Mechanisms for Taste Sensation of Carbonation

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ABSTRACT

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Carbonation, or the presence of carbon dioxide (CO_2) dissolved in solution, is a commonly encountered feature of beverages in the contemporary human diet. While the popularity of carbonation may be attributed to its distinct sensory qualities, the specific orosensory pathways mediating CO_2 detection in mammals have not previously been delineated. This dissertation describes the identification of specific cellular and molecular mechanisms that mediate taste sensation of carbonation, using the mouse as a model system.

The mammalian gustatory system is sensitive to CO_2, and these responses are sensitive to inhibition of carbonic anhydrases, enzymes that catalyze the interconversion of carbon dioxide with carbonic acid. Through gene expression profiling I discovered that the gene carbonic anhydrase IV (Car4), encoding an extracellular enzyme, is specifically expressed in acid sensing taste receptor cells (TRCs). Genetic ablation of the Car4 locus resulted in a major deficit in gustatory CO_2 sensation that is stimulus specific, not affecting responses to acid. Ablation or silencing of acid sensing TRCs likewise produced a profound deficit in taste responses to CO_2. These studies identified a primary pathway of the gustatory carbonation response, substantiating acid sensing TRC and the Car4 enzyme as key mediators.

A smaller gustatory neural response to carbonation remains even in the absence of sour-sensing TRC and/or Car4. To identify additional carbonation sensing pathways, I

applied an *in vivo* calcium-imaging assay to define the ensemble of primary gustatory neurons activated by CO_2. These studies revealed that in addition to robust activation of sour sensing neurons, a secondary gustatory pathway for CO_2 detection is mediated by subpopulations of bitter and sweet responsive neurons. I identified carbonic anhydrase VII (Car7) as an intracellular carbonic anhydrase specifically expressed by sweet, bitter and umami sensing TRC. Pharmacological and gene expression data support a role for Car7 in transducing the secondary CO_2 sensing pathway.

These studies suggested that carbonation acts as a complex gustatory stimulus, stimulating sour, sweet and bitter taste qualities simultaneously. The rules governing peripheral encoding of multi-modal taste stimuli are not well understood. To address this issue, I examined the peripheral gustatory response to binary mixtures of taste qualities. I found that most combinations of taste qualities are represented as a superimposition of the component responses. However, neural responses to attractive stimuli, including natural sugars, artificial sweeteners and umami tastants, are selectively suppressed by simultaneous co-stimulation with a sour (acidic) stimulus. Acid-mediated suppression of sweet is cell autonomous, occurring even in the absence of gustatory acid sensing. Remarkably, carbonation stimulates sour signaling without suppressing sweet taste response. These studies suggest that cross-modal interactions at the periphery modulate the sensory response to complex taste stimuli

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Materials presented in this dissertation have been included in the publications below. License was obtained from the publishers for inclusion.

Chandrashekar J., Yarmolinsky, D.A., von Buchholtz, L., Oka, Y., Sly, W., Ryba, N.J., Zuker, C.S. (2009). The taste of carbonation. Science 326: 443-445

Yarmolinsky, D.A., Zuker, C.S., Ryba, N.J. (2009). Common sense about taste: from mammals to insects. Cell 139: 234-244

Chapter I: General Background

1.1 Introduction

In 1772, the chemist Joseph Priestley published a method for impregnating water with 'fixed air', the contemporary term for what is now known as carbon dioxide (CO_2), noting the 'delicate agreeable flavor' thus produced (Priestley, 1772). In years hence, carbonated beverages have come to enjoy immense global popularity. Remarkably, carbonated soft drinks constitute the single largest source of calories in the contemporary American diet (Block, 2004). While Priestley's aim was to replicate the purported tonic qualities of naturally occurring effervescent water, the current popularity of sugary, carbonated drinks has been assigned a share of the blame for the epidemics of obesity and metabolic syndrome in the developed world (James and Kerr, 2005). What underlies the widespread popularity of carbonation? A reasonable assumption is that the sensory qualities of CO_2 play a significant role; however, the mechanisms by which CO_2 elicits the distinct and familiar sensory percepts associated with carbonation are not well understood. The aim of the work described in this dissertation is to uncover the cellular pathways and molecular processes that generate the 'flavor' of carbonation. I have focused in particular on gustatory reception of CO_2, as the sense of taste is a key regulator of nutrient selection.

Carbon dioxide has been present in the atmosphere prior to the genesis of life, and is constantly being produced within organisms by fundamental metabolic processes. Not surprisingly, systems for CO_2 detection exist in every type of biological organism, from

bacteria to plants, fungi and animals (Cummins et al., 2013). Detailed sensory transduction mechanisms are known only for a select set of these systems. Of particular medical significance are the mammalian peripheral and central chemoreceptors, which sense internal CO_2 levels and regulate respiration accordingly. While the primary goal of the research presented here is to explain orosensory detection of carbonation, placing this work in the broader context of CO_2 sensing by animals may illustrate principles, and perhaps even molecular components, which could be relevant to other such systems.

1.2. CO_2 Sensing in Invertebrates

Invertebrate animals display a wide range of responses to CO_2 in their environment. While diverse invertebrate species are responsive to CO_2, mechanisms of CO_2 sensing in the model organisms *Caenorhabditis elegans* and *Drosophila melanogaster* have been particularly well studied. Genetic tools available in these organisms have facilitated identification of genes, neurons, and circuitry involved in detection of CO_2.

CO_2 Sensing in Caenorhabditis elegans

Although the roundworm *C. elegans* is most familiar to biologists as residing within the laboratory in a petri dish, natural populations are most abundant in rotting fruit and plant matter, an environment containing abundant biogenic sources of CO_2 (Félix and Duveau, 2012). *C. elegans* are highly reliant on chemosensation to navigate this

environment in search of food, mates and safety. The *C. elegans* nervous system consists of a stereotyped set of 302 neurons. A significant proportion of these are involved in chemosensation of the worm's environment (Bargmann, 2006). The main chemosensory structures are the bilaterally symmetrical amphids, located at the anterior end of the worm's body, which each contain eleven chemosensory neurons and one thermosensory neuron. Two phasmids at the posterior end each contain two chemosensory neurons, and additional chemosensory neurons are distributed within the body.

C. elegans display avoidance behavior to CO_2, chemotaxing away from sources of the gas (Hallem et al., 2008; Bretscher et al., 2008). Elevation of environmental CO_2 rapidly increases the rates of reversal of locomotion and turning, while removal of CO_2 suppresses these behaviors (Bretscher et al., 2011). These responses mediate efficient navigation away from CO_2 sources. While this behavior is robust in adult worms of the standard N2 genotype, the avoidance response is strongly modulated by intrinsic and extrinsic factors, to the extent that it can be completely suppressed under certain conditions. This behavioral plasticity, combined with the experimental accessibility of the worm, makes CO_2 sensing in this organism an attractive model in which to study sensory integration and decision making.

A number of sensory neuron types contribute to CO_2 sensing. The first identified and most essential appear to be the BAG neurons, located in the anterior body outside of the amphids (Hallem et al., 2008). Ablation of the BAG neurons results in a marked deficit in CO_2 avoidance, and BAG neurons respond to elevations in CO_2 with rapid and sustained calcium transients (Hallem et al., 2011). Interestingly, BAG neurons are also activated by hypoxia (Zimmer et al., 2009). CO_2 is also sensed by the thermosensory

AFD neurons located in the amphids (Bretscher et al., 2011). In contrast to BAG neurons, AFD neurons are most sensitive to decreases in CO_2, responding with a rapid increase in intracellular calcium. Increased CO_2 evokes a rapid decrease in intracellular calcium in AFD neurons, followed by a slow rise. Analysis of behavior after AFD ablation suggests that these neurons might play a role in promoting straight-line locomotion upon sensing decrease in CO_2, thereby coordinating with BAG to optimize traversal of CO_2 gradients. There are likely additional sensors, as ablation of all BAG and AFD neurons leaves a residual locomotive response to CO_2 (Bretscher et al., 2011). Calcium imaging of a range of other neurons reveals slower and less robust calcium responses in several populations. The most sensitive of these is the gustatory salt-sensor ASE, which exhibits a slow and sustained calcium response to CO_2 (Bretscher et al., 2011).

The molecular pathways by which CO_2 activates BAG and AFD neurons are incompletely understood. Sensory transduction in BAG neuron relies on cyclic nucleotide mediated signaling, requiring function of a cyclic guanosine monophosphate gated (CNG) channel encoded by the tax-2 and tax-4 genes and a receptor-type guanylyl cyclase enzyme encoded by *gcy-9* (Hallem et al., 2008; Bretscher et al., 2008; Hallem et al., 2011). CNG channels are also necessary for CO_2 sensing by AFD neurons, which express a range of guanylyl cyclase enzymes (Bretscher et al., 2011). Intriguingly, both BAG and AFD neurons express carbonic anhydrase, an enzyme family involved in CO_2 sensing in other sensory systems, as described below.

The CO_2 avoidance response is potently regulated by internal state and environmental cues. Starvation of worms leads to a complete loss of avoidance, and in extreme cases, weak attraction to CO_2 (Bretscher et al., 2008). Ablation of the BAG and

AFD neurons suggest that these neurons play differential roles depending on the presence or absence of food (Bretcher et al., 2011). Avoidance is also complexly regulated by environmental O_2 concentration. Sustained hypoxia suppresses avoidance through a transcription dependent mechanism (Bretscher et al., 2008). Conversely, elevated oxygen also inhibits avoidance behavior, in this case through a circuit mechanism dependent on the O_2 sensing URX neuron (Carrillo et al., 2013).

Studies in *C. elegans* highlight the complexity of CO_2 responses, even in a relatively simple animal. Multiple sensors respond to CO_2 in the environment, and this information is synthesized with a range of internal and external cues to determine behavior. Despite the elucidation of multiple cell types and molecular pathways involved in CO_2 detection, the precise mechanism by which CO_2 is sensed at the molecular level has not yet been uncovered; for example, it is not known whether guanylyl cyclase is directly activated by CO_2 in any of the responsive neurons. The genetic accessibility of the worm provides a promising platform for future work dissecting the details of this process.

CO_2 Sensing in Drosophila melanogaster

The insect *Drosophila melanogaster,* also known as the vinegar or common fruit fly, feeds on fermenting food sources that produce CO_2, and follows plumes of concentrated CO_2 while flying, yet displays aversion to CO_2 in walking chemotaxis assays (Cummins et al., 2013). Two distinct sensory systems are responsible for these responses: olfaction, mediated by olfactory receptor neurons (ORNs) located on the

antennae, and gustation, mediated by gustatory receptor neurons (GRNs) distributed primarily in and around the mouthparts. The olfactory receptor neurons send projections to the antennal lobe of the fly brain, which comprises 43 olfactory glomeruli. Each glomerulus is a stereotyped neuropilar structure receiving projections from a defined subset of olfactory receptor neurons. Olfactory cues regulate innate behaviors such as courtship and foraging through activation of genetically determined circuitry, while also serving as substrates for associative learning. Taste information from GRNs is routed primarily to the suboesophageal ganglion and plays a primary role in regulation of feeding behavior, in particular regulating extension and retraction of the proboscis, the feeding organ of the fly (Vosshall and Stocker, 2007).

Olfactory responses to CO_2 are mediated in *Drosophila* by neurons co-expressing two members of the insect gustatory receptor gene family, Gr21a and Gr63a, which project to a single, ventral-most glomerulus in the antennal lobe (Suh et al., 2004; Jones et al., 2007). Calcium imaging of the antennal lobe demonstrates that this one glomerulus responds to CO_2 (Suh et al., 2004). Optogenetic activation of Gr21a-expressing neurons is sufficient to mediate avoidance, while silencing neural transmission from these neurons results in indifference to CO_2 (Suh et al., 2004, Suh et al., 2007). Misexpression of Gr21a together with Gr63a in a normally CO_2 insensitive ORN population confers sensitivity to CO_2, an effect not observed when only one of the two is expressed (Kwon et al.,, 2007). Responses to CO_2 are reduced by transcriptional knock-down of mediators of G-protein coupled receptor signaling (Yao and Carslon, 2010; Deng et al., 2011). These data argue for a model of olfactory detection of CO_2 in which Gr21a and Gr63a function together as

a G-protein coupled receptor for CO_2 in ORNs, which are hard wired to circuitry that mediates an innate avoidance response.

Complicating this model, there is evidence to suggest that additional olfactory pathways for CO_2 exist. In contrast to the behavioral avoidance exhibited while walking, *Drosophila* are attracted to high concentrations of gaseous CO_2 when in flight. This response appears to be olfactory, as occlusion of the antenna eliminates this attraction, yet remains upon silencing of output from Gr21a expressing olfactory CO_2 neurons. The identity of the olfactory receptor mediating attraction is unclear. Orco, an olfactory co-receptor that is required for responses mediated by members of the insect olfactory receptor family (but not gustatory receptors such as Gr21a and Gr63a), is necessary for attraction. However, if other olfactory pathways mediate CO_2 sensing, one would expect to see CO_2 generated activation of the antennal lobe outside of the V glomerulus. The absence of such a response might mean that this pathway is suppressed during the imaging assay. Surprisingly, attraction also requires Ir64a, an ionotropic receptor that functions in the olfactory system to sense acid, but which does not need orco to function. The necessity of both elements suggests that attractive responses might involve multiple independent elements, an Ir64a mediated pathway and an as yet uncovered olfactory pathway active only during flying behavior (Wasserman et al., 2013).

In the context of walking within a novel environment, the avoidance response to CO_2 might serve to warn walking flies of approaching predators or other threats; in fact, stressed flies emit an increased level of CO_2 which can act as a social signal to mediate conspecific avoidance behavior. In contrast, CO_2 in a feeding context promotes attraction and food acceptance. These responses are mediated by a specific population of CO_2

sensitive gustatory neurons that are sensitive to low concentrations of aqueous CO_2, such as produced by yeast growing in liquid, as well to higher concentrations of gas phase CO_2 (Fischler et al., 2007). Neither Gr21 nor 63a appear to be expressed in the gustatory system, and mutants for Gr63a retain normal attractive responses to CO_2, implying that some other molecular mechanism accounts for CO_2 sensing in these neurons. It is interesting to note that flies devote an entire class of taste receptor neuron to the detection of CO_2, meaning that carbonation defines a unique taste quality in the fly, analogous to bitter or sweet in the mammalian taste system.

Given conflicting gustatory and olfactory pathways, how do flies choose the correct behavioral response to CO_2? Several mechanisms have been proposed to be involved in this decision. At the molecular level, compounds found in *Drosophila* food sources such as ripening fruit directly antagonize the Gr21a+Gr63a CO_2 sensor, which may explain how the animal overcomes the avoidance response to its preferred, CO_2 rich, food sources (Turner et al., 2009). CO_2 responses may also be inhibited by other odors through a non-synaptic, activity dependent mechanism that generates lateral inhibition between neighbouring olfactory sensing neurons; indeed, this phenomenon has been demonstrated to be relevant to olfactory CO_2 responses in the mosquito, which are inhibited by stimulation with 1-octen-3-ol, a ligand for a neighbouring neuron (Su et al., 2012).

At the circuit level, additional complexity in CO_2 response has been identified. Two distinct neuronal outputs project from the CO_2 sensitive V glomerulus to higher brain regions, one displaying high sensitivity to CO_2, and the other low sensitivity. The high sensitivity output is inhibited by activity of a third projection neuron type that is

stimulated by high concentrations of CO_2 and by food odors, which does not inhibit the low sensitivity output (Lin et al, 2013). This means that at high CO_2 concentrations or in the presence of other odors, such as food, the low sensitivity output is preferentially engaged. Interestingly, another study reported a neuron projecting from the V glomerulus (resembling and possibly identical with the high sensitivity output neuron) that is specifically required for CO_2 avoidance in starved but not fed flies (Bräcker, et al., 2013). Differential modulation of V glomerulus outputs by environmental context and by internal state thus provides an additional substrate for orchestration of CO_2 responses during feeding.

With regards to the disparity observed between CO_2 response in walking and flying *Drosophila*, it appears that neurons releasing the neurotransmitter octopamine are necessary for attraction during flight, as flying animals avoid CO_2 upon inhibition of these neurons (Wasserman et al., 2013). Reconfiguration of the CO_2 response circuitry by release of octopamine during flight may thus account for the behavioral switch, although the relevant targets of this transmitter have yet to be described.

How do the lessons learned from flies translate to other insect species? From the standpoint of human health, an important issue is the mechanism of CO_2 sensing by mosquitoes, where it is a key element in location of human hosts by these disease vectors. Interestingly, the malaria vector *Anopheles gambiae* possesses orthologs of Gr21a and Gr63a, but these are expressed in the maxillary palps (mouthparts) rather than in the antennal ORNs as in flies (Jones et al., 2007). This anatomical difference may in part explain why mosquitoes are attracted rather than averse to CO_2. Interestingly, mosquitos in which olfactory receptor function is abrogated by genetic ablation of orco lose their

attraction to human host odor, but this phenotype is rescued when odor is co-presented with CO_2, suggesting that CO_2 may synergistically interact with orco independent olfactory mechanisms to mediate host attraction (DeGennaro et al., 2013).

Invertebrate studies highlight some salient aspects of CO_2 detection; perhaps most intriguing, with respect to the focus of this dissertation, is the finding that *Drosophila* utilize a dedicated gustatory pathway to detect CO_2 in the context of feeding. Interestingly, CO_2 may be an innately attractive or aversive cue depending on concentration, internal state, or behavioral context. It is also clear that CO_2 detection may be mediated by multiple sensory modalities, and that organismal response to CO_2 requires circuitry to synthesize these disparate inputs. At the molecular level some important elements have been identified, including cyclic nucleotide and GPCR, signaling, but further work is required to elucidate complete transduction pathways.

1.3. CO_2 Sensing in Mammals

Carbonic Anhydrases

A recurring feature of mammalian CO2 detection systems is the involvement of carbonic anhydrase activity. Carbonic anhydrases are a family of enzymes that catalyze the inter-conversion of carbon dioxide and water with bicarbonate and a free proton. This family can be subdivided into five distinct classes, of which only one, the α-class, is found in mammals. The mammalian genome contains diverse isoforms of α-class carbonic anhydrases that may be cytosolic, mitochondrial, membrane-linked or secreted,

(Imtaiyaz Hassan et al., 2013) (Figure 1.5). In the mouse, there are thirteen distinct genes encoding active carbonic anhydrase enzymes, along with three encoding homologous but inactive carbonic anhydrase related proteins. Each isoform has a characteristic tissue distribution, supporting a wide range of physiological functions. For example, carbonic anhydrase isoforms I and II are expressed in red blood cells, where their activity is required to hydrate tissue derived CO_2 to form bicarbonate, allowing it to be transported to the lungs and excreted. Key roles for carbonic anhydrase have also been identified in the kidney, brain and in certain cancers (Frost, 2014). As described below, carbonic anhydrases are specifically involved in the sensory detection of CO_2 as well.

Respiratory regulation

Mammalian species make use of both interoceptive and exteroceptive systems for carbon dioxide sensing. Internal carbon dioxide detection is of particular importance for the regulation of breathing. The mammalian circulatory system carries CO_2 from the tissues where it is produced by respiration, to the lungs, where it is exhaled. The concentration of CO_2 in the blood is thus a critical input for systems controlling ventilation, and is monitored at both peripheral and central sites. Peripheral chemoreceptors reside in the carotid body, a densely vascular sensory organ positioned at a branch point of the carotid artery. The sensory cells of the carotid, referred to as type I or glomus cells, respond to decreases in arterial P_{O2} or pH, and to increases in PCO_2. Activity in these cells is transmitted by primary afferents in the carotid sinus nerve to the

brainstem, to regulate the central pattern generator controlling ventilation (Jonz and Nurse 2010; Kumar and Prabhakar, 2012).

While the precise mechanism of CO_2 detection by carotid body glomus cells is unclear, carbonic anhydrase activity has been shown to play a key role (Jonz and Nurse 2010). In carotid body glomus cells, carbonic anhydrase inhibitors suppress physiological responses to hypercapnia (Black et al., 1971). Given the function of carbonic anhydrases, either intracellular acidification or production of bicarbonate ion might be a proximate stimulus responsible for depolarization of the chemoreceptive neuron. Although the molecular pathway from carbonic anhydrase to activation of the sensory cell is not completely characterized, inhibition of hyperpolarizing currents through the acid sensitive channel Task-1 is likely to play a role (Trapp et al., 2008).

Respiratory control is achieved by cooperation and interaction of carotid body chemoreceptors with central chemoreceptors believed to be located at the ventral medullary surface, where they monitor the composition of cereberospinal fluid. In contrast to the carotid body, where physiological response is dominated by O_2 sensing, this system is primarily CO_2 sensitive. Despite a large body of literature investigating central chemoreception, it is not yet clear which populations of neurons are directly involved in CO_2 sensing, or even whether CO_2, HCO3- and/or H+ are the relevant input for one or more classes of sensory neurons in the central nervous system (Huckstepp and Dale, 2011).

Olfactory CO_2 detection

Although humans perceive CO_2 to be odorless, other mammalian species are keenly sensitive to the smell of CO_2. Rodents make use of a dedicated olfactory subsystem that is highly sensitive to CO_2, detecting concentrations just above atmospheric background. Mice display innate aversion to the smell of CO_2. This response is mediated by a population of specialized olfactory receptor neurons that project to a the necklace glomeruli, a distinct set of neuropilar structures at the border of the olfactory bulb. The neurons are molecularly distinct from conventional olfactory receptor neurons in expressing the enzymes guanylyl cyclase D (GC-D) and carbonic anhydrase II (Car2) (Hu et al., 2007).

How do these neurons detect CO_2 in the nose? Responses in these olfactory neurons, as well as the behavioral aversion of mice, are dependent upon carbonic anhydrase activity. A proposed molecular pathway for olfactory detection begins with conversion of CO_2 to carbonic acid by Car2 in the cytoplasm, which rapidly dissociates to bicarbonate ion and a proton. Biochemical studies demonstrate that GC-D cyclase activity is dose-dependently stimulated by bicarbonate (Sun et al. 2009). This provides a mechanism for activation of GC-D, thus leading to a rise in cGMP and opening of cyclic nucleotide gated channels, depolarizing the olfactory receptor neuron. This mechanism is strikingly similar to that used in *C. elegans* BAG neurons, as described above. Notably, the GC-D gene has degenerated into a non-functional pseudogene in the primate lineage, perhaps explaining the inability of humans to detect CO_2 by smell (Young et al., 2007).

