modalities: sugar, bitter, salt and water. Previous work in our lab and others has identified members of the Gustatory Receptor (GR) gene family as gustatory receptors in sugar and bitter neurons (Hallem et al., 2006; Ebbs and Amrein, 2007). Here, we identify ppk28 as the water taste receptor. Thus, several gene families mediate taste detection. This is similar to what is seen in the mammalian taste system, where two different families of G-protein coupled receptors mediate the detection of sugars or bitter compounds and ion channels mediate sour-sensing (Yarmolinsky et al., 2009).

How does a fly discriminate water from other taste substances? When water is the only stimulus, ppk28 taste neurons will report its presence and allow for water detection. However, when the fly encounters a sugar solution, both the water cell and the sugar cell would respond, with the water cell response decreasing and the sugar cell response increasing with sugar concentration. Thus, the fly may integrate the activity of the water cell and other taste cells for the detection of solutes in aqueous solutions. The identification of ppk28 provides a molecular basis to examine this simple form of taste integration by dissecting the contribution of water cell activity to the detection of different tastes.

How is water consumption regulated? Both water and sugars mediate ingestion, suggesting that they impinge on common neural pathways that elicit feeding behavior. However, water and sugar fulfill different needs for the animal in terms of maintaining fluid or energy levels. Are there different internal states of the fly for thirst and hunger that regulate these two taste pathways independently? Our studies of the cellular basis for water and sugar taste detection in the periphery provides a starting point for long-term studies to dissect how these tastes are processed higher in the brain, whether they are differentially modulated by internal states and how they both elicit feeding behavior.

Finally, although the taste of water has received relatively little attention as a classic taste modality, water-responsive taste neurons have been identified in many other insects, such as the blowfly and mosquitoes, as well as in mammals, such as cats and rats (Evans and Mellon, 1962; Werner-Reiss et. al., 1999; Lindemann, 1996; Gilbertson et. al., 2002). To what extent do diverse animals taste water and how does this relate to water consumption? What is the molecular mechanism of water taste detection in other organisms? What are the ecological pressures that may have necessitated the evolution of a water taste modality? For example, are *ppk28* mutants more sensitive to drought conditions due to reduced water consumption? The identification of ppk28 as a water taste receptor provides a framework for examining water taste detection in other animals, including humans.

ENaC/Deg family members, ppk23 and CG13568, are co-expressed in a putative novel population of taste neurons

Two previously uncharacterized ENaC/Deg family members, ppk23 and CG13568, were identified from a microarray screen (chapter 2) and are co-expressed in a putative novel population of taste neurons distinct from water, sugar, and bitter sensing. What is the ligand specificity of these receptors and neurons? Preliminary mutant analysis suggests that ppk23 and CG13568 are not involved in salt detection, suggesting their possible involvement in pheromone detection or another undefined stimulus, such as fat taste, acid sensing, or even amino acid sensing. Indeed, there has been very little attention paid to these other taste modalities, though they are (or have been suggested to be) important in mammalian gustation (Yarmolinsky et. al., 2009). The fact that ppk related ASIC channels have a well-established role in mediating acid detection further bolsters this possibility (Bianchi and Driscoll, 2002). Future work will aim at determining the ligand specificity and potential chemosensory roles of ppk23 and CG13568 through calcium imaging, misexpression, and behavioral experiments. Indeed, it is a very exciting prospect to further elucidate the diversity of ENaC/Deg family member chemosensory functions.

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