Somatosensory CO_2 sensation

Carbonation evokes a distinct 'prickly' sensation in the mouth that is mediated by somatosensory neurons innervating the oral cavity and nasopharynx. In human studies, this sensation remains even when atmospheric pressure is raised to eliminate the formation of bubbles, suggesting that the response is of chemogenic origin rather than a mechanical effect (Wise, 2013). Furthermore, inhibition of carbonic anhydrase activity on the tongue suppresses neural responses to CO_2 in the trigeminal nerve of the rat, which carries somatosensory information from the face (Komai and Bryant, 1993). Consistent with this finding, treatment of the tongue with carbonic anhydrase inhibitors eliminates the prickly sensation of soda for human subjects (Simons et al. 1999).

Recently, an *ex vivo* study of responses to CO_2 in dissociated somatosensory neurons has shown that neurons expressing the channel TrpA1 respond to CO_2, and require this channel for CO_2 response (Wang et al., 1010). TrpA1 responds to chemical irritants, including allyl isothiocyanate (mustard oil), and cinnamaldehyde, the pungent component of cinnamon. This study additionally demonstrated the TrpA1 channel itself is activated by intracellular but not extracellular acidification. This supports a model in which CO_2 first diffuses across the plasma membrane of TrpA1 expressing nerve endings, where it causes intracellular, carbonic anhydrase dependent acidification to evoke depolarizing currents through the TrpA1 channel. This neuronal population likely provides the distinctive 'bite' generated by oral stimulation with CO_2.

In summary, mammals possess diverse neuronal systems for CO_2 detection within the body and in the environment. Olfactory CO_2 detection by mice is currently the best understood of these, with a substantiated pathway from CO_2 molecule to the olfactory bulb. Although CO_2 detection is critical for the regulation of breathing, the relevant

mechanisms remain poorly understood. A common theme in mammalian sensation of CO_2 is the involvement of carbonic anhydrase enzymes. What about mammalian taste sensation of CO_2? Does the gustatory system play a role in evoking the 'flavor' of carbonation? I will next review the organization of the mammalian taste receptors cells and receptors, and present a few lines of evidence supporting a role for gustatory carbonation detection.

1.4 The Mammalian Gustatory System

The sense of taste provides information regarding the risks and rewards presented by food and drink. For example, the sweet taste elicited by nutritious sugars promotes consumption of energy-dense food items, while the repulsive bitterness of toxins such as strychnine ensure the rejection of potentially dangerous items. Taste buds, the sensory organs for taste, are distributed on the tongue and palate. This location allows the gustatory system to sample the chemical composition of food as it enters the oral cavity, but before the final decision to ingest, thereby serving as a gatekeeper for feeding behavior.

Humans, and most other mammalian species, respond to five distinct classes of taste stimuli corresponding to the five primary taste qualities: sweet, sour, salty, bitter and umami. These stimuli are detected by five genetically and functionally defined populations of taste receptor cells (TRCs). Sweet cells detect simple sugars and artificial sweeteners, umami cells respond to certain L-amino acids, bitter cells to a wide range of noxious chemicals, salty cells to sodium salts, and sour cells to acidity. These five

distinct receptor types are intermingled within taste buds, along with supporting cells and the terminals of afferent nerves. Taste buds are housed within papillae, epithelial specializations of which there are three types: (1) dozens of taste buds are distributed across the anterior surface of the tongue in fungiform papillae, (2) hundreds are located in the trenches of circumvallate papillae at the back, and (3) dozens to hundreds more localize to the sides of the tongue in foliate papillae (Fig 1.1). Many isolated taste buds are also distributed on the soft palate.

Taste signals from the fungiform taste buds and palate are transmitted to neurons in the geniculate ganglion via the chorda tympani and greater superficial petrosal nerve, respectively, whereas the circumvallate and foliate papillae are innervated primarily by the glossopharyngeal nerve, composed of fibers initiating from the petrosal ganglion (Figure 1.1). Notably, TRCs actively regenerate during adult life, with taste cells living an average of only 2 weeks before dying and being replaced by newly born cells (Lindemann, 2001); this poses the interesting challenge of ensuring that the correct newly born TRC connects to the appropriate afferent nerve fibers. Taste information from sensory ganglia converges onto the rostral portion of the nucleus of the solitary tract in the brainstem, from where it is routed through the parabrachial nucleus in mice or directly to the ventral posteromedial nucleus of the thalamus in primates. From the thalamus, projections connect to the primary gustatory cortex in the insula. Local projections from the nucleus of the solitary tract (NST) within the brainstem mediate low-level (i.e., noncortical) behavioral responses, such as salivation and gaping induced by bitter taste (Spector and Travers, 2005).

Figure 1.1

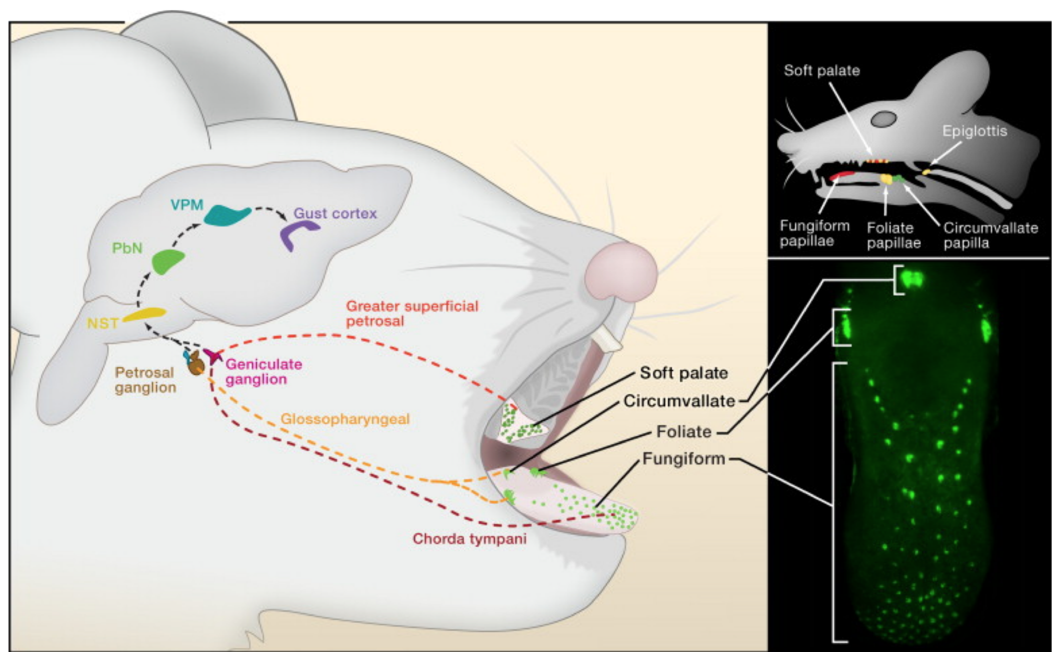


Figure 1.1
The anatomy of taste. Taste buds are broadly distributed on the tongue and soft palate. On the tongue, taste buds are localized to three classes of papillae: In mice, the single circumvallate papilla is found at the very back of the tongue; foliate papillae are at the posterior lateral edge, and fungiform papillae are distributed over the anterior two thirds of the tongue; these three classes of papillae can be highlighted in mice engineered to express green fluorescent protein in taste bud areas (lower right panel). The taste buds on the tongue and palate are innervated by three afferent nerves: the chorda tympani, greater superficial petrosal, and glossopharyngeal. These nerves carry taste information from the taste receptor cells to the nucleus of the solitary tract (NST) in the brain stem. From the NST, taste responses are transmitted (and processed) through the parabrachial nucleus (PbN) and the thalamus (VPM) to the primary gustatory cortex in the insula. Behavioral responses to food (and perceptions of flavor) are ultimately choreographed by the integration of gustatory information with other sensory modalities (such as olfaction, texture, etc.)

Taste receptor genes and cells

The selective tuning of TRCs to a particular set of chemical species is dependent upon specific expression of genes encoding taste receptor proteins. The attractive tastes, sweet and umami, are sensed by heterodimeric G protein-coupled receptors (GPCRs) assembled by the combinatorial arrangement of T1R1, T1R2, and T1R3 subunits (Nelson et al., 2001; Li et al., 2002; Nelson et al., 2002; Zhao et al., 2003). The key role of these receptors in mediating mammalian sweet and umami taste was uncovered from a range of studies, including heterologous expression in cell-based assays (Nelson et al., 2001; Nelson et al., 2002; Li et al., 2002) and the engineering of mice with ablated or genetically altered T1R subunits (Damak et al., 2003; Zhao et al., 2003). Together, these studies validated T1R1+3 (a heteromeric receptor composed of the T1R1 and T1R3 subunits) as the mammalian umami receptor (Li et al., 2002; Nelson et al., 2002; Zhao et al., 2003) and T1R2+3 as the mammalian sweet taste receptor (Nelson et al., 2001; Li et al., 2002; Damak et al., 2003; Zhao et al., 2003). The T1R2+3 sweet receptor recognizes simple sugars, a wide range of artificial sweeteners, D-amino acids, and even intensely sweet proteins (Figure 1.2). How does a single receptor accommodate this broad range of tastants? Recent structure-function studies have begun to dissect the fine-grained details of the T1R receptor complexes and identified several discrete sites on each of the three subunits that participate in ligand binding (Cui et al., 2006; Jiang et al., 2004; Jiang et al., 2005; Winnig et al., 2007); the presence of multiple sites in each receptor complex may help explain their remarkable breadth of tuning.

Figure 1.2

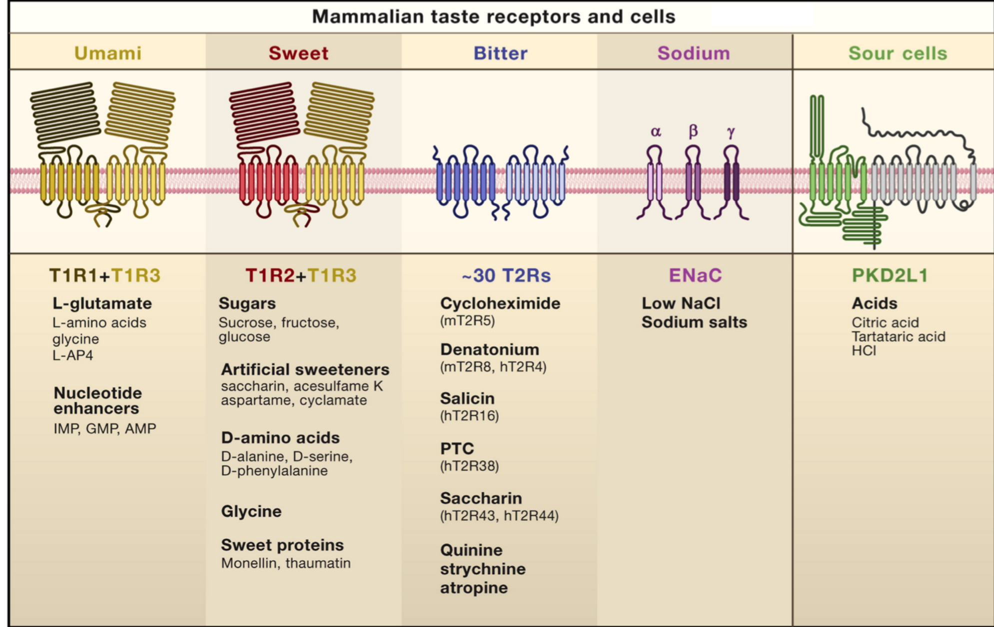


Figure 1.2
Mammalian taste receptors, cells and ligands. Detection of the gustatory world is mediated by several distinct classes of taste receptors and taste receptor cells. Sweet and umami compounds are sensed by T1R heterodimers, while bitter compounds activate T2R receptors. Salt is detected via several mechanisms, one of which relies upon the sodium channel ENaC. Sour-sensing cells are defined by the expression of PKD2L1.

Mammalian taste receptors show markedly more sequence divergence between species than do typical GPCRs (Adler et al., 2000; Nelson et al., 2001). This diversity is the substrate for functional differences reflecting the adaptation of different species to distinct ecological niches and diet. For example, mice and humans display a number of differences in the range of compounds stimulating sweet and umami taste. Umami is strongly stimulated in humans only by L-Glutamate (MSG) and L-Aspartate, whereas mice display robust attraction and neural responses to the majority of L-amino acids (Iwasaki et al., 1985; Nelson et al., 2002; Zhao et al., 2003). Similarly, humans taste as sweet several compounds to which mice are indifferent (e.g., aspartame; Nelson et al., 2001). Notably, these differences in selectivity are perfectly matched by the tuning of the respective T1R subunits, such that exchanging T1R components between the human and mouse receptors generate the corresponding altered taste selectivity both in cell-based assays and in vivo (Nelson et al., 2001; Nelson et al., 2002; Li et al., 2002; Zhao et al., 2003). This strict correlation between receptor function and behavioral selectivity and sensitivity across species strongly implies that T1R receptors are a major determining factor in species-specific taste preferences. Indeed, two extreme examples illustrate this principle: (1) introduction of the human T1R2 gene into mice humanizes sweet taste preferences (Zhao et al., 2003), and (2) the Felidae family acquired a loss-of-function mutation in the T1R2 gene early in their evolution and have consequently loss all sweet taste; this nicely explains the behavioral indifference of all cats to sugars (Li et al., 2005).

Orthologs of the three T1Rs are present in the genomes of all vertebrates thus far examined. T1Rs have not been identified in any invertebrate species, including the chordates amphioxus and *Ciona intestinalis*. Importantly, all members of the T1R family

are present in fish, where they also function as heteromeric receptors (Oike et al., 2007; Yasuoka and Abe, 2009). However, fish T1R2+3 responds to L-amino acids rather than prototypical sweet tastants (Oike et al., 2007; Yasuoka and Abe, 2009). This suggests that the mammalian T1R2+3 complex was remodeled to recognize sugars at some point during the transition of vertebrates from oceans to land.

The role of sweet and umami taste is to help identify food sources rich in sugar and protein. As such, the T1Rs are low-affinity receptors mediating behavioral preference thresholds in the millimolar range (Damak et al., 2003; Zhao et al., 2003); such low affinity helps the receptors distinguish between different potential sugar and protein sources without reaching saturation below nutritionally relevant concentrations.

Bitter recognition faces a different challenge. Not only is the chemical diversity of bitter substances orders of magnitude greater, but in addition these toxic compounds must be detected at much lower concentrations in order to avoid potentially lethal dietary mistakes. To accomplish this task, mammals are endowed with a family of GPCRs encoding the T2R bitter receptors (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000). The T2Rs have a highly variable structure with few regions of extended conservation; this sequence diversity reflects the need to recognize a disparate chemical universe. T2Rs are both necessary and sufficient for bitter taste. On the one hand, knockout (Mueller et al., 2005) or genetic alterations (Kim et al., 2003; Bufe et al., 2005) of specific T2Rs leads to changes in bitter taste sensitivity and selectivity. On the other, introduction of novel T2Rs expands the bitter taste repertoire (Mueller et al., 2005).

Ligands for several mouse and human T2Rs have been identified in cell-based assays, and as expected, all are bitter to humans or aversive to mice (Chandrashekar et al.,

2000; Bufe et al., 2002; Pronin et al., 2004; Meyerhof et al., 2005). Given that there are far fewer T2Rs (ranging from about 10 to 40 members, depending on the species) than chemically distinct bitter-tasting chemicals, it is not surprising that any given T2R actually recognizes a wide repertoire of ligands (Meyerhof et al., 2005). Interestingly, some compounds, for example acesulfame K and saccharin, evoke sweetness at low concentrations but bitter responses at high concentrations. What underlies this duality of response? As it turns out, not only do these two artificial sweeteners activate the sweet taste receptor (Nelson et al., 2001; Li et al., 2002), but in addition they also activate specific T2Rs at high concentration (Kuhn et al., 2004; Pronin et al., 2007). This observation nicely illustrates the concept that a single chemical species may elicit more than one taste (i.e., through the activation of multiple receptors) and may explain the characteristic "aftertaste" associated with these tastants.

Why do chemically diverse compounds generate a common sensation of bitterness? Studies of the expression of *T2R* transcripts in TRCs showed that each bitter-sensing cell coexpressess the majority of the *T2R* genes (Adler et al., 2000; Mueller et al., 2005; Meyerhof et al., 2005). Given this lack of selectivity in the expression of T2Rs, Adler et al. proposed that bitter TRCs detect a wide range of toxic chemicals but do not discriminate between them. Indeed, subsequent behavioral studies demonstrated that rodents are unable to discriminate between bitter compounds (Spector and Kopka, 2002), and molecular studies showed that taste-blind animals engineered to restore bitter taste function under the control of single T2R promoters recovered taste recognition to the entire repertoire of bitters (Zhang et al., 2003; Mueller et al., 2005). This is exactly the type of sensor needed to warn against the ingestion of noxious substances and provides a

nice biological underpinning to the observation that many human cultures use a single word to define diverse bitter-tasting compounds.

Sour-sensing TRCs are characterized by the expression of PKD2L1, a TRP ion channel proposed to function as a component of the acid-sensing machinery (LopezJimenez et al., 2006; Ishimaru et al., 2006; Huang et al., 2006). Genetic ablation of these cells via targeted expression of diphtheria toxin fragment A (DTA) subuspecifically and completely abolishes taste responses to acids, without affecting the other four taste qualities (Huang et al., 2006).

How might PKD2L1-expressing TRCs sense acid? Several candidate receptors have been proposed for sour taste, including PKD2L1, PKD1L3, HCN1, and HCN4 (Stevens et al., 2001; Ishimaru et al., 2006; LopezJimenez et al., 2006; Huang et al., 2006); knockout of Pkd2L1 and/or Pkd1L3 result in only moderate reduction in neural responses to acids, suggesting that other mechanisms contribute to acid transduction; genetic ablation studies are needed to determine the role, if any, of the other putative acid sensors in vivo (Horio et al., 2011).

On the basis of rodent studies, the taste of salts has typically been divided into two components based on taste preferences to salt-containing solutions and the sensitivity of salt responses to the channel blocker amiloride (Breslin et al., 1993; Spector et al., 1996). At low concentrations (10–150 mM NaCl), mice will consume salt, but the behavior (and neural responses in this range) are largely blocked by amiloride (Bachmanov et al., 2002). At high concentrations of salt, however, mice exhibit innate aversion, and these responses are unaffected by amiloride. Recent work has demonstrated the cellular and molecular basis of these two opposing effects of salt. The epithelial

sodium channel ENaC, which is strongly inhibited by amiloride, has long been proposed to participate in salt taste (Heck et al., 1984; Brand et al., 1985). This has been validated through genetic ablation of the alpha subunit of ENaC specifically in TRCs, which results in loss of the amiloride sensitive component of the neurophysiological response to salts as well as behavioral attraction to salt (Chandreshekar et al. 2010). On the aversive side, high concentrations of sodium chloride as well as other salts have been show to stimulate aversion through combined stimulation of T2R expressing bitter cells and Pkd2L1 expressing sour cells. Mice in which both these pathways have been genetically silenced are not averse to high concentrations of salts, and when salt-depleted will consume NaCl at concentrations that are repulsive to wild type mice (Oka et al, 2013).

Logic of taste coding at the periphery

The expression of bitter, sweet, umami, and sour receptors in segregated TRCs implies that these tastes are mediated by distinct, dedicated receptor cells, each tuned to a single taste modality (Figure 1.3). Indeed, a series of studies in genetically engineered mice have now substantiated this logic of taste coding and provided evidence of a labeled-line organization for the taste system at the periphery (Chandrashekar et al., 2006). For example, specific taste receptor cell populations can be genetically ablated by expression of the diphtheria toxin A fragment (DTA) , and the resulting animals exhibit a deficit only in that modality while other responses remain intact (Huang et al., 2006). In addition, the innate nature of taste preferences strongly suggests that TRCs are hardwired to behavioral programs for acceptance and rejection. If this is true, activation of selective

Figure 1.3

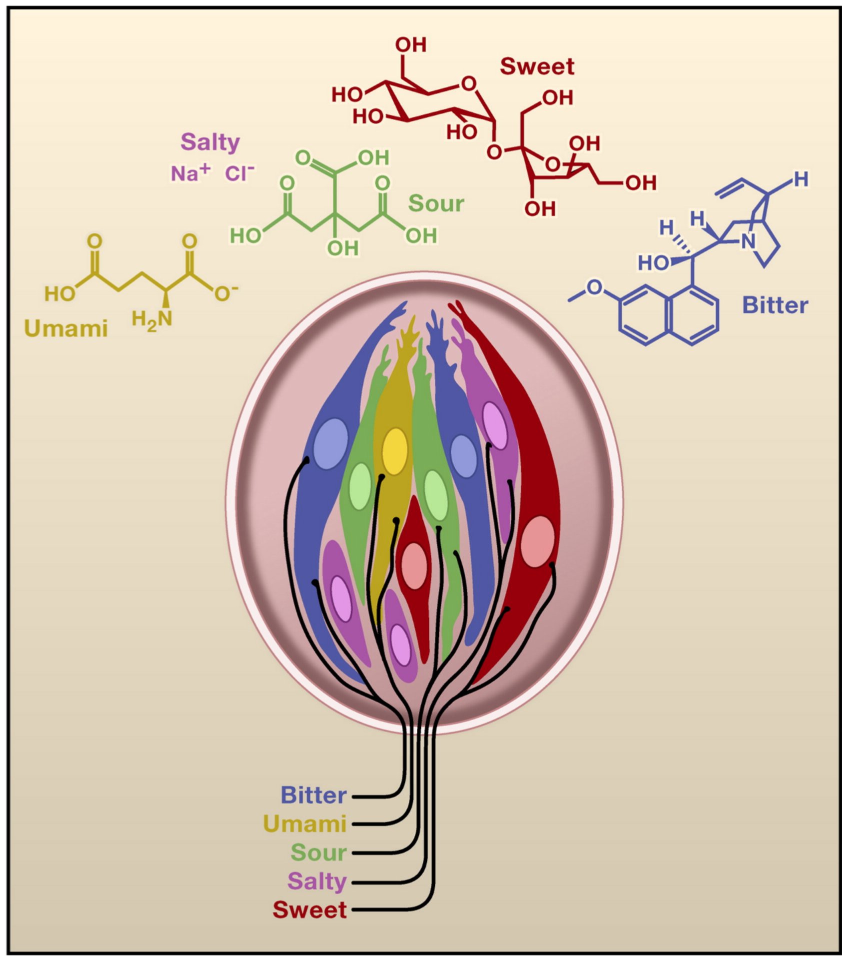


Figure 1.3
Labeled inputs mediate taste sensation. Taste compounds and receptor cells are categorized into five classes under our current model of coding. The tastes of sweet (sucrose), bitter (quinine), sour (citric acid), umami (glutamate), and salty (sodium chloride) are mediated by separate populations of selectively tuned taste receptor cells. Notably, taste buds from all regions of the oral cavity contain cells that respond to the five basic modalities. Thus, contrary to popular belief, there is no topographic map (i.e., a tongue map) of taste qualities on the tongue.

TRC populations should be sufficient to drive taste behavior. Indeed, expression of a non-taste receptor in sweet or bitter TRCs did allow taste cells to be activated, and a strong specific behavior elicited, by an ordinarily tasteless ligand (Zhao et al., 2003 and Mueller et al., 2005). As Figure 1.4 shows, if this receptor (RASSL, Coward et al., 1998) is expressed in sweet-sensing cells under the control of the T1R2 promoter, these mice are strongly attracted to solutions containing the normally tasteless ligand (Zhao et al., 2003). If, on the other hand, the very same RASSL receptor is expressed in bitter cells, these mice now exhibit strong repulsion (Mueller et al., 2005). Similarly, expression of a bitter receptor in sweet-sensing cells produces animals that exhibit strong attraction to the cognate bitter ligand, that is, mice respond to bitter compounds as if they were sweet (Mueller et al., 2005). These behaviors do not involve learning, as receptor expression is absent during development and is induced only immediately prior to the behavioral tests. Taken together, these experiments demonstrate that behavioral responses to taste stimuli are determined by the identity of the stimulated cell type, and not by the properties of the taste receptor molecule or even the tastants; they also illustrate how the functional segregation of taste modalities endows the taste system with a refined engine to drive innate behaviors. It will be an interesting challenge to understand the genetic program and mechanism(s) by which each taste cell type is hardwired to the appropriate neural circuitry and to explore if one can also alter taste behavior by manipulating the wiring scheme.

How do TRCs transmit information to primary afferents? Remarkably, only a few cells in each taste bud, namely the PKD2L1-expressing sour cells, possess conventional synapses as defined by ultrastructural studies (Yang et al., 2000). Yet, ablation of these

Figure 1.4

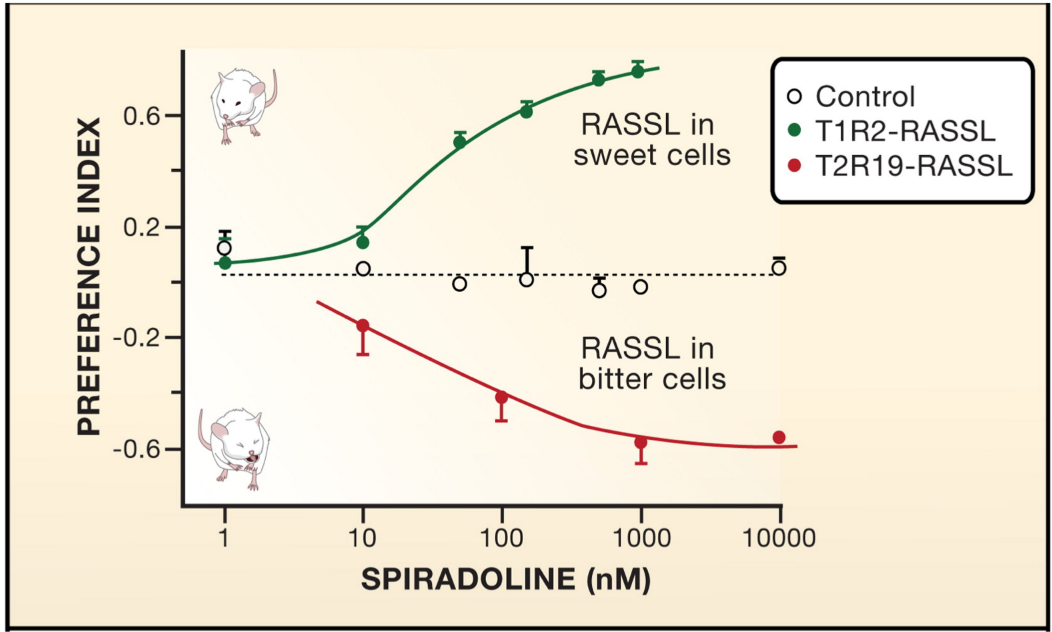


Figure 1.4
Behavioral attraction and aversion are hardwired. Dedicated cells tuned to selective taste qualities are hardwired to trigger specific behavioral responses. The synthetic opiate spiradoline is normally tasteless to mice (A, open circles). However, after targeted expression of the spiradoline receptor (RASSL) to sweet cells, mice exhibit dose-dependent attraction to spiradoline. In marked contrast, directing expression of the very same RASSL receptor to bitter cells results in strong aversion to the ligand (Mueller et al., 2005); preference index = (tastant − control)/total. Data are taken from Zhao et al., 2003 (sweet), and Mueller et al., 2005 (bitter).

cells selectively eliminates responses to sour tastants without affecting the other modalities (Huang et al., 2006). This has led to several models of atypical synaptic signaling, including the proposal that sweet, bitter, umami, and salty taste responses are transmitted to primary afferents through some nonconventional connection, such as release from subsurface cisternae (Royer and Kinnamon, 1988) or neurotransmitter release through pannexin/connexin hemichannels (Huang et al., 2007 and Romanov et al., 2007). The most likely neurotransmitter to communicate taste information to primary afferents is adenosine triphosphate (ATP), as combined knockout of the purine receptors P2x2 and P2x3 results in taste blindness in mice (Finger et al., 2005). The membrane protein Calhm1 has been implicated in mediating tastant evoked ATP release from bitter sweet and umami TRCs, but not sour or salty (Taruno et al., 2013). Subcellular localization of Calhm1 may provide important clues as to the structural basis of specific signaling between TRCs and afferent fibers.

Regardless of the mechanism of transmission, it would be extremely useful to be able to follow the connectivity of defined TRCs to second-order and higher neurons. Three groups have reported the transmission of the purportedly transneuronal tracer wheat germ agglutinin from genetically labeled TRCs to higher stations (Sugita and Shiba, 2005, Ohmoto et al., 2008 and Damak et al., 2008). Although these approaches have provided limited and somewhat conflicting information about taste pathways, when combined with functional studies, they may help determine how taste signals are transformed, and how organization at the periphery compares to representation of taste in the higher neuronal stations (ganglia, brain stem, thalamus, and primary cortex).

Peripheral encoding of taste: outstanding questions

If peripheral taste sensation is defined as the process by which our gustatory system transforms a chemical stimulus into a pattern of neural activity, the key questions fall into three classes: 1) What are the molecular mechanisms by which a given chemical stimulus generates a physiological response in TRCs? 2) How are these responses distributed across TRC; i.e. how many distinct cell populations define labeled gustatory inputs? And 3) How is activity in TRCs translated into a pattern of electrical activity in the primary sensory neurons which transmit taste signals from the tongue to the central nervous system? While recent work has significantly clarified our understanding of these processes, some uncertainties remain.

The molecular reception of sweet, bitter and umami and salty taste stimuli appear to be essentially resolved. but some questions of molecular mechanism remain: 1) Molecular receptors of sour remain elusive. Many molecules have been proposed to function as acid sensors mediating sour taste, yet at this stage, none have been substantiated by *in vivo* studies. 2) Although ENaC is the receptor for the salty taste of sodium, and ENaC expression defines the cells mediating sodium taste in mice, human salt perception does not appear to be affected by amiloride (Halpern, 1998). This could be due to post-transcriptional modification of the ENaC complex in humans, affecting amiloride sensitivity, or to the existence of alternate salt sensors in humans. 3) No candidate receptors have been proposed that might mediate the very high threshold salt responses in T2R or Pkd2L1 expressing TRC. While the responses of these cells to very high concentrations of salt may simply reflect a non-specific effect of high ionic strength

on cellular physiology, it is formally possible that a bonafide receptor mediates these responses.

While five classes of TRCs define the fundamental complement of taste qualities, it is also possible receptors for additional taste modalities remain to be discovered; possibilities include the taste of water, or of fat. At this stage there is weak and limited evidence substantiating such possibilities, although a putative mechanism for gustatory detection of fat has been proposed (Laugerette et al., 2005). If additional taste modalities do indeed exist, they would be mediated by additional classes of TRC. In this regard it is worth noting the possibility of functional diversity within the T1R and T2R expressing TRC populations. Two atypical T1R expressing cell types have been reported: one expressing T1R3 but not T1R1 or T1R2, and one expressing all three subunits. Each could potentially define a distinct labeled line mediating attraction to nutrients (Nelson et al., 2001, Kusuhara et al., 2013). However, these populations are relatively rare, and might simply represent transitional stages of TRC development, rather than functional TRCs. Similarly, the large size of the T2R bitter receptor family means that the individual bitter receptor cells could be tremendously diverse. Although a number of lines of evidence suggest that these cells function as generalized bitter sensors, it is unlikely that each cell expresses the entire complement of T2R receptors at precisely equivalent levels. Indeed, *ex vivo* studies of TRC physiology suggests functional heterogeneity within the bitter population (Caicedo and Roper, 2001; Hacker et al., 2008). While mice do not distinguish between bitter compounds in behavioral assays, it is still possible that diversity of sensitivity across bitter TRC might provide meaningful information to the animal.

The transmission of information from TRC to primary afferent remains something of a mystery, despite the elucidation of a few molecular elements as described above. The structural nature of the 'handshake' between receptor cell and afferent fiber is unclear, as only the sour taste receptor cells possess recognizable synapses as assayed by electron microscopy. This problem is intimately linked to the developmental processes by which new TRCs are specified and wired to the appropriate circuits. As TRCs are continously generated throughout adult life, each newly born cell must form appropriate connections to ensure fidelity of taste coding.

Taste encoding in living organisms may involve processing beyond the simple detection of chemical stimuli. One consideration is that natural taste stimuli are chemically complex, and typically activate multiple populations of TRC simultaneously. Taste sensations as studied in the laboratory are usually evoked by stimulation with prototypical tastants chosen to activate only a single quality. Examining representations of more complex taste stimuli might reveal context dependent signal transformation. Another potential modulator of peripheral transduction is internal state (e.g. hunger). Some studies have suggested roles for systemic hormonal signal in regulating peripheral taste sensitivity (Sinclair et al., 2010, Shin et al., 2010).

Our current understanding of taste transduction at the periphery supports a simple but elegant model in which five populations of TRC encode taste stimuli by activating 'labeled lines', segregated pathways in the brain that each evoke a distinct taste percept. This stands in stark contrast to models of taste coding favored in the past, in which patterns of activity across broadly tuned inputs are synthesized to generate taste percepts and appropriate feeding behavior ('across-fiber' coding) (Erickson, 2008). The evidence

presented above strongly supports spatial segregation of taste quality into distinct cellular populations at the earliest stages of encoding. While there remains some ambiguity in the precise number of labeled lines, this principle of organization nicely explains the discrete sensation of the five traditional taste modalities. Therefore, an understanding of the taste any novel stimulus, such as carbonation, depends on defining the set of receptor populations that it activates.

1.5 Taste Sensation of Carbonation

Surprisingly, given the economic importance of carbonated soft drinks, taste sensation of carbonation may be the least understood aspect of CO_2 detection. The gustatory qualities of CO_2 are somewhat controversial in the psychophysical literature; while some reports support a role for taste in sensing carbonation, other describe CO_2 as entirely tasteless to humans and purely a somatosensory stimulus (Cowart, 1998; Cornetto-Muniz et al., 1987). Indirect evidence for human taste sensation of carbonation is provided for a phenomenon known as the 'champagne blues'. This malady affects mountain climbers taking carbonic anhydrase inhibitors for altitude sickness, causing carbonated beverages (e.g. celebratory champagne) to acquire an altered, repulsive taste quality. A controlled study of this effect revealed altered taste of carbonated water with either systemic or topical lingual administration of acetazolamide, the drug responsible for the champagne blues. Strikingly, the changes in taste profile affected multiple primary taste qualities, suggesting a complex pattern of gustatory activation by CO_2 (Graber and Kelleher, 1988). Additionally, some physiological studies support the existence of a taste

response to CO_2, although none were able to link this to sensation of a particular taste quality (Kawamura and Adachi, 1967; Komai and Bryant, 1994; Lyall et al, 2001). These data suggest that in addition to somatosensory detection, carbonation in the mouth could be sensed via one or more gustatory labeled lines, with carbonic anhydrase activity likely playing some role in the transduction mechanism.

How can we define the taste of carbonation more clearly? In my thesis work I proposed to study the basis of gustatory CO_2 sensing in the mouse. This organism offers genetic tools to manipulate taste receptor genes and each class of TRC, as well as established physiological assays for peripheral taste function. It should therefore be possible to define with great precision the mechanisms by which carbonation is sensed on the tongue. The remainder of this dissertation describes efforts to identify cellular and molecular elements of gustatory CO_2 sensation.

Figure 1.5

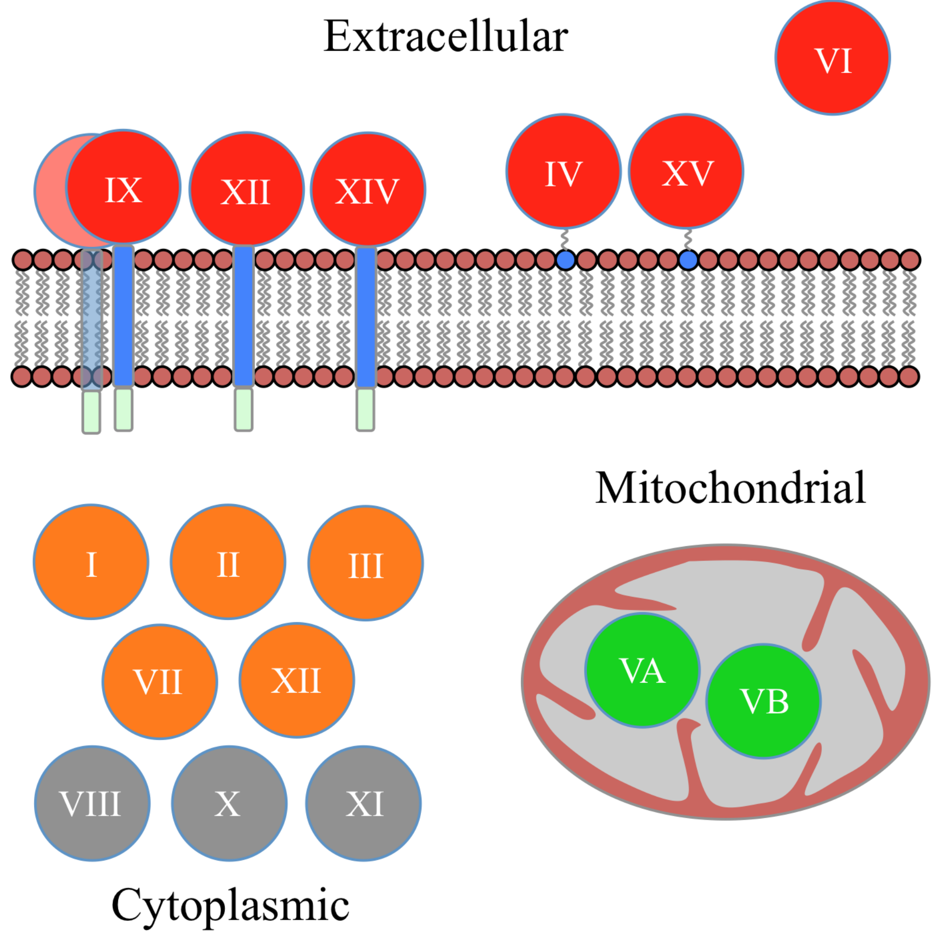


Figure 1.5
Mammalian carbonic anhydrase enzymes. The mouse genome encodes sixteen distinct proteins in the carbonic anhydrase gene family (humans lack the gene encoding CAXV). Each isozyme has a distinct subcellular localization, as depicted. Isozymes VIII, X and XI are catalytically inactive and referred to as 'carbonic anhydrase related proteins'. CAIV and CAXV are linked to the membrane by a glycophosphotydilinsotitol modification, while CAIX, XII and XIV are integral membrane proteins. CAIX exists as a homodimeric complex. CAVA and CAVB localize to the mitochondrial matrix.

Chapter 2 Car4 and Sour-Sensing Cells Mediate Taste Responses to CO_2

2.1 Abstract of this Chapter

Carbonated beverages are commonly available and immensely popular, but little is known about the cellular and molecular mechanisms underlying the perception of carbonation in the mouth. In mammals, carbonation elicits both somatosensory and chemosensory responses, including activation of taste neurons. We have identified the cellular and molecular substrates for the taste of carbonation. By targeted genetic ablation and the silencing of synapses in defined populations of taste receptor cells, we demonstrated that the sour-sensing cells act as the taste sensors for carbonation, and showed that carbonic anhydrase 4, a glycosylphosphatidylinositol-anchored enzyme, functions as the principal CO_2 taste sensor. Together, these studies reveal the basis of the taste of carbonation as well as the contribution of taste cells in the orosensory response to CO_2.

2.2 Introduction

Humans perceive five qualitatively distinct taste categories: bitter, sweet, salty, sour, and umami (a savory sensation characterized by the taste of monosodium glutamate). Sweet and umami are sensed by members of the T1R family of heterotrimeric guanine nucleotide binding protein (G protein)–coupled receptors (GPCRs) (Nelson et al., 2001; Nelson et al., 2002; Li et al., 2002); bitter stimuli are detected by T2R GPCRs

(Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000; Mueller et al., 2005); salty by the heterotrimeric epithelial sodium channel (Chandrashekar et al., 2010), and sourness is sensed by cells expressing the ion channel PKD2L1 (Huang et al., 2006; Ishimaru et al., 2006; LopezJiminez et al., 2006). In the tongue, these receptors function in distinct classes of taste cells, each tuned to a specific modality (Mueller et al., 2005; Huang et al., 2006; Zhang et al., 2003; Zhao et al., 2003).

In addition to these well-known categories of stimuli, the taste system appears to be responsive to CO_2 (Kawamura, 1967; Komai et al., 1994; Lyall et al., 2001). Mammals have multiple sensory systems that respond to CO_2, including nociception (Dessirier et al., 2001, Simons et al., 1999), olfaction (Hu et al., 2007), and chemoreception essential for respiratory regulation (Lahiri and Forster, 2003). Thus, we wondered how taste receptor cells (TRCs) detect and respond to carbonation.

We studied the electrophysiological responses of TRCs to CO_2 by recording tastant-induced action potentials from one of the major nerves innervating TRCs of the tongue [chorda tympani (Lyall et al., 2001)]; this physiological assay monitors the activity of the gustatory system at the periphery and provides a reliable measure of TRC function (Zhao et al., 2003; Dahl et al., 1997). Indeed, the taste system displayed robust, dose-dependent, and saturable responses to CO_2 stimulation. The responses were evident for carbonated drinks (e.g., club soda), CO_2 dissolved in buffer, and even direct stimulation of the tongue with gaseous CO_2 (Fig. 2.1). In contrast, stimulation with pressurized air did not elicit any gustatory response.

Figure 2.1

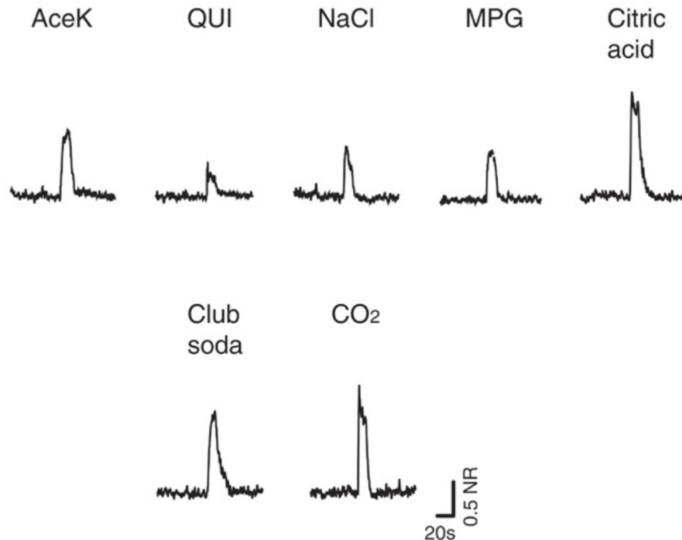


Figure 2.1
Taste responses to CO_2. (A) Wild-type mice exhibit neural responses to carbonated solutions and carbon dioxide. Shown are chorda tympani nerve responses to control sweet (30 mM acesulfame K, AceK), bitter (10 mM quinine, QUI), salty (120 mM NaCl), amino acid (30 mM monopotassium glutamate + 0.5 mM inosine monophosphate, MPG), and sour (50 mM citric acid) stimuli as well as carbonated water (club soda) and gaseous CO_2 normalized to the responses to 250 mM NaCl (0.5 NR = 50% of NaCl response).

2.3 Pkd2l1-expressing Cells are Required for Gustatory CO_2 Responses

To define the identity of the TRCs needed to taste carbonation, we examined CO_2 responses from engineered mice in which specific populations of TRCs were genetically ablated by targeted expression of attenuated diphtheria toxin [e.g., sweetless, sourless mice, etc. (Huang et al., 2006; Chandrashekar et al., 200)] and determined whether their taste systems remained responsive to CO_2. Selective ablation of sour sensing (i.e., PKD2L1-expressing) cells not only abolished gustatory responses to acidic stimuli, but also profoundly reduced responses to gaseous or dissolved CO_2 (Figure 2.2). These results show that PKD2L1-expressing cells are essential for CO_2 detection.

2.4 Identification of a Candidate CO_2 Receptor

To identify candidate CO_2 receptors, we carried out gene expression profiling of sour cells. We reasoned that transcripts for genes involved in carbonation sensing should be enriched in PKD2L1-expressing cells, but that such transcripts would be relatively rare in taste tissue in which PKD2L1 cells have been ablated. Thus, we conducted complementary microarray experiments using mRNA isolated from hand-picked green fluorescent protein (GFP)–labeled sour TRCs, and from taste buds of animals in which defined subsets of TRCs were ablated by expression of DTA (See Appendix A for details). One gene, *Car4*, was particularly attractive: It was highly specific for PKD2L1-expressing cells versus other TRC types (Fig. 2.3), and moreover it encodes carbonic anhydrase 4, a member of a large family of enzymes implicated in sensing, acting on, and

Figure 2.2

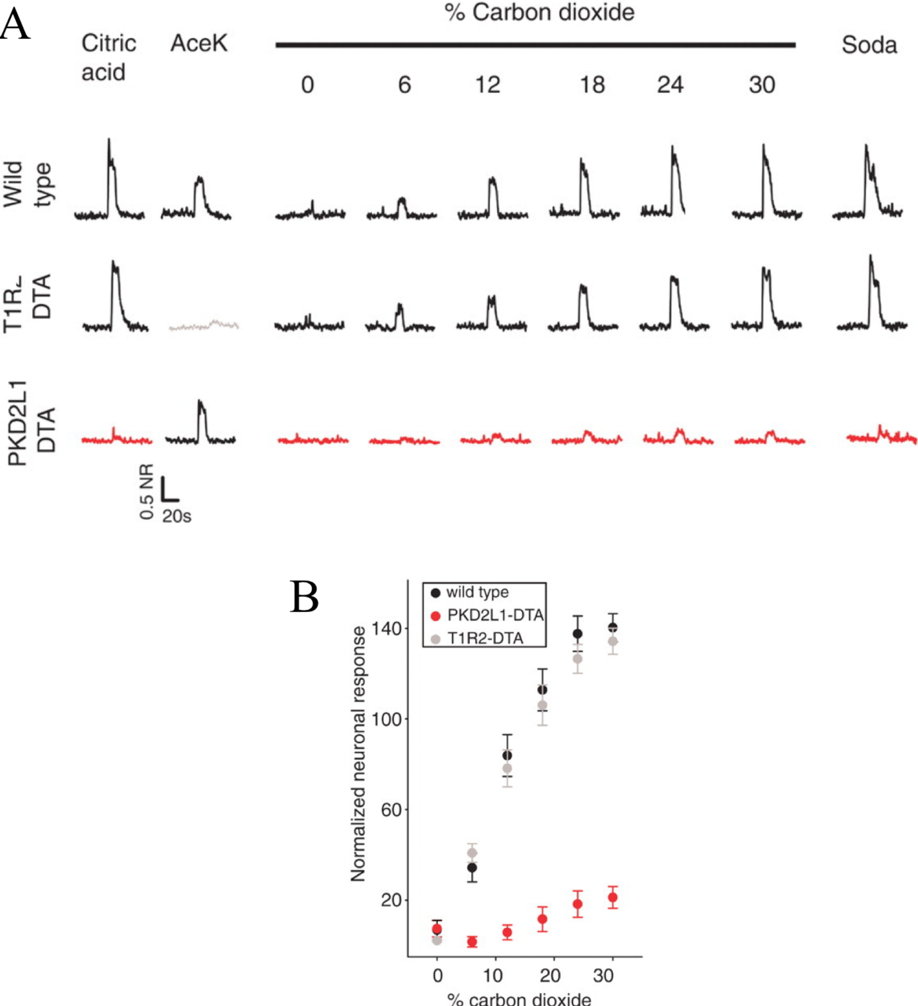


Figure 2.2
PKD2L1-expressing cells mediate taste responses to carbonation. (A) Dose response to CO_2 in wild-type mice or in animals lacking sour-sensing cells (PKD2L1-DTA) and in control animals lacking sweet-sensing cells (T1R2-DTA). (B) Quantitation of carbon dioxide responses in wild-type (n = 6), T1R2-DTA (n = 4), and PKD2L1-DTA (n = 5) animals. Values are means ± SEM of normalized chorda tympani responses.

Figure 2.3

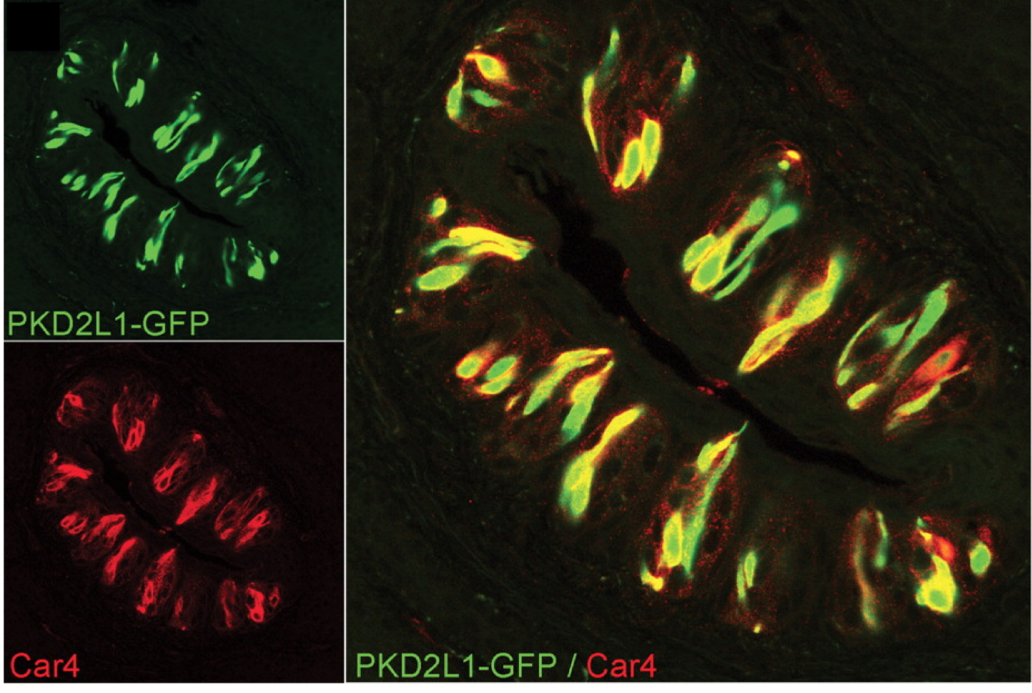


Figure 2.3
Selective localization of carbonic anhydrase 4 to sour cells.
Immunohistochemical staining of Car4 expression (lower panel, red) in taste buds of transgenic mice in which sour-sensing cells were marked by GFP fluorescence (PKD2L1-GFP; upper panel, green); large panel shows the superimposed double labeling.

responding to CO_2 in various systems, including chemosensation (Simons et al., 1999; Hu et al., 2007; Lahiri and Forster, 2003).

Carbonic anhydrases (CAs) reversibly catalyze the conversion of CO_2 into bicarbonate ions and free protons (Supuran, 2008; Sly and Hu, 1995). Car4 is a mammalian carbonic anhydrase that functions as an extracellular, glycosylphosphatidylinositol–anchored enzyme (Sly and Hu, 1995; Okuyama et al., 1995). To determine the contribution of Car4 to sensation of CO_2, we examined taste responses in mice in which the Car4 gene had been genetically ablated (Shah et al., 2005). Gustatory responses to CO_2 in the chorda tympani nerve were indeed severely reduced in mutants, whereas responses to other taste stimuli, including sour, were unaltered (Figures 2.4 and 2.7). Thus, Car4 is selectively required for taste reception of carbonation.

2.5 Mechanism of Car4 Mediated CO_2 Reception

How does CO_2 activate the taste system? Bicarbonate does not stimulate TRCs (Figure 2.5); thus pointing to protons as the relevant signal. Each of the basic taste modalities is mediated by distinct TRCs, with taste at the periphery proposed to be encoded via labeled lines [i.e., a sweet line, a sour line, a bitter line, etc. (Chandrashekar et al., 2006)]. Given that Car4 is specifically tethered to the surface of sour-sensing cells, and thus ideally poised to provide a highly localized acid signal to the sour TRCs, we reasoned that carbonation might be sensed through activation of the sour-labeled line. A prediction of this postulate is that prevention of sour cell activation should eliminate CO_2

Figure 2.4

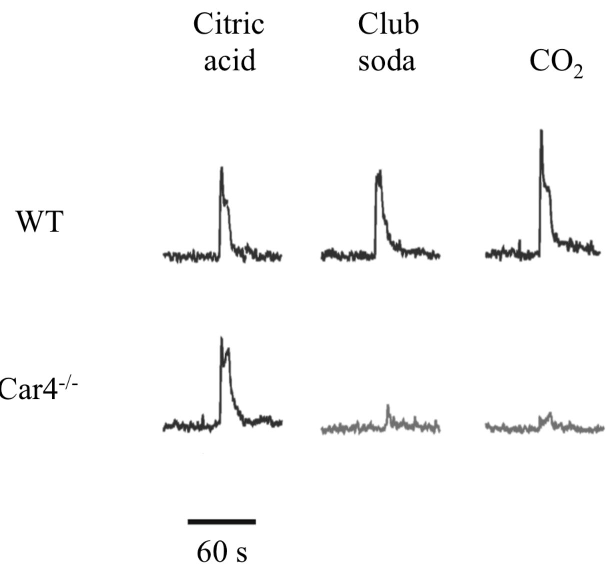


Figure 2.4
Requirement of carbonic anhydrase 4 for taste responses to CO_2.
Representative integrated chorda tympani responses to citric acid and aqueous or gaseous CO_2 stimulation in wild-type or Car4$^{-/-}$ animals. Responses to sweet, bitter, sour salty and umami were not significantly different from wild type in the Car4$^{-/-}$ animals (see figure 2.7).

Figure 2.5

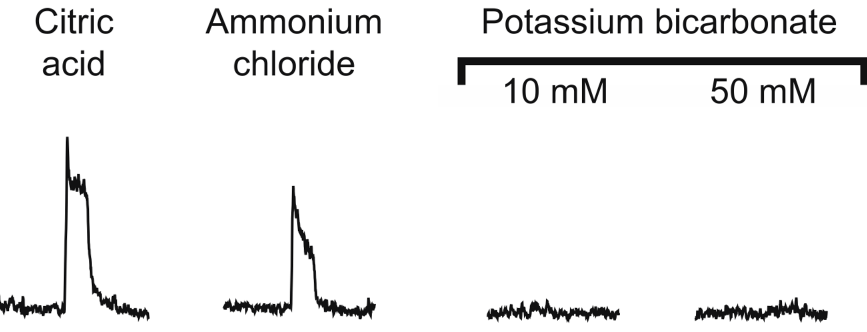


Figure 2.5
Bicarbonate does not elicit taste responses. Potassium bicarbonate does not elicit any taste response in the chorda tympani nerve; for comparison responses to 50 mM citric acid and 100 mM ammonium chloride are shown.

detection, even in the presence of wild-type Car4 function. To test this hypothesis, we engineered animals in which the activation of nerve fibers innervating sour-sensing cells was blocked by preventing neurotransmitter release from the PKD2L1-expressing TRCs. In essence, we transgenically targeted expression of tetanus toxin light chain [TeNT, an endopeptidase that removes an essential component of the synaptic machinery (Yamamoto et al.; Yu et. al, 2004; Zhang et al., 2008) *34–36*)] to sour-sensing TRCs, and then monitored the physiological responses of these mice to sweet, sour, bitter, salty, umami and CO_2 stimulation. As predicted, taste responses to sour stimuli were selectively affected, whereas responses to sweet, bitter, salty and umami tastants remained unaltered (Figures 2.6 and 2.7). However, these animals also displayed a dramatic loss of taste responses to CO_2 even though they still expressed Car4 on the surface of PKD2L1 cells. Together, these results implicate the extracellular generation of protons, rather than intracellular acidification (Lyall et al., 2001), as the primary signal that mediates the taste of CO_2, and demonstrate that sour cells not only provide the membrane anchor for Car4 but also serve as cellular sensors for carbonation.

2.6 Discussion

Why do animals need gustatory CO_2 sensing? CO_2 detection could have evolved as a mechanism to recognize CO_2-producing sources (Hu et al., 2007; Suh et al., 2004); for instance, to avoid fermenting foods. This view would be consistent with the recent

Figure 2.6

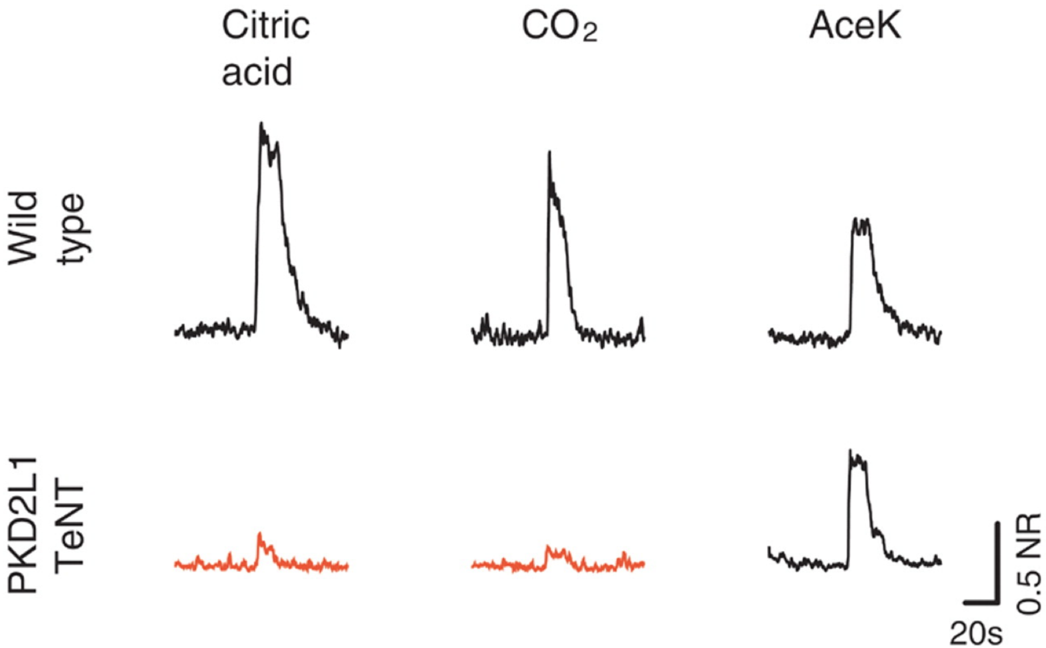


Figure 2.6
Requirement of PKD2L1-sour cells for the taste of carbonation.
Representative integrated chorda tympani responses to sour, sweet, and CO2 stimuli in wild-type mice or in animals expressing tetanus toxin (TeNT) in PKD2L1 sour-sensing TRCs.

Figure 2.7

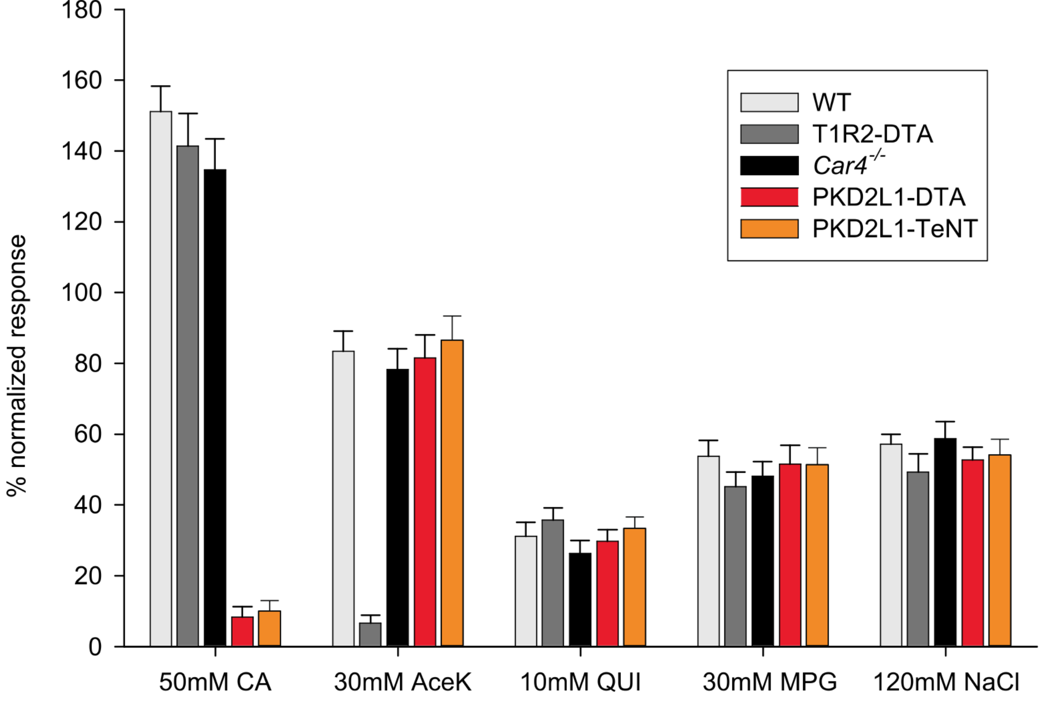


Figure 2.7
Chorda tympani responses to sour, sweet, bitter, umami and salty stimuli.
Average responses across all five taste modalities for each genotype used in this study. The values are mean±s.e.m. (n=4), normalized to response for 250mM NaCl. CA = Citric Acid; AceK = Acesulfame Potassium; QUI = Quinine; MPG = Monopotassium Glutamate; NaCl = Sodium Chloride.

discovery of a specialized CO_2 taste detection in insects where it mediates robust innate taste behaviors (Fischler et al., 2007). Alternatively, Car4 may be important to maintain the pH balance within taste buds, and might gratuitously function as a detector for carbonation only as an accidental consequence. Notably, mice lacking Car4 from birth do not have any detectable deficits in taste response to any of the primary taste qualities, including sour (Figure 2.7).

How does CO_2 activate sour sensing? Given our current understanding of Car4 function and sour TRC physiology, I propose the following model (Figure 2.8): 1) Car4 catalyzes hydration of CO_2, producing bicarbonate and proton. 2) Protons produced at the extracellular surface activate the sour TRC through its acid sensing mechanism. Electrophysiological studies of isolated sour cells support a key role for a depolarizing proton conductance (Chang et al., 2010). 3) Depolarization opens voltage dependent sodium and calcium channels, generating action potentials and calcium influx into the cytoplasm (Chang et al., 2010; Roberts et al., 2009). 4) Calcium entry initiates release of ATP containing vesicles, signaling to primary afferents through P2x3/P2x3 purinergic receptors (Finger et al., 2005).

What is the molecular receptor for Car4 generated protons? The molecular identity of the sour receptor remains unclear. Although proton uncaging experiments support a role for an unidentified apical proton permeable channel (Chang et al., 2010), it has also been argued that intracellular acidification is the proximate stimulus for sour taste (Lyall et al., 2001). However, the results outlined here demonstrate that specific inhibition of extracellular proton production on the tongue, by genetic ablation of Car4, is

Figure 2.8

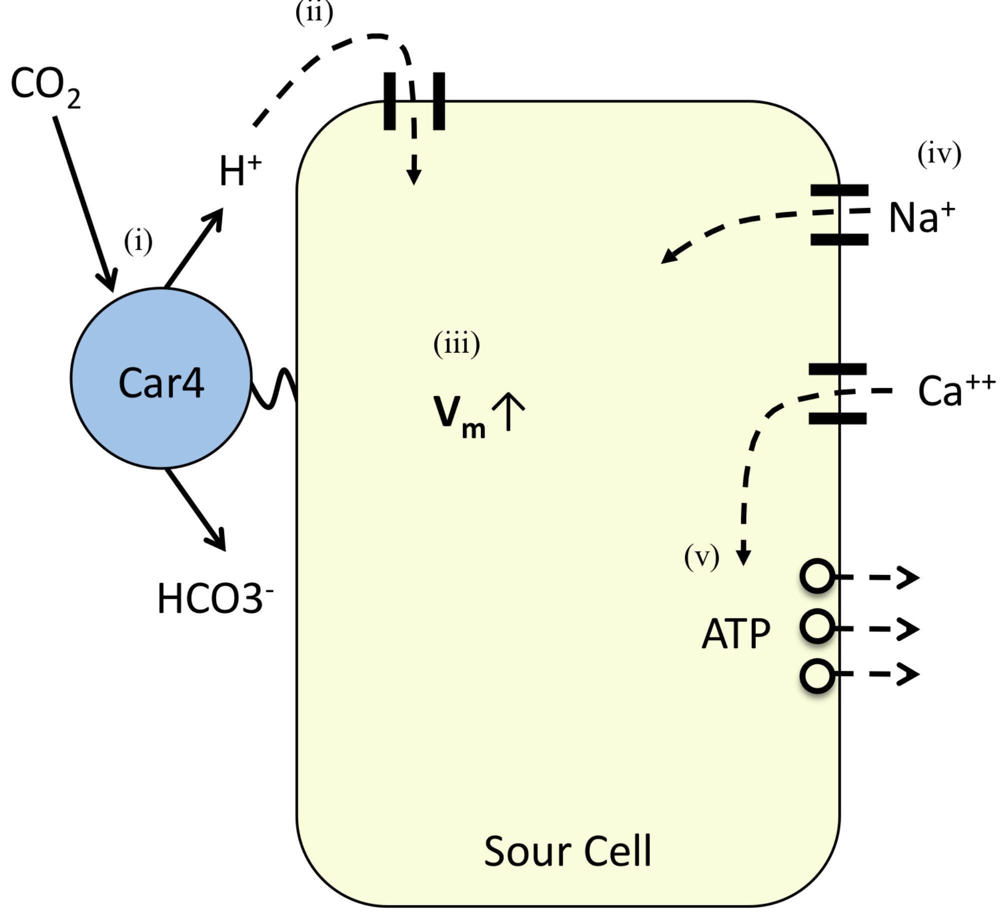


Fig. 2.8
Model of CO_2 reception by sour taste receptor cells. The taste response to CO_2 is dependent on Car4 and synaptic transmission from Pkd2l1-expressing sour cells. I propose the following mechanism of CO_2 transduction: i) Membrane tethered Car4 catalyzes the hydration of CO_2, yielding bicarbonate and a proton. ii) Protons produced at the cell membrane enter the cell through an apical proton conductance, thus (iii) depolarizing membrane potential. Continued depolarization generates action potentials through voltage dependent sodium channels (iv), and calcium influx through voltage activated calcium channels, initiating calcium dependent release of neurotransmitter (ATP) onto afferent terminals (v) .

sufficient to inhibit the activation of sour cells. This is most consistent with molecular transduction of sour taste occurring outside the plasma membrane of the sour TRC, a model supported by proton uncaging experiments (Chang et al., 2010). Thus, the molecular machinery for sour transduction most likely includes proteins localized to the plasma membrane, an important clue for future efforts to identify this elusive receptor.

Is Car4 itself a CO_2 receptor? Canonical sensory receptors like the T1R and T2R taste receptors change conformation upon binding a ligand, initiating a series of signal transduction events inside the cell. Rather than transmitting a signal across the plasma membrane, Car4 accelerates a chemical reaction outside the cell, thereby eliciting a cellular response by altering the extracellular environment. Thus, Car4 might be most accurately described as a sensory 'transponder' molecule, as opposed to receptor. (Frommer, 2010).

Although CO_2 activates the sour-sensing cells, it does not simply taste sour to humans (Cowart, 1998). CO_2 (like acid) acts not only on the taste system but also in other orosensory pathways, including robust stimulation of the somatosensory system (Simons et al., 199;, Komai and Bryant, 1993); thus, the final percept of carbonation is likely to be a combination of multiple sensory inputs. Nonetheless, the "fizz" and "tingle" of carbonated water is often likened to mild acid stimulation of the tongue, and in some cultures seltzer is even named for its salient sour taste (e.g., saurer Sprudel or Sauerwasser).

2.7 Experimental Procedures

Mouse strains and immunostaining

Car4$^{-/-}$-mice (Shah et al., 2005) and *Rosa26-flox-STOP-TeNT* (Zhang et al., 2008) were as described; PKD2L1-TeNT mice (Figure 4) expressed TeNT in more than 90% of PKD2L1 cells (data not shown). All other mice including double positive Cre-driver/reporter lines PKD2L1-DTA (sourless),PKD2L1-GFP and T1R2-DTA (sweet-less) animals were as described and characterized before (Huang et al., 2006). Antibodies to PKD2L1 (Huang et al., 2006) and Car4 (Waheed et al., 1992) were described previously; immunocytochemistry used standard techniques (Huang et al., 2006; Zhang et al., 2003). Images were obtained using a Leica SP2 TSC confocal microscope; 1-2 μm optical sections were recorded to ensure that any overlapping signal originated from single cells.

Identification of Car4 as a candidate CO_2 receptor

We used a strategy that combined bioinformatics, differential gene array screening, and in situ hybridizations to identify candidate CO_2 receptors. Taste buds from control mice, from GFP-labeled TRCs, and from mice lacking sour cells (PKD2L1-DTA animals) were used to prepare cDNA for hybridization to Affymetric Mouse Gene 1.0 ST microarrays. Bioinformatic analysis of genes containing at least 1 transmembrane domain (Gene Ontology Cellular Component 0016020) identified *Car4* as the most dramatically underexpressed gene in the sample derived from PKD2L1-DTA taste tissue (>20 fold reduced signal relative to wild type). Examination of expression of other membrane-

bound carbonic anhydrases (Car9 and Car14) showed no significant expression in PKD2L1-expressing cells. Further details of the screen are included in Appendix A of this dissertation.

Nerve Recording and Stimulus Delivery

Lingual stimulation and recording procedures were performed as previously described (Nelson et al., 2002; Zhao et al., 2003). All data analyses used the integrated response over a 15 s period immediately after the application of the stimulus. Tastants used for nerve recordings were: 30mM, 60mM acesulfameK (AceK); 30mM mono potassium glutamate + 1mM inosine mono phosphate (Glu); 10mM quinine hydrochloride (Qui); 120mM, 250mM sodium chloride (NaCl); 10mM, 50mM citric acid. Carbonated solutions were made using a Soda-Club home soda maker and gaseous CO_2 stimulation was done at a constant flow rate by mixing different ratios of air and CO_2. The mean response to 250mM NaCl was used to normalize responses to each experimental series in the wild type, PKD2L1-DTA/TeNT, T1R2-DTA and *Car4*-/- animals.

Chapter 3: Carbonation is a Multi-Modal Taste Stimulus

3.1 Abstract of this Chapter

Acid and CO_2 each stimulate a common gustatory pathway, yet elicit distinct sensory percepts. Notably, a small gustatory neural response to carbonation remains even in the absence of signaling from sour-sensing TRCs (Figures 2.2 and 2.6). To investigate whether additional gustatory pathways contributed to the taste of carbonation sensing, I used an in vivo calcium imaging assay to define the ensemble of primary gustatory ganglion neurons activated by CO_2. These studies revealed that in addition to robust activation of acid sensing neurons, CO_2 also activates sub-populations of bitter, sweet and umami sensing neurons. These responses are carbonic anhydrase dependent, suggesting a a role for additional carbonic anhydrases in the response to CO_2. Indeed, I identified carbonic anhydrase VII as an intracellular carbonic anhydrase specifically expressed by sweet, bitter and umami sensing taste receptor cells. Pharmacological and gene expression data suggest a role for Car7 in transducing CO_2 in TRCs. These studies illustrate that a singular molecular species is capable of broadly activating multiple TRC populations, and support a widespread role for carbonic anhydrase activity in CO_2 sensing.

3.2 Introduction

As discussed in the previous chapter, acid and carbonation are both detected by Pkd2l1-expressing TRCs, yet the sensory percepts evoked by each of these stimuli are clearly distinguishable. Although this may be explained in part by differential recruitment of other sensory modalities (e.g.. olfaction and somatosensation), another possibility is that additional gustatory pathways, not sensitive to acid, are recruited by carbonation. Consistent with distinct sensory quality, functional imaging in human subjects demonstrates differential activation of insular (taste) cortex by CO_2 versus acid (Di Salle, 2013). Furthermore, psychophysical studies suggest that although CO_2 and acid have overlapping characteristics, with CO_2 displaying a more complex taste profile. (Cowart et al., 1998). In this chapter, I explore the contribution of additional taste pathways to the unique flavor of carbonation.

3.3 Residual Response to CO_2 in the Absence of Sour Signaling

If Pkd2l1-expressing TRCs were the sole detectors of CO_2 in the gustatory system, we would expect ablation or silencing of Pkd2l1-expressing TRC to eliminate CO_2 responses completely. However, we observed small but clearly distinguishable increases in firing rate of the chorda tympani nerve in response to CO_2 in animals engineered to eliminate sour signaling (Figure 2.2, 2.6). At high CO_2 concentrations this corresponded to ~15% of the response observed in wild type mice. Where does this residual response originate? Is it simply an experimental artifact (e.g. incomplete genetic ablation or silencing of Pkd2l1-expressing TRCs), or does it represent CO_2 evoked activity in additional populations of TRC? As bulk extracellular recording lacks the

resolution to address this question directly, I employed an alternate assay capable of identifying the source of gustatory responses with greater precision and specificity.

3.4 Experimental Strategy

Ideally, we could apply an *in vivo* physiological assay to directly monitor the responses of TRC to various taste stimuli on the tongue, thus determining the tuning properties of individual TRCs to prototypical tastants. However, my efforts to develop an *in vivo* platform for calcium imaging of TRC responses failed to produce a reliable functional assay. As an alternative approach to characterize the peripheral gustatory response to CO_2 *in vivo*, I chose to assay the responses of primary gustatory neurons in the geniculate ganglia of anesthetized mice.

All taste information from the tongue passes through either the geniculate or petrosal cranial ganglia on its way to the brainstem. The geniculate ganglion receives taste information from fungiform papillae on the anterior tongue, from the anterior-most of the foliate papillae, and from the palate. Importantly, this means that the geniculate ganglion receives inputs from all five classes of TRC. ENaC expressing sodium sensing cells are not present in taste buds in the posterior tongue, therefore the petrosal ganglion does not process this information (Chandrashekar et al., 2010). Previous studies employing single unit extracellular recording from cell bodies and fibers of the geniculate ganglion support the existence of neurons tuned to single taste modalities, as well as more broadly tuned neurons (Frank et al., 2008). I reasoned that narrowly tuned neurons must receive input from a single class of TRC (e.g. T2R-expressing bitter receptors,

Pkd2l1-expressing sour receptors), and therefore each such neuron could serve as a readout for the activity and tuning properties of the corresponding TRC population on the tongue.

3.5 Imaging Assay for Primary Taste Responses

Previously, a number of studies have examined response properties of primary taste neurons via single fiber electrophysiology of gustatory afferents (Frank et al., 2008). Applied to rodents, this approach limits data acquisition on average to one or two responsive neurons per animal (e.g. Lundy and Contreras 1999). In order to survey taste responses over a representative population, I chose to employ a novel calcium imaging strategy to examine coding of carbonation. Neuronal calcium imaging has the advantage over electrophysiology of allowing multiplex characterization of large neuronal populations simultaneously (Svoboda et al., 1997, Chen et al., 2011).

Two technical requirements for calcium imaging are: 1) Introduction of a calcium indicator into the target neurons, and 2) Optical access to the area of interest. To target calcium indicator to the geniculate ganglion I utilized mice expressing the genetically encoded calcium indicators GCaMP3 or GCaMP6s, introduced either by transgenesis or by viral infection with a modified adeno-associated virus (Tian et al., 2009; Chen et al., 2013; Peel and Klein, 2000). As the geniculate ganglion is located deep within the skull, optical access was achieved by implantation of a microendoscope on the ventral surface of the ganglion, allowing visualization of indicator-expressing neurons by 2-photon microscopy (Barretto et al., 2009). This configuration permitted efficient optical

recording of neural responses to taste stimuli perfused into the oral cavity of the anesthetized mouse. (Figure 3.1)

3.6 Taste Coding in the Geniculate Ganglion

In order to identify which labeled lines are activated by CO_2, I first needed to determine how sweet, sour, bitter, salty and umami are represented at the level of the geniculate ganglia. I surveyed the tuning of GCaMP3 expressing geniculate ganglia neurons with a panel of prototypical tastants representing the five basic taste qualities. I observed that the majority of GCaMP3 expressing neurons are narrowly tuned, selectively responding to just one of the sweet, bitter, sour or salty classes of stimulus. These tuning properties suggest that the genetically defined populations of TRC connect to primary gustatory neurons in a predominantly 1:1 fashion. One notable exception is that most umami sensitive neurons also responded to a sweet stimulus, with umami-only neurons outnumbered 3:1 by sweet-umami. These doubly-tuned neurons may represent convergence at the ganglia of inputs from narrowly tuned sweet and umami TRC, or it might be a result of broadly tuned sweet/umami TRC on the tongue, as has recently been proposed (Kusuhara et al., 2013) (Fig 3.2). It is worth noting that sweet and umami are mediated by members of the same gene family (T1R1+T1R3 versus T1R2+T1R3).

A second apparent exception to strict labeled-line coding was that many (43%) of the bitter sensing neurons assayed with the five tastant panel responded to the sour stimulus as well. These neurons could receive convergent input from sour and bitter

Figure 3.1

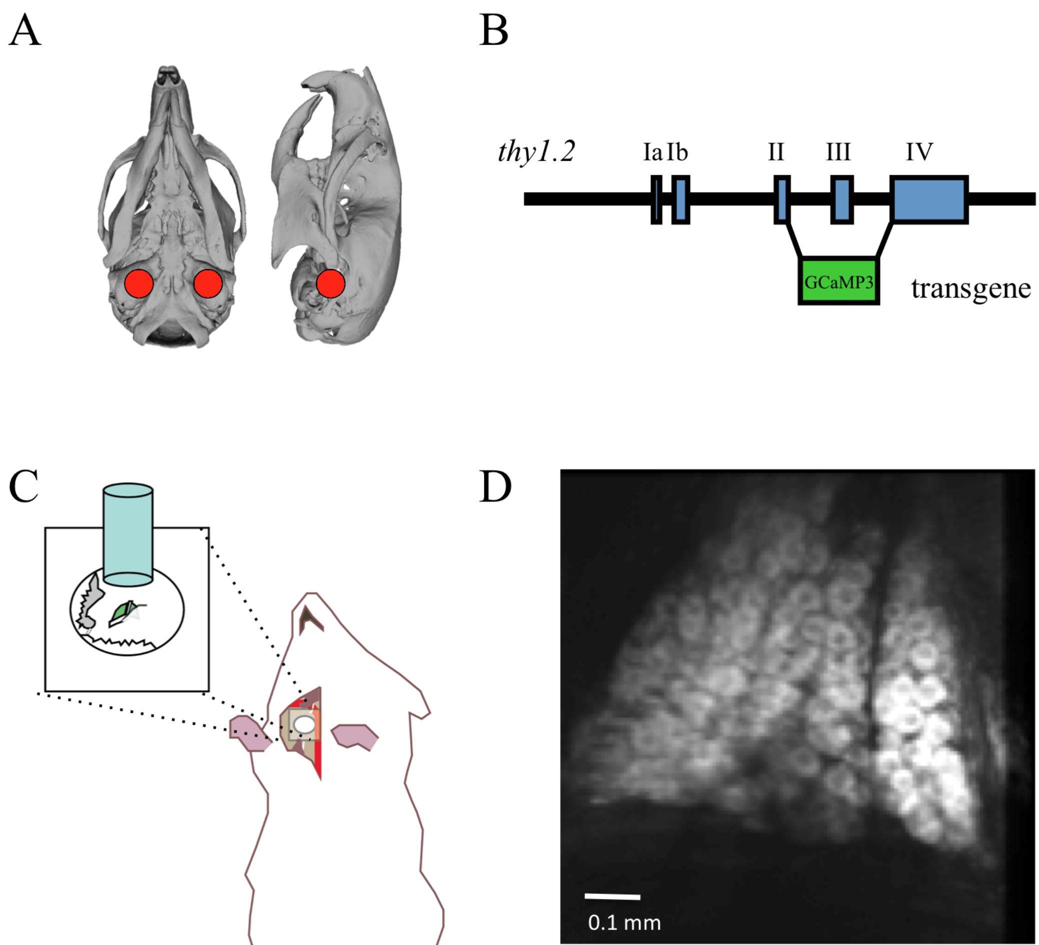


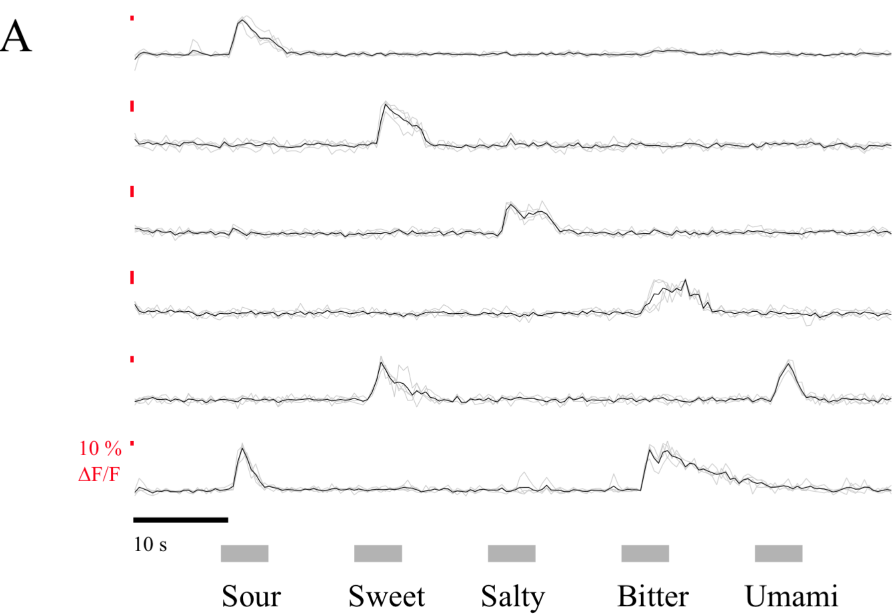
Figure 3.1
Preparation for calcium imaging of geniculate ganglion. The geniculate ganglia (red circles in A) are located within the tympanic bulla, deep within the skull. We delivered the genetically encoded calcium sensor GCaMP3 by generating transgenic mice with a construct driving expression under the control of Thy1 regulatory sequence (B). GCaMP expressing ganglia were exposed from the ventral side, allowing implantation of an endoscopic lens (blue cylinder in C, inset). In (D), an example field of view is shown, visualized by two-photon microendoscopy in an anesthetized animal. Cell bodies appear as donut shapes due to nuclear exclusion of GCaMP.

Figure 3.2

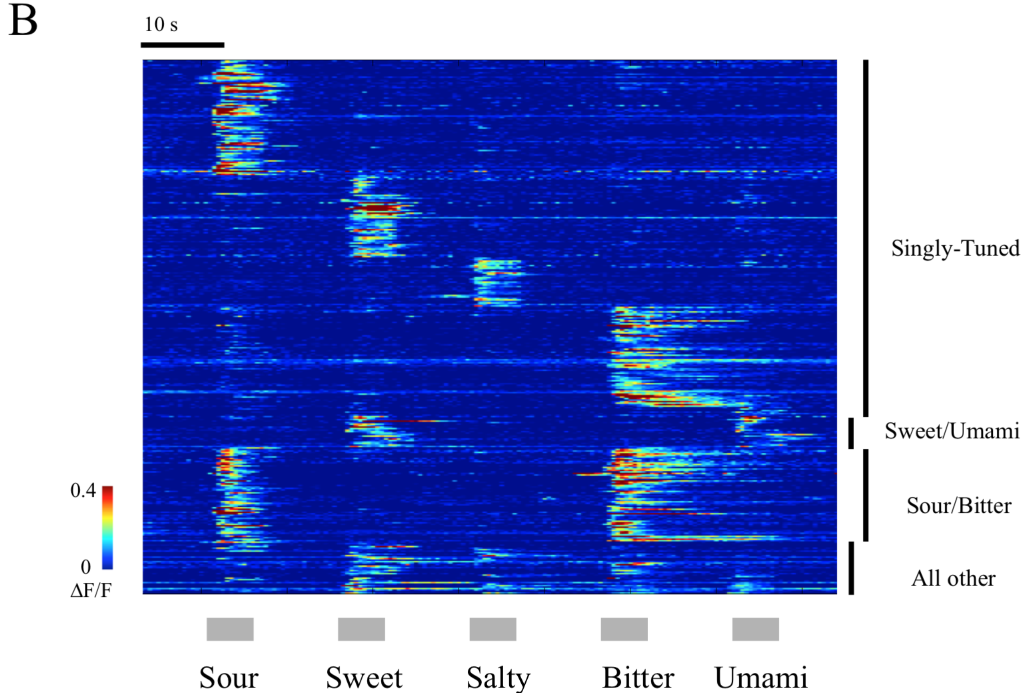
Figure 3.2
Coding of taste quality in geniculate ganglion neurons. Calcium imaging of geniculate taste neurons *in vivo* reveals that they are predominantly tuned to sour, sweet, salty, bitter, sweet-umami or sour-bitter. Panel (A) depicts examples of high signal-to-noise fluorescence time courses for each of these categories of neuron. The trace in black is the mean response averaged across trials; it is overlaid on four individual trial traces shown in grey. Responses are highly consistent across trials. Red bars represents 10% $\Delta F/F$ and grey bars below the bottom trace indicate each stimulus delivery window. B) Automated classification of response profiles for primary taste neurons supports the existence of 'labeled lines' encoding sour, salty, sweet and bitter taste. Each row represents the averaged fluorescence of a single GCaMP3 expressing taste neuron in the geniculate ganglia.

Figure 3.2

A



B



TRCs, or alternately represent the activation of T2R bitter cells by acid stimuli. To distinguish between these possibilities, I imaged taste responses in mice in which Pkd2l1 expressing cells had been ablated by transgenic expression of diphtheria toxin A fragment (DTA) (Huang et al., 2006). indeed, a substantial population of acid responsive neurons remained in these mice, despite complete loss of Car4-expressing sour TRCs on the tongue (Figure 3.3a,b). Notably, nearly all of these responses derive from bitter cells (Figure 3.3c). This strongly suggests that a subpopulation of T2R expressing cells are themselves acid sensitive, perhaps through one or more acid-sensitive T2R GPCRs. This would explain bitter/sour responses in manner consistent with labeled-line coding.

Taken together, the singly tuned, sweet-umami and bitter-sour categories account for 90% of all taste-responsive neurons. Small populations were distributed across the remaining categories. However, as the great majority of geniculate neurons are unambiguously singly tuned, the calcium imaging assay provides an efficient means to determine the output of bitter, sweet, salty and sour sensing TRC on the tongue.

3.7 CO_2 Activates Multiple Populations of Taste Neurons

Next, I identifed and characterizied responses to CO_2 in the geniculate ganglion. I observed robust activation of geniculate ganglion neurons by stimulation with carbonation. As expected, sour neurons responded robustly to carbonation (Figure 3.4a). The amplitudes of CO_2 responses in sour neurons were on average 87.1% ± 3.0% (s.e.m) of the calcium response to 50 mM citric acid, a strongly sour taste stimulus. Consistent

Figure 3.3

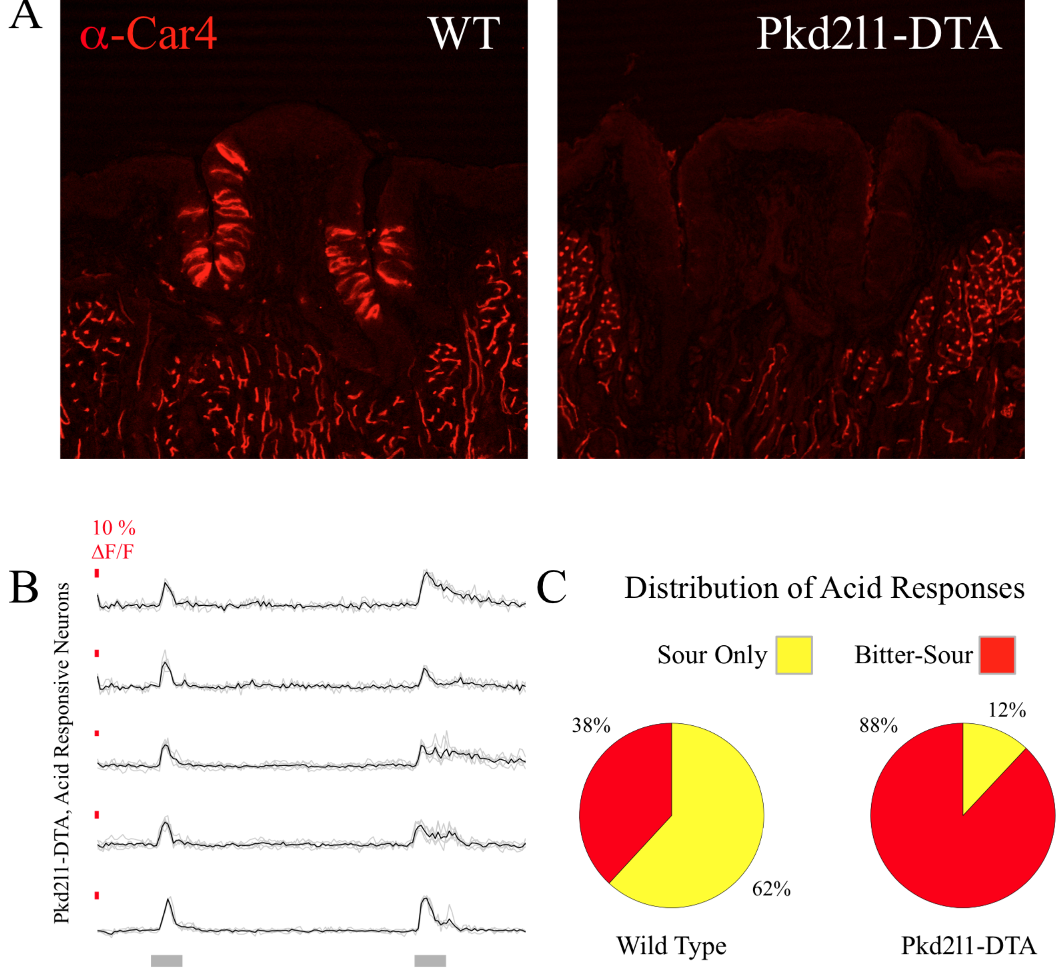


Figure 3.3
Acid sensing remains after ablation of Pkd2l1-expressing cells. Mice lacking sour TRC were generated by crossing a Pkd2l1-Cre line to a conditional diphtheria toxin (Pkd2l1-DTA). Loss of sour TRC was confirmed by immunochemistry for Car4 (A). These mice retained responses to acid (B). The proportion of all sour responsive that exhibited bitter sensitivity was greatly increased in this genotype (C). N= 118 cells in wild type, 50 in Pkd2l1-DTA

Figure 3.4

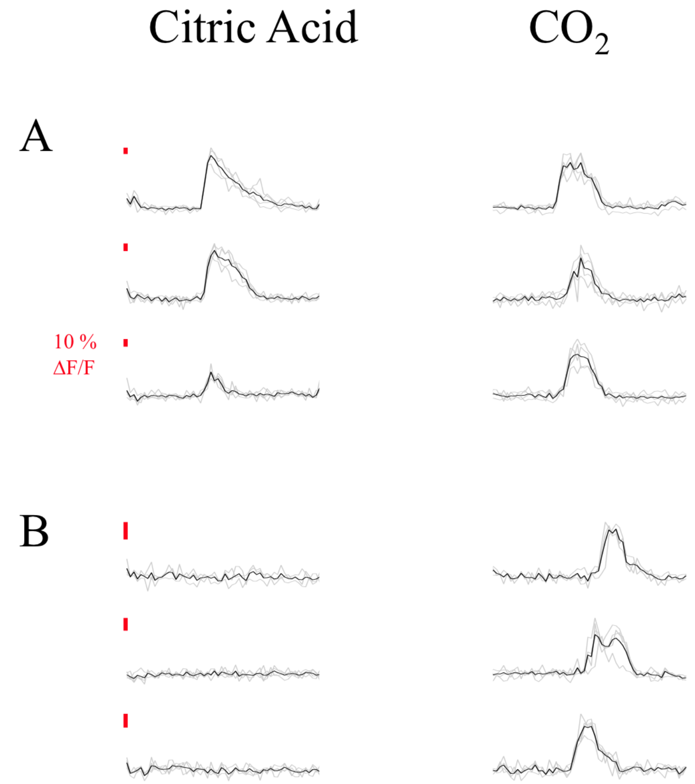


Figure 3.4
Both sour and non-sour neurons respond to CO_2. Examples of calcium responses to carbonation and acid in sour (A) and non-sour (B) neurons. Responses to carbonation were observed in 41 of 42 acid sensitive neurons and 36 of 67 acid insensitive neurons in the geniculate ganglia of GCaMP3 expressing mice.

with the CO_2 response in extracellular recordings from the chorda tympani nerve, these responses were suppressed by inhibition of carbonic anhydrase (Figure 3.6a). Unexpectedly, I also observed that carbonation elicited robust responses in a substantial set of neurons outside of the sour population (Figure 3.4b).

To characterize the identity of these novel CO_2 responsive neurons with maximal sensitivity, I conducted imaging experiments in mice expressing GCaMP6s, the most sensitive genetically encoded calcium indicator currently available (Chen et al., 2013). The overall tuning properties of geniculate ganglia neurons assayed with GCaMP6s are consistent with those determined using GCaMP3 imaging (i.e. singly tuned, together with sour/bitter and sweet/umami double responders make up 85% of all responsive neurons). Sour neurons were detected relatively less frequently; this may be due to high basal activity of acid sensing neurons saturating GCaMP6s response at baseline (Breza et al., 2010).

Imaging of taste responses to carbonation with GCaMP6s confirmed widespread activation of otherwise acid-insensitive neurons by CO_2. Strikingly, all such neurons were responsive to either bitter or sweet (Figure 3.5a,c). A high proportion of bitter neurons (84%) responded to CO_2, as compared to just over half (53%) of the sweet and sweet-umami responsive neurons. Notably, the population of salt-responsive neurons was unresponsive to the carbonation stimulus, with no responses observed across salty tuned cells identified by GCaMP6s imaging (Figure 3.5b). The insensitivity of salty neurons to carbonation suggests that a specific cellular mechanism is required to explain the activity evoked in the sweet and bitter taste populations by CO_2.

Figure 3.5

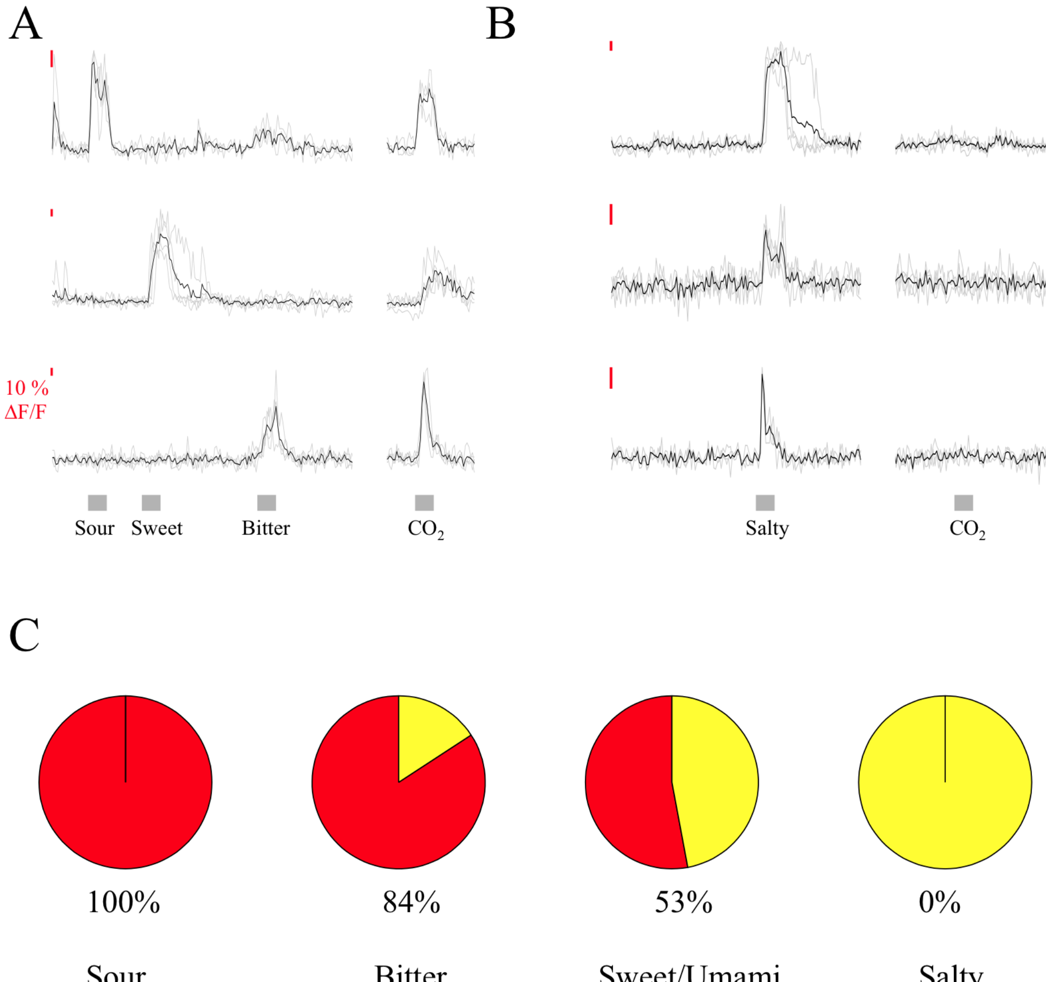


Figure 3.5
CO_2 stimulates multiple taste pathways. Taste neurons expressing GCaMP6s were assayed for responses to carbonation. In addition to sour, sweet and bitter selective taste neurons respond to carbonation as well (A) whereas salt sensing neurons do not (B). CO_2 sensitivity is distributed across sour, bitter and sweet/umami neurons, as quantified in (C). N=7 sour, 38 bitter, 34 sweet/umami and 12 salty neurons

3.8 A Candidate Transducer for the Secondary Carbonation Pathway

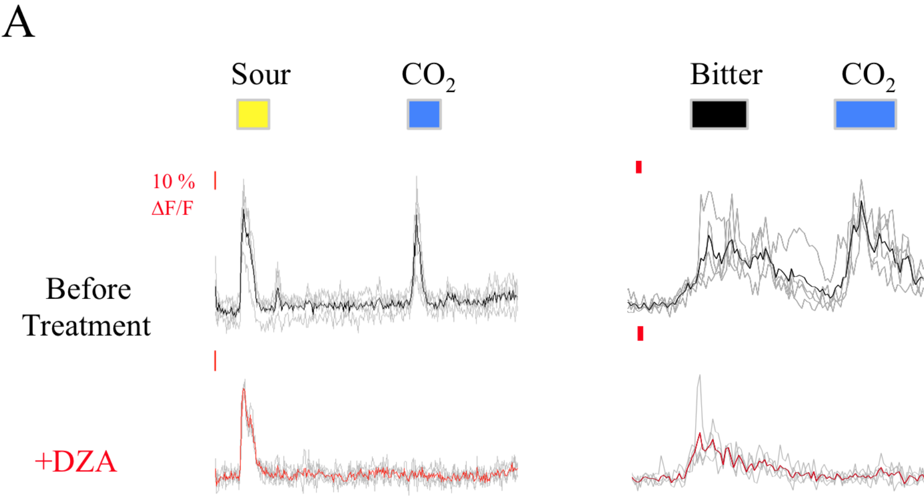
How might bitter, sweet and umami TRC respond to CO_2? Given that taste sensing of carbonation by sour cells relies on a carbonic anhydrase dependent mechanism, I wondered if the same could be true for the remaining CO_2 sensors. As noted before, Car4 knockout mice retain a small residual chorda tympani response to carbonation (Figure 3.6b). Importantly, this response is unaffected by a membrane impermeant inhibitor of carbonic anhydrase, arguing that no other extracellular carbonic anhydrases are involved in taste sensing of CO_2. However, the residual responses are completely eliminated by the membrane permeant carbonic anhydrase inhibitor dorzolamide (Figure 3.6a). This pharmacology suggests that intracellular carbonic anhydrase activity mediates the residual response to CO_2.

What carbonic anhydrases could contribute to Car4-independent taste sensation of CO_2? Several lines of evidence point to carbonic anhydrase VII (Car7), a soluble, cytosolic and catalytically active enzyme (Lakkis et al., 1996). By microarray analysis of gene expression, I identified Car7 as highly enriched in taste tissue as compared to the surrounding lingual epithelium, and significantly reduced in taste tissue lacking bitter, sweet and umami cells (see Appendix A). Furthermore, RNAseq profiling of fluorescently labeled bitter/sweet/umami cells or of sour cells confirmed that Car7 and Car4 are respectively highly selective for these two cell types, and are by far the most abundant carbonic anhydrase transcripts in TRCs (Figure 3.7a). By double label immunohistochemistry-*in situ* hybridization, Car7 expression precisely overlaps with a marker for the

Figure 3.6

Figure 3.6
Sour and non-sour CO_2 responses require carbonic anhydrase. Shown in (A) are GCaMP fluorescence traces from one sour neuron, on left, and one bitter neuron, on right. Both neurons respond to CO_2 (upper traces), but in each case the response is eliminated by topical blockade of carbonic anhydrase with dorzolamide (lower traces). Panel (B) illustrates the effect of carbonic anhydrase inhibition on whole-nerve response to CO_2 and citric acid, in wild type and Car4 KO animals. The membrane impermeant carbonic anhydrase inhibitor benzolamide mimics the effects of Car4 ablation in wild type mice (upper traces), but has no effect in the knockout (lower traces). The membrane permeant drug dorzolamide eliminates the residual response in the Car4 knockout, suggesting the involvement of intracellular carbonic anhydrase in gustatory CO_2 sensing. Neither drug affects responses to acid.

Figure 3.6



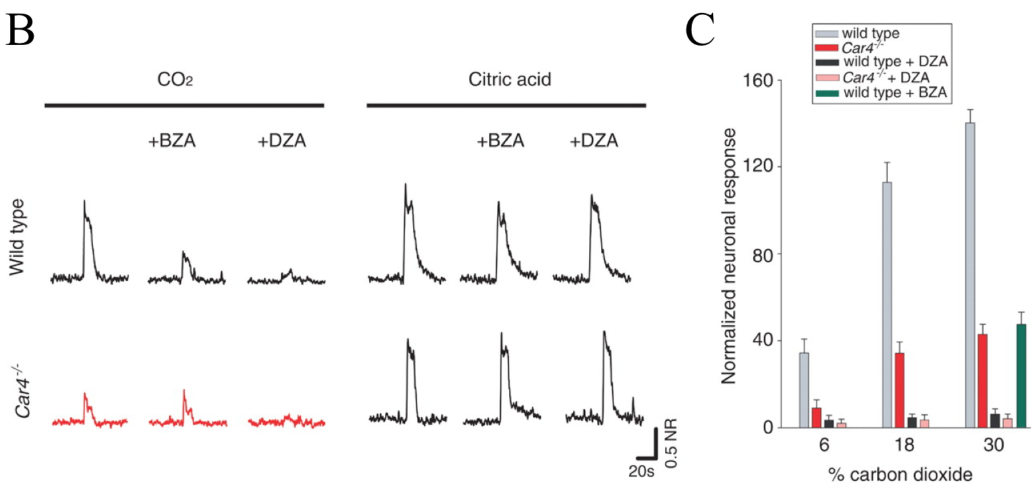
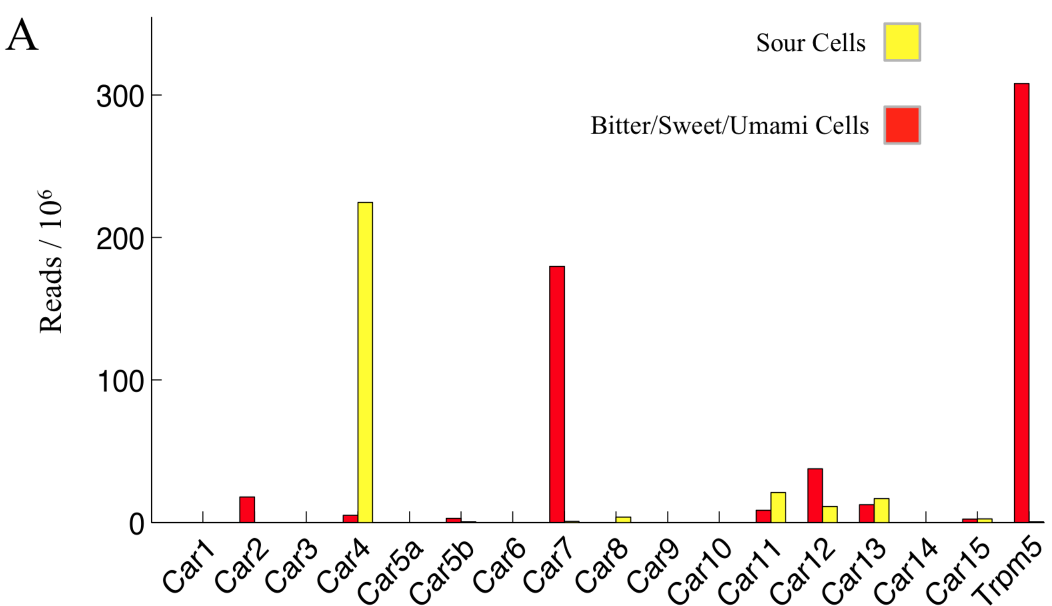


Figure 3.7

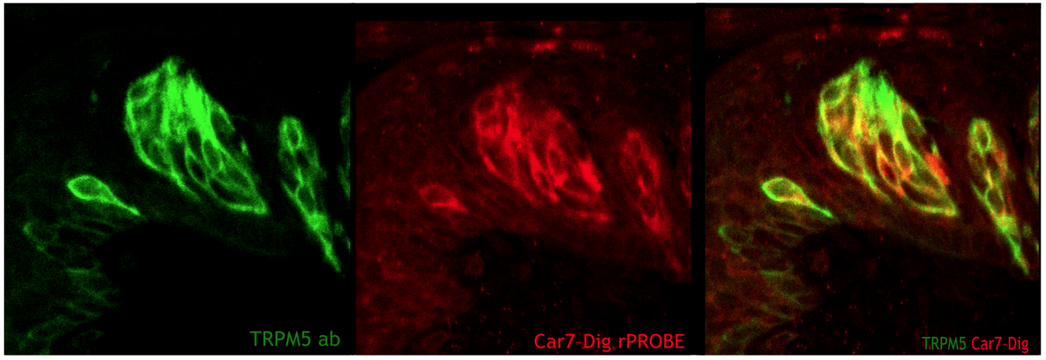
Figure 3.7
Car7 is expressed in the bitter/sweet/umami receptor population. Analysis of sequencing data from sorted TRCs identify Car7 as the principal carbonic anhydrase expressed in the population of bitter, sweet and umami sensing TRC, and confirm the selectivity of Car4 to sour sensing TRCs (A). Specific expression of Car7 is confirmed by precise overlap of Car7 mRNA detected by fluorescent *in situ* hybridization with immunoreactivity for Trpm5, a channel specifically expressed by bitter, sweet and umami cells (B).

Figure 3.7

A



B



bitter/sweet/umami population of TRCs (Figure 3.7b). Thus, Car7 is in the correct population of cells, and in the right subcellular location, to contribute to the secondary carbonation detection pathway.

3.9 Discussion

In the previous chapter, I concluded that taste sensation of carbonation occurs primarily through the sour sensing population of TRCs. Here, by examining carbonation sensing with a novel calcium imaging assay, I have been able to define with greater precision the ensemble of primary gustatory neurons that are stimulated by carbonation as compared to prototypical taste stimuli. These studies reveal that carbonation additionally activates both attractive (sweet) and aversive (bitter) labeled lines. The existence of these secondary pathways may provide an explanation for the observation that CO_2 is not perceived as an unambiguously 'sour' stimulus by human subjects (Cowart, 1998). Rather, the unique percept of carbonation is generated at the periphery by combinatorial activation of receptor populations.

A Second Carbonic Anhydrase Dependent Mechanism for CO_2 Sensing

How could the activity of an intracellular carbonic anhydrase contribute to CO_2 sensing? One possible mechanism is provided by olfactory sensation of CO_2, where bicarbonate produced by carbonic anhydrase II activates guanylyl cyclase D (GC-D) activity, opening cyclic nucleotide gated channels (Hu et al., 2007, Sun et al., 2009).

However, I found no evidence for GC-D expression in TRCs. Production of intracellular bicarbonate could alternatively drive a depolarizing bicarbonate current out of the cells through anion selective channels (Figure 3.8). This would be consistent with the role of Car7 in the developing nervous system, where it contributes to excitation through bicarbonate permeable GABA-A receptors (Ruusuvuori et al., 2013). It is also possible that intracellular acidification plays a role, perhaps through a mechanism similar to that proposed for carotid body chemoreceptors, in which acidification excites the cell by inhibiting hyperpolarizing potassium currents (Jonz and Nurse, 2012). Notably, bitter responsive neurons exhibited relatively greater sensitivity to carbonation than do sweet cells, suggesting that T2Rs might play a role in CO_2 sensing.

Novel aspects of taste coding

In my efforts to define the set of gustatory inputs activated by carbonation, I identified some additional unexpected features of taste coding at the periphery. Two observations are particularly noteworthy. First, the discovery of bitter cells sensitive to acid is unexpected. Previous studies relying on nerve recording did not detect residual acid sensitivity upon ablation of the Pkd2l1 cells, or find any effect on acid detection when bitter signaling is abolished (Huang et al., 2006; Zhang et al., 2003). This discrepancy highlights the sensitivity of the imaging approach as compared to bulk extracellular recording.

Figure 3.8

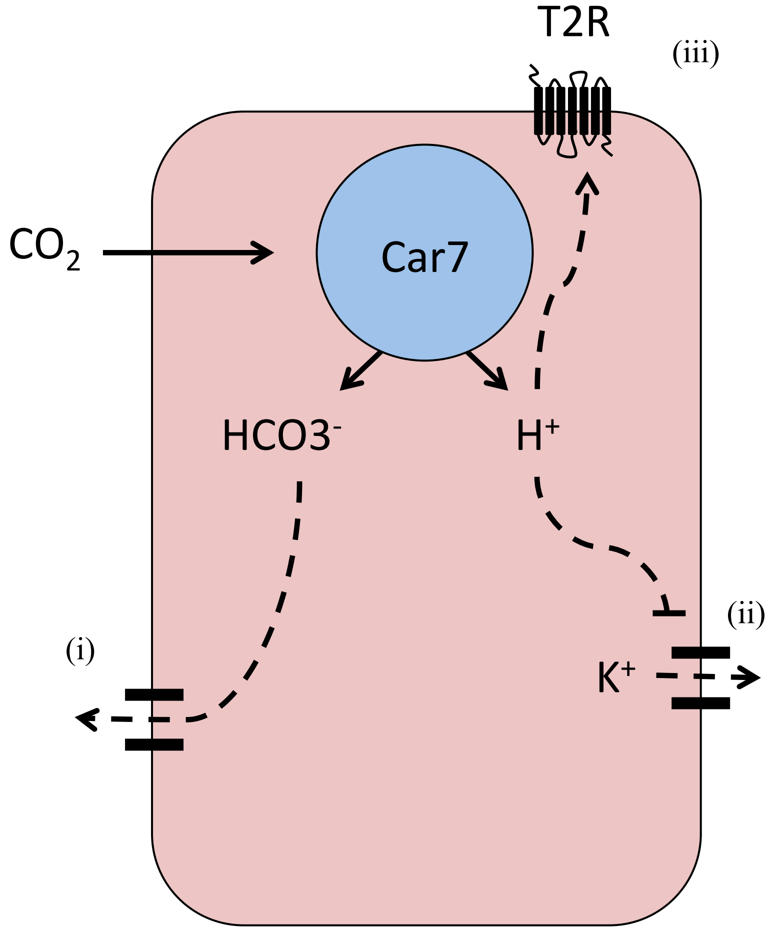


Figure 3.7
Hypothesized mechanisms for Car7 mediated CO_2 sensing. Carbonic anhydrase activity is required for CO_2 detection through sweet/bitter/umami TRC. Potentially, signal transduction could involve either or both of $HCO3^-$ and H+. Car7 could generate a depolarizing current through bicarbonate production, driving a depolarizing efflux of $HCO3^-$ through anion permeable channels (i). Alternatively, intracellular acidfication could inhibit an acid sensitive potassium leak conductance, as has been proposed for central chemoreceptors (ii). The bitter pathway is particularly sensitive to carbonation, and bitter neurons display acid sensitivity, suggesting Car7 generated protons could activate bitter TRC through direct activation of T2R GPCRs (iii).

The second notable finding is the apparent convergence of sweet and umami signaling onto doubly tuned primary gustatory neurons, together with the rarity of neurons singly tuned to umami. This is in contrast to the representation of these tastes on the tongue, where several lines of evidence suggest that separate populations of TRC are selective for sweet or for umami; perhaps most compelling, genetic ablation of T1R2 expressing TRCs eliminates neural responses to sweeteners while responses to umami stimuli remain intact (Chandrashekar et al., 2009). It will be interesting to address this disparity, and determine if there is indeed convergence of information from sweet and umami TRC at the level of the primary afferent. Importantly, the mouse appears capable of extracting an umami signal from the overlapping selectivity observed in the ganglia, as umami responses in the primary taste cortex are selective for only umami and not sweet (Chen et al., 2011).

3.10 Experimental Procedures

Transgenic Animals and Viral Gene Delivery

Thy1-GCaMP mice were generated by pronuclear injection of a construct in which GCaMP3 coding sequence (Tian et al., 2009) was cloned into the XhoI sites in the mouse Thy1 vector (Feng, 2000). Multiple founders were generated; progeny of a single founder displaying robust fluorescence in sensory neurons was used for all experiments.

Viral delivery of GCaMP6s was achieved by stereotaxic injection into the nucleus of the solitary tract. The virus used was AAV1-hSyn-GCaMP6s (UPenn Vector Core). Mice were allowed to recover for at least 2 weeks after injection before imaging.

Surgical Preparation

Experimental animals were anesthetized by intraperitoneal injection of Ketamine (100mg/Kg) and Xylazine (10mg/Kg), with booster injections applied as necessary to maintain surgical plane of anesthesia. Body temperature was monitored and maintained with a closed loop system. Mice were head fixed to a post with dental acrylic and then tracheotomized.

Surgery was performed under a stereoscope. The lower belly of the digastricus muscle was retracted caudally and the hypoglossal nerve was cut. The tympanic bulla was exposed by retracting overlying muscle. The ventral surface of the tympanic bulla was removed, after which the tensor tympani muscle was cut and retracted. The cochlea was exposed and aspirated, after which temporal bone directly above the ganglia was carefully removed. Upon visualization of the ganglia, a singlet microendoscopic probe (1 mM diameter, 0.42 pitch, 700µM working distance, GrinTech) was placed over the target and fixed in place with 2% low melting point agarose.

Calcium Imaging and Stimulation

Following endoscope implantation, mice were placed under an Olympus 20x LMPlanFl objective for imaging with an upright two-photon microscope (Prairie Technologies). GCaMP fluorescence was excited using a Ti:Sapphire laser tuned to 920nm. Images were acquired at 2 Hz at a resolution of 128 x 128 pixels. A polyethylene tube was inserted approximately 8mm into the oral cavity between the tongue and palate for tastant delivery. Taste solutions were presented at a constant flow rate of ~30ml/minute for 5 seconds, interleaved with 10 second washes of artificial saliva. All solutions were made up in artificial saliva. The following taste stimuli were used: 300mM sucrose (sweet), 50mM citric acid (sour), 100mM NaCl (salty) 5mM quinine (bitter) and 30mM monopotassium glutamate plus 1mM inosine monophosphate (umami). Solutions were carbonated using a Soda Club home soda maker. All taste solutions were made up in an artificial saliva buffer (5mM KCl, 3mM $NaHCO_3$, 3mM $KHCO_3$, 2mM NaCl, 250µM $CaCl_2$, 250µM $MgCl_2$, 120µM K_2HPO_4, 120µM KH_2PO_4, pH 7.4).

Pharmacology

Dorzolamide hydrochloride was dissolved 0.5% W/V in artificial saliva and applied to the tongue at 1ml/minute for 10 minutes.

Analysis of Calcium Imaging Data

Imaging time series were first corrected for in-plane motion by automated registration to the first frame collected for each field of view using the Turboreg plug-in

for ImageJ (Thevanaz et al., 1998). Fields of view were then manually segmented into regions of interest (ROIs) exhibiting changes in fluorescence, each corresponding to a single neuron. Subsequent processing and analysis was performed in Matlab. Overlap between ROIs was automatically parsed out. Raw traces were calculated as the average difference in fluorescence across each ROI from its average value, normalized to its average fluorescence to calculate $\Delta F/F$, and corrected for any drift in baseline due to photobleaching.

Responses of each ROI to each taste stimulus were quantified by maximal increase in fluorescence during tastant presentation and the following 2.5 seconds. Cells were scored as responsive to a tastant if maximal response reached a criterion of 3σ above median fluorescence in at least 75% of trials, where σ is estimated by: median absolute deviation for each trial, multiplied by 1.4826. Automatically classified traces were manually screened for discrepancies in response assignment. In cases where the cause for misclassification was clear (e.g. neurons in which the response to a bitter tastant extended into the presentation window of a subsequent umami stimulus), cells were reclassified; otherwise they were excluded from further analysis.

Double Label Immunohistochemistry/In Situ Hybridization

Double-label detection of TrpM5 and Car7 were performed essentially as described previously (Zhang et al., 2003). Digoxigenin labeled antisense probe targeting Car7 was detected by an alkaline phosphatase conjugated anti-digoxigenin antibody, and visualized by Fast Red/HNPP (Roche). TrpM5 was detected with a rabbit polyclonal

antibody (Zhang et al., 2003) and Cy3-labeled secondary antibody (Jackson Immunoresearch). Images were acquired on a Zeiss confocal microscope.

FACS and RNAseq analysis of TRC

Lingual epithelium from Pkd2l1-Cre/Ai9 or Trpm5-Cre/Ai-9 mice was dissociated for 15 minutes at 37°C in 0.25% Trypsin-EDTA, filtered through 70µm and 40µm filters, and sorted by fluorescence intensity using a BD FACSAria Cell Sorter. Total RNA was extracted, amplified for sequencing using the Ovation RNA-Seq system (Nugen), and whole transcriptome analysis was performed on a Hi-Seq system (Illumina).

Chapter 4: Representation of Multi-Modal Taste Stimuli

4.1 Abstract of this chapter

Gustatory responses to prototypical taste stimuli are transduced by distinct, segregated populations of taste receptor cells. In contrast, natural taste stimuli are often complex, stimulating two or more taste modalities simultaneously. In this chapter I examine the output of the peripheral gustatory system in response to mixtures of primary taste stimuli. By *in vivo* calcium imaging at single neuron resolution, I find that most taste combinations are represented in the ganglia by a simple superimposition of the component responses. However, neural responses to attractive stimuli, including natural sugars, artificial sweeteners and umami tastants, are selectively suppressed by simultaneous co-stimulation with a sour (acidic) stimulus. Acid suppression of sweet is cell autonomous, occurring even in the absence of acid sensing taste receptor cells. Notably, carbonation in the mouth, which stimulates sour taste transduction through Car4 dependent acidification, does not suppress responses to sweet. These studies illustrate that cross-modal interactions are important at the earliest stages of taste detection in the generation of flavor.

4.2 Introduction

Taste stimuli are detected by several distinct populations of taste receptor cells (TRCs) on the tongue and palate, each of which is narrowly tuned to detect chemical

stimuli corresponding to the five primary taste modalities: bitter, sweet, salty, sour and umami. Many natural foods and drinks are complex chemical mixtures capable of simultaneously stimulating two or more of these populations. In the case of carbonation, the previous chapter showed that a single chemical species can activate at least three (sour, bitter and sweet) TRC populations. Thus, the decision to accept or reject such a polymodal stimulus will depend on the synthesis and integration of attractive and aversive cues.

Recently, some physiological studies of TRCs have suggested a role for interactions between the primary taste modalities at the periphery. The close proximity within the taste bud of cells responsive to distinct taste qualities may allow lateral interactions to occur, while still maintaining segregated outputs. Under this scenario the taste bud is thus a functional unit, an integrated "taste organ." Indeed, multiple potential neurotransmitters and their receptors are expressed in selective taste receptor cell populations, hinting that intra-taste bud interactions may occur (Herness et al., 2005; Roper, 2006; Roper, 2009; Herness and Zhao, 2009). For example, sour-sensing PKD2L1 cells produce serotonin and GABA (Huang et al., 2005; Cao et al., 2009) and have been reported to release serotonin in an activity-dependent manner (Huang et al., 2005). Such release could potentially modulate the activity of sweet, umami, or bitter receptor cells or fibers and in the process alter the saliency, and thus the behavioral output, elicited by taste mixes containing both attractive and aversive tastants. In this chapter, I describe the application of *in vivo* assays of gustatory function to investigate the possibility that sensory integration begins peripherally, at the initial stage of taste reception.

4.3 Experimental Approach

Taste receptor cells transmit information to the brainstem via sensory afferents with cell bodies in the geniculate and petrosal cranial ganglia. Therefore, I reasoned that monitoring responses of taste neurons in the ganglia would be a direct way to determine if the output of the taste bud is transformed by cross-modal interaction - i.e., is the signal relayed from the tongue to the ganglion, and ultimately the brain, more or less than the sum of the individual qualities present in a mixed stimulus?

4.4 Suppression of Sweet and Umami by Sour

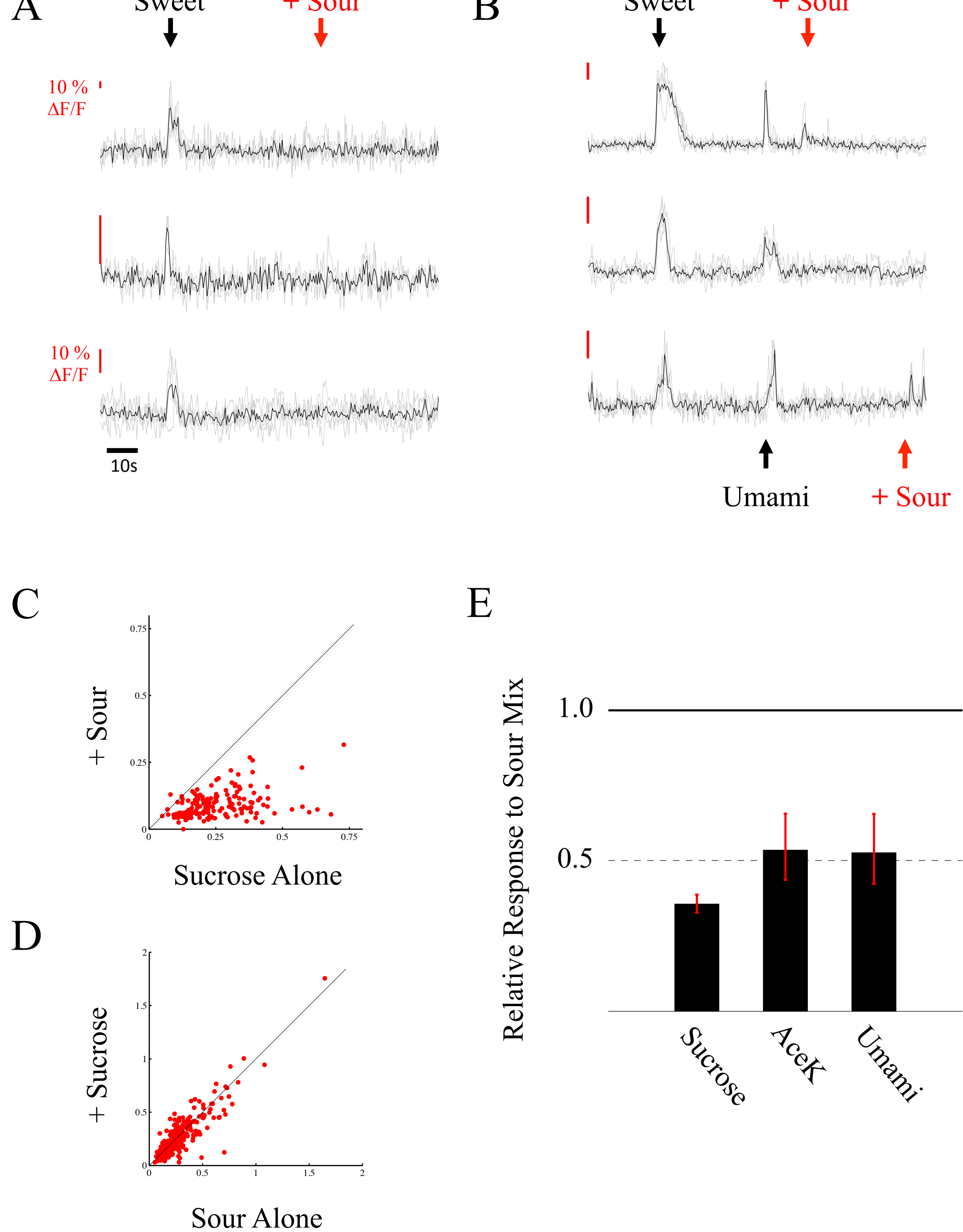
I observed robust mixture interactions at the level of the geniculate ganglion. Sour responses are equally robust when the stimulus is presented alone or in a mixture, however, neural responses to sweet and umami stimuli are dramatically suppressed when either is co-presented with the acidic stimulus (Figure 4.1). Amplitudes of calcium transients were suppressed on average by 47.4% (34.6-57.8 95% CI) for artificial sweeteners, 46.4% (34.5-56.4) for an umami stimulus, and 64% (61.3-67.3) for the natural sugar sucrose.

The binary mixes exhibiting suppression consisted of the sweet or umami stimulus, combined with 50 mM citric acid, at pH 2.2. Titrating a sweet-sour mixture to neutral pH entirely rescued the sweet response, indicating that suppression is dependent on pH and not the citrate ion, or a chemical effect of low pH on the sweet tastant itself

Figure 4.1

Figure 4.1
Sweet and umami are suppressed by co-stimulation with sour. Sweet neurons (A) and sweet/umami neurons (B) respond reliably to their cognate ligand when presented alone, but these responses are reduced when it is presented as a component of a binary mixture with sour (red arrows). Each data point in panel C represents the trial-averaged $\Delta F/F$ for a single neuron in response to sucrose or a sucrose-citric acid mixture. Nearly all points are below the 1:1 line, indicating widespread incidence of suppression. In contrast, sour responsive neurons cluster around the line, (D). Values for mean relative suppression by acid (average across responsive population) are shown for sucrose, the artificial sweetener Acesulfame K, and an umami stimulus in (E). A value of one corresponds to no suppression, while zero indicates complete suppression of response. Error bars represent 95% confidence intervals.

Figure 4.1



(Figure 4.2a). Presentation of a pH-response curve with a fixed concentration of sucrose demonstrated dose-dependent suppression of responses (Figure 4.2b,c). Sour sensing neurons presented with the same stimulus series responded with graded amplitudes of calcium response (Figure 4.2d). Therefore, acid suppression of sweet taste, and acid activation of sour sensing, are evoked within a largely overlapping range of stimulus intensity.

Does sour suppress other taste qualities? I observed no significant suppression by acid of bitter taste responses to cycloheximide or to quinine, two unrelated bitter compounds (Fig 4.3a,b). Similarly, salty taste responses were not significantly inhibited by co-stimulation with sour (Figure 4.3e). As one early study suggested suppression of chorda tympani response to mixtures of sweet with electrolytes in general (i.e. salty and sour); (Hyman and Frank, 1980), I examined responses to sweet-salty mixtures. I found no suppressive effect for either sweet or salty in these mixtures (Figure 4.3c,d). Suppression of sweet and umami by sour thus appears to be a selective phenomenon. Importantly, sour-sensing neurons exhibit robust responses to binary mixtures of acid together with any of the other four taste qualities (Figure 4.3f).

4.5 Taste Suppression is Cell-Autonomous

A number of possible mechanisms could generate cross-modal suppression. One possibility is that activation of sour sensing cells generates an inhibitory signal that specifically affects sweet and umami signaling. Several recent models of taste bud function posit extensive interaction between TRCs, and *in vitro* studies have suggested

Figure 4.2

Figure 4.2
Binary mixture suppression is selective. Bitter tastants (quinine and cycloheximide) evoke equally robust responses in bitter cells when presented in a mixture with a sour stimulus or alone(A,B). Population average responses to bitter were not significantly reduced ($p>0.05$). Sweet is not suppressed by salty (C), nor is salty suppressed by sweet or sour (D,E). Sour responses are unaffected by co-presentation with, left to right: sweet, salty, bitter or umami stimuli; + signs mark stimulus delivery (F).

Figure 4.2

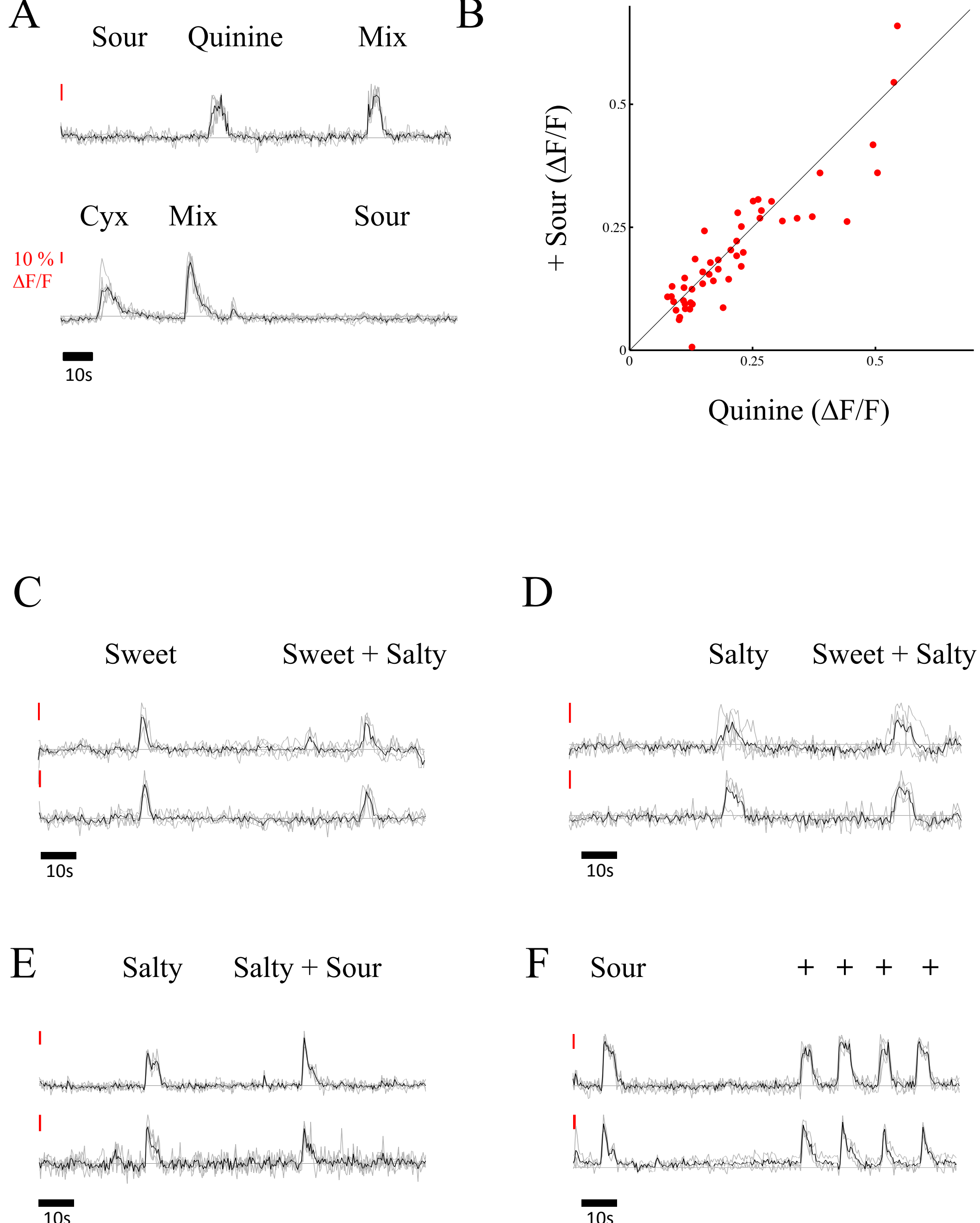


Figure 4.3

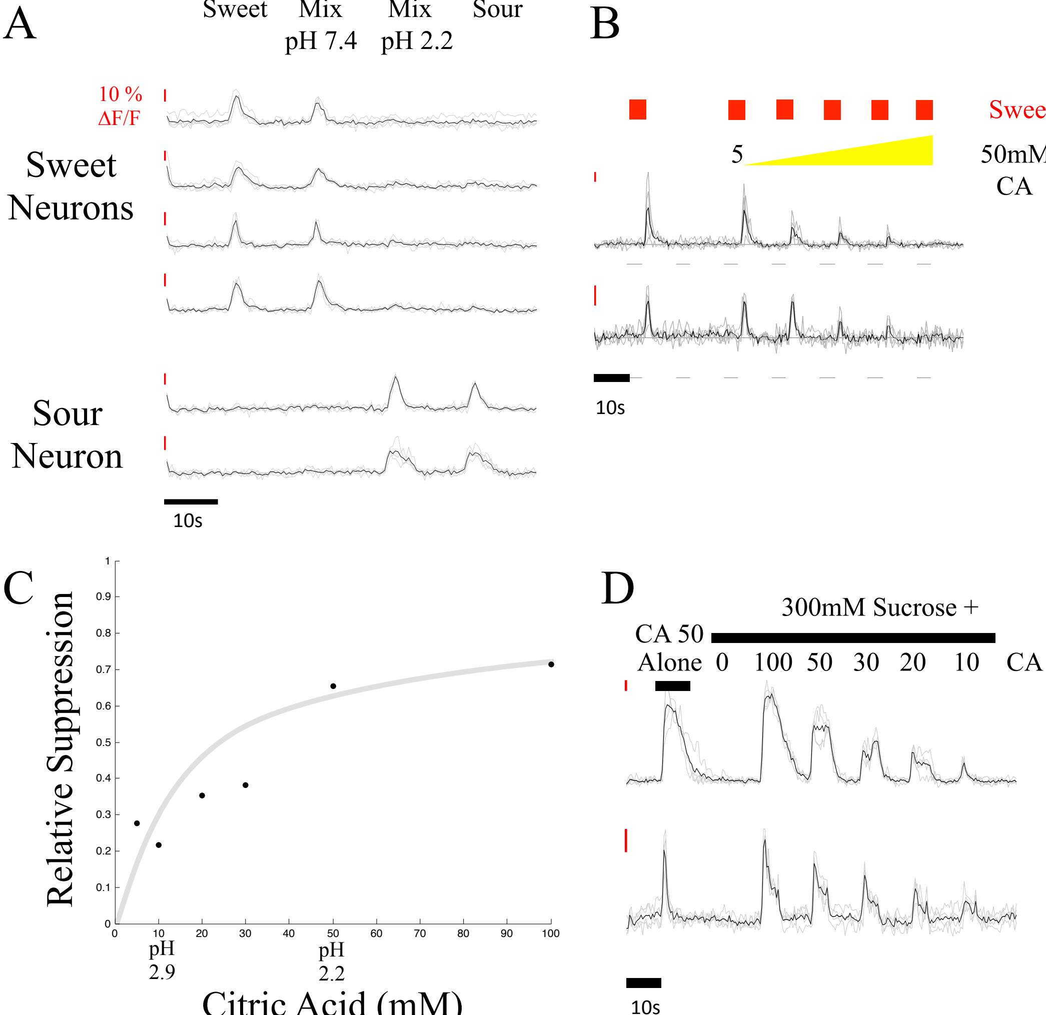


Figure 4.3
Suppression is pH dependent. Binary mixtures of sucrose and citric acid evokes responses in sweet neurons when pH is adjusted to neutral (A), but these responses are suppressed when the mixture is acidic (pH 2.2). Suppression is progressively enhanced by reduction of pH with increasing concentrations of citric acid (B,C). From left to right, red bars in B indicate sucrose with 0, 10, 20, 30 and 50 mM citric acid. Acid sensitive cells display graded responses within this stimulus range (D).

that GABAergic and serotinergic signaling from Pkd2l1-expressing sour receptor cells may shape taste responses (Huang et al., 2011). To directly test the necessity of sour TRC for suppression *in vivo*, I genetically ablated this population by expressing diphtheria toxin fragment A (DTA) under the control of the Pkd2l1 promoter. We confirmed complete loss of sour cells in taste tissue of experimental animals by immunostaining for Car4. If intercellular signaling from Pkd2l1-expressing cells is responsible for suppression, we would expect sweet sensing neurons in these mice to respond robustly to sweet-acid mixes. In contrast, we found that even in the absence of sour cells, sweet responses remain strongly suppressed by acid (Figure 4.4).

Could acid suppression of sweet taste be mediated by another TRC population? In addition to stimulating Pkd2l1-expressing cells, acid activates a subset of the bitter responsive neurons in the geniculate ganglia (see section 3.4 of this dissertation). Does bitter suppress sweet? Co-stimulation of sweet neurons with sucrose and the bitter compound cycloheximide has no suppressive effect on the activity of sweet ganglion neurons, showing that bitter signaling does not inhibit sweet at the periphery (Figure 4.5a). Furthermore, blockade of bitter with allyl isothiocyanate, a pharmacological inhibitor of bitter taste signaling (Oka et al., 2013), did not affect suppression of sweet by acid, yet eliminated responses in bitter sensing neurons (Figure 4.5b). Given that suppression is retained in the absence of signaling by acid sensing by Pkd2l1- or T2r-expressing TRCs, I concluded that suppression must be a result of a cell-autonomous mechanism; with acid likely acting directly on targets in sweet (and umami) cells to suppress signaling.

Figure 4.4

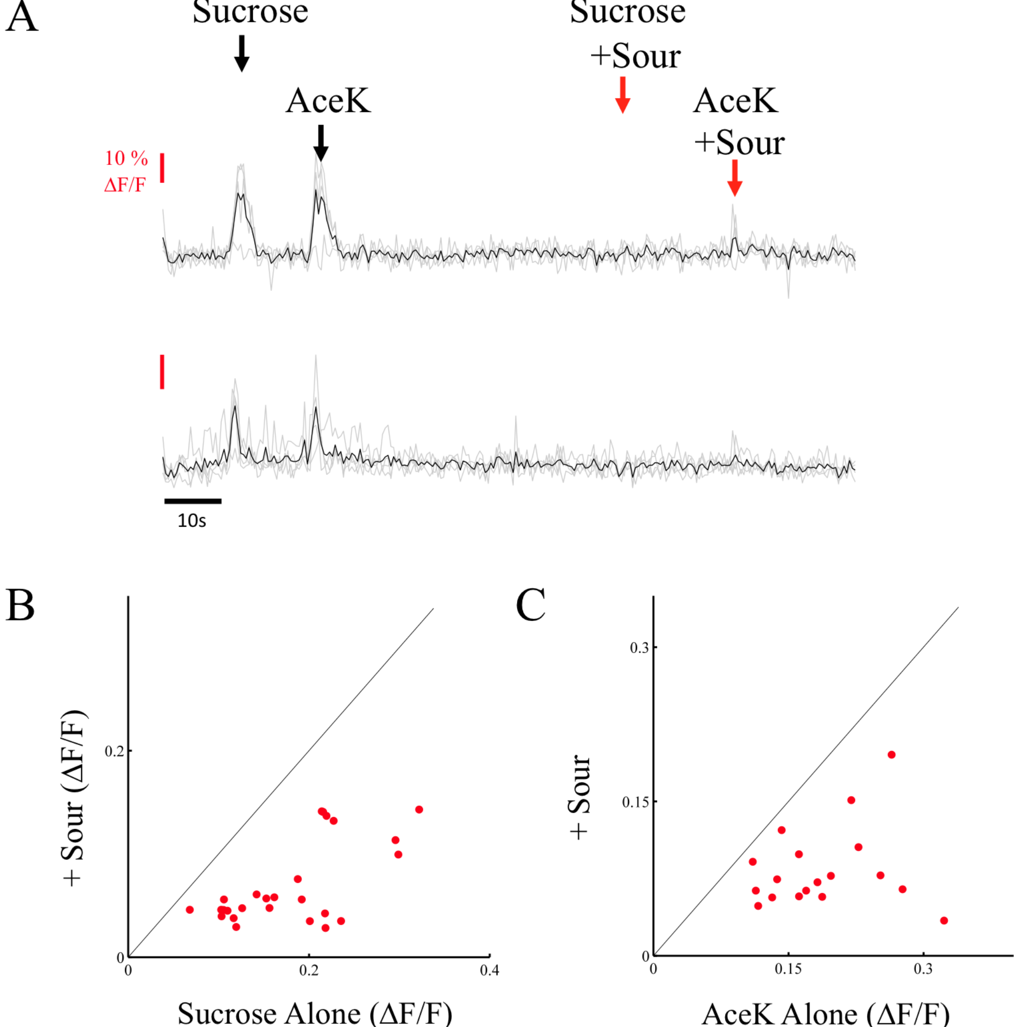


Figure 4.4
Acid mediated suppression does not require sour cells. Strong suppression of sweet by acid remains in Pkd2l1-DTA mice lacking sour TRC. (A) Example traces from sweet neurons in mice lacking Pkd2l1-expressing sour cells and distribution of maximal ΔF/F responses to sugar and artificial sweetener mixes in these mice (B,C). Suppression ratio of sweet tastants by acid is not significantly altered in Pkd2l1-DTA mice versus wild type ($p>0.05$).

Figure 4.5

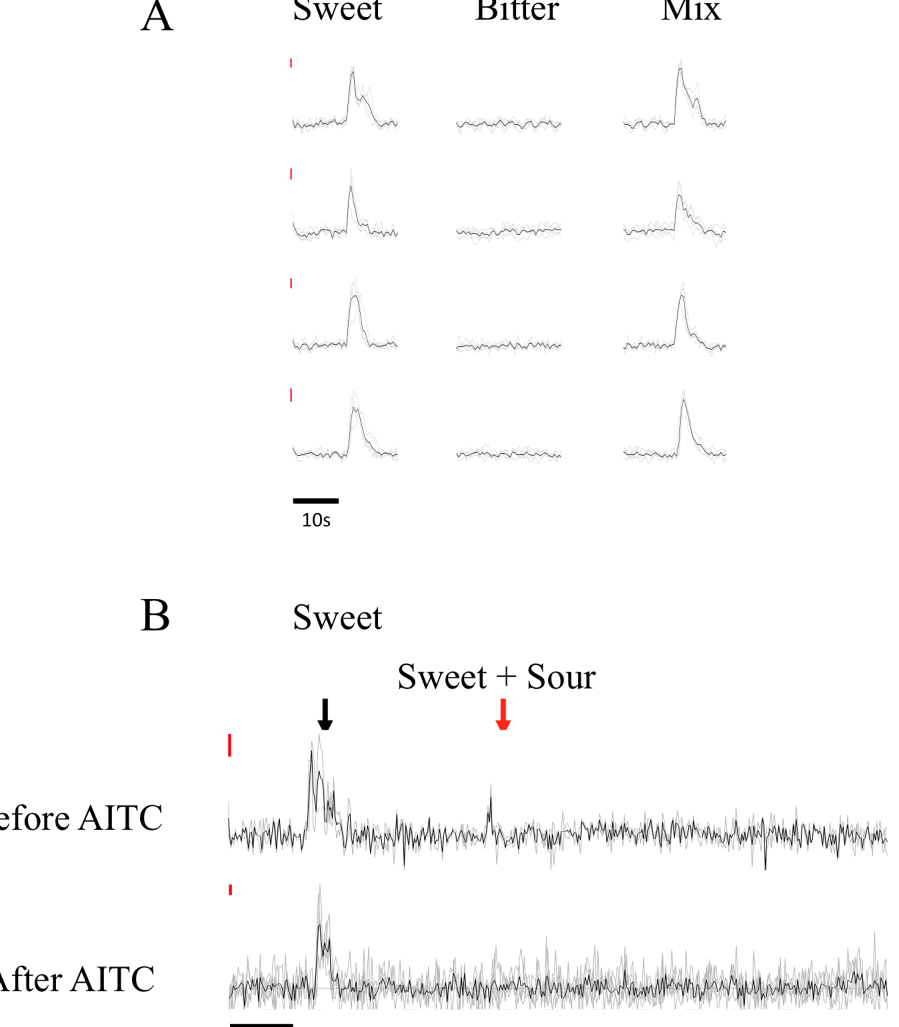


Figure 4.5
Bitter signaling does not suppress peripheral sweet responses. (A) Co-stimulation with the bitter compound cycloheximide does not suppress responses in sweet neurons. Population average responses to sweet were not significantly reduced ($p>0.05$). (B) Elimination of bitter signaling by lingual application of allyl isothiocyanate (AITC) does not reduce suppression of sweet by sour.

4.6 Representation of a Sweet-CO_2 Mixture

CO_2 is commonly encountered as a component of sweetened soft drinks, raising the question of whether CO_2 modulates sweet taste perception. Carbonation applied to the tongue acidifies TRCs in a carbonic anhydrase dependent manner, as measured in an *ex vivo* taste bud preparation (Figure 4.6A). The localization of carbonic anhydrase enzymes within taste tissue suggests that CO_2 dependent acidification would be catalyzed at the extracellular surface of sour TRCs by Car4, and intracellularly within sweet, bitter and umami TRCs by Car7. If the molecular target of acid mediated suppression is engaged by CO_2, I would expect carbonation to also suppress responses to sweet. To test this proposition, I imaged sweet responsive ganglion neurons while presenting a sweet solution either alone, mixed with acid, or carbonated. Surprisingly, I only observed suppression for the acid mixture (Figure 4.6B). This held true whether or not the sweet sensing neuron was itself CO_2 responsive. Thus, there is a clear distinction between the suppressive effect of acid applied directly to the tongue, versus the restricted and carbonic anhydrase dependent acidification elicited by CO_2.

4.7 Discussion

These results suggest that TRCs act as integrators of attractive (sweet and umami) and aversive (sour) chemical stimuli. While suppression does not require gustatory acid sensing, it is not yet clear what molecular target is inhibited by acid in the sweet and

Figure 4.6

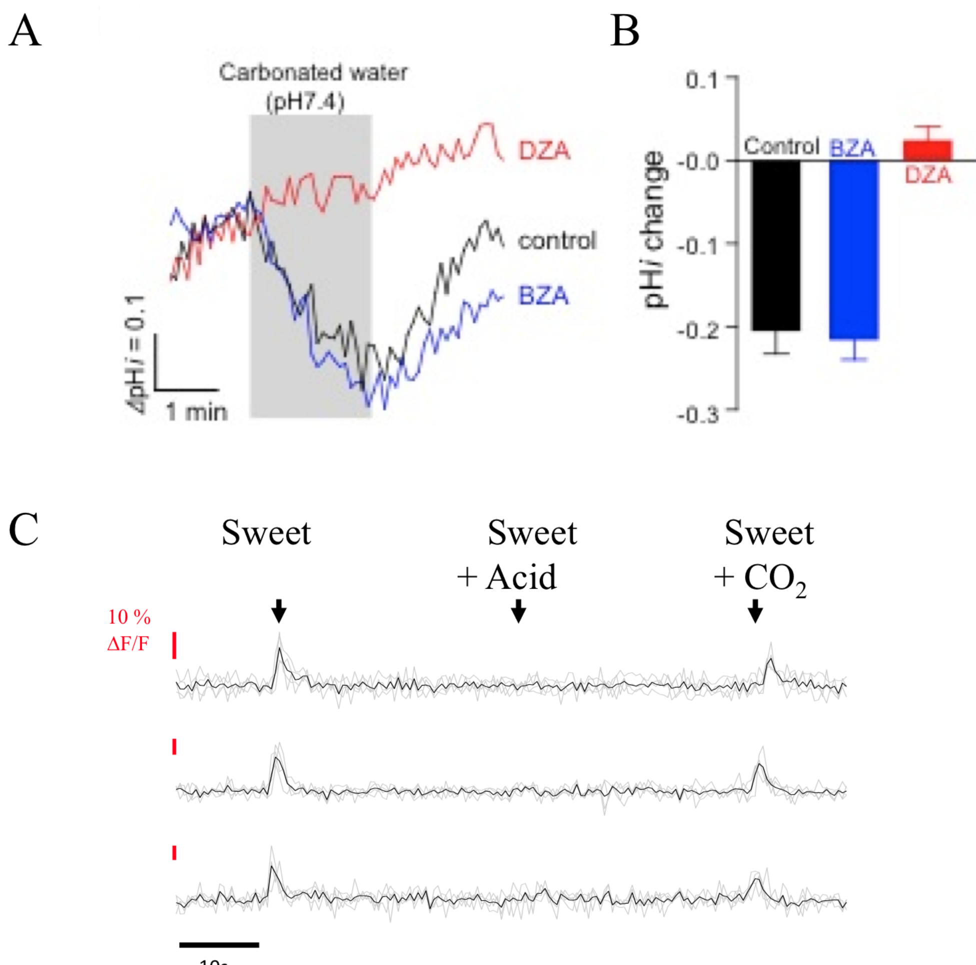


Figure 4.6
CO_2 acidifies taste receptor cells but does not suppress sweet taste.
Carbonation decreases intracellular pH (pHi) in TRC, dependent on intracellular carbonic anhydrase activity (A). Averaged pHi changes in 9 TRC are shown in (B); values are mean ± SEM, n=9 cells. BZA=benzolamide, DZA= dorzolamide. Despite acidifying TRC, carbonation of sweet solutions does not suppress response in sweet neurons, while citric acid does (C). Carbonation did not significantly suppress amplitudes of response to sweet for CO_2 insensitive sweet neurons (n=17) or CO_2 sensitive sweet neurons (n=24).

umami cells themselves. The insufficiency of CO_2 to elicit suppression, despite generating intracellular acidification, points to a target at the extracellular surface of sweet and umami TRCs. One attractive possibility is that acid acts directly on T1R1+T1R3 and T1R2+T1R3 receptors. The most likely site of action might be the T1R3 subunit, as it is required for both sweet and umami sensation (Nelson et al., 2001, Nelson et al., 2002). A logical next step will be to examine the function of T1R3 containing receptors in a heterologous system, to determine whether pH indeed modulates the function of the receptor itself.

Notably, ablation of sour cells does not alter the representation of binary taste mixtures containing acid. Models of taste information processing by paracrine neurotransmitter signaling within the taste bud would predict that suppression of sweet, bitter and umami responses by acid would be dependent on the function of the sour receptor population (Huang 2009; Roper, 2013). Support for these models has been derived using *ex vivo* assays of TRC function. In contrast, the results obtained here *in vivo* argue that cross-quality taste interactions seem to be limited to the cell-autonomous effect of acid on sweet and umami. This suggests that lateral neurotransmitter signaling within the bud does not significantly alter the relative saliency of taste responses, and supports independent function of the labeled lines at the periphery.

Is the physiological phenomenon of suppression important for taste-mediated behaviors? Notably, sugars, acids and bitter alkaloids are frequently found together in natural food sources; grapefruit is a familiar example. In these and many other fruits, acidity decreases and sugar increases as ripening progresses (Prasanna et al., 2007). In this context, suppression of sweetness could sharpen the distinction between ripe and

unripe. In contrast, suppression of a bitter (toxic) signal by acid would be deleterious. The broad effects of acid on the gustatory system suggest that low pH is a highly relevant signal in the context of nutrient selection. The modern diet includes items that simultaneously stimulate both sweet and sour taste pathways, with carbonated soft drinks an obvious example. It is remarkable that CO_2 stimulates sour taste signaling through carbonic anhydrase mediated acidification, yet does not suppress sweet taste signaling in the same manner as an equivalently 'sour' acid stimulus. The ability of carbonation to evoke robust sour signaling without concomitantly suppressing attractive taste transduction is an additional feature differentiating the taste of carbonation from the purely sour taste of acids, and may thus play a role in its widespread popularity.

4.8 Experimental Procedures

In Vivo Calcium Imaging

Calcium imaging and analysis were performed as described in Chapter 3. Taste stimuli tested included: 300mM sucrose, 12.5mM acesulfameK, 40mM monopotassium glutatamate + 1mM inosine monophosphate, 100mM NaCl, 5mM quinine, 0.1mM cycloheximide, all tested ± 50mM citric acid, 5, 10, 20, 30, 50 and 100mM citric acid + sucrose, carbonated artificial saliva and carbonated 300mM sucrose. Taste solutions were made up in artificial saliva buffer immediately prior to each experiment. Relative suppression for mixture combinations was calculated as the geometric mean over responsive neurons of the ratio of mean mixture response amplitude to mean single

component amplitude, with confidence intervals calculated on the basis of geometric standard error.

Pharmacology

To block bitter signaling, 25ml of 10mM allyl isothiocyanate in artificial saliva was applied to the oral cavity through the stimulus delivery tube at 5ml/minute for 10 minutes (Oka et al., 2013). Analysis of identified bitter responding ROIs prior to drug treatment confirmed subsequent loss of bitter responses.

pH imaging in taste cells

Freshly-peeled taste epithelium (Lyall et al., 2001) was pre-incubated with benzolamide (100 μM), dorzolamide (0.5%) or control buffer for 6 min. prior to CO_2 stimulation. Intracellular pH (pHi) was monitored with SNARF-1 dextran (Invitrogen) as described (Swietach et al., 2009) using a 5-Live confocal microscope (Zeiss). Carbonated water was buffered to pH 7.4 using 72 mM NaHCO3 and applied to the epithelium for 2 min. ΔpHi was calculated as the difference in pHi between the beginning and end of stimulation.

Chapter 5: Concluding Remarks

5.1 Summary

My primary motivation for the line of research presented in this dissertation came from an intuitive curiosity about where the familiar and unique 'flavor' of carbonation comes from. The results presented here represent progress towards answering this question, principally by defining the populations of receptor cells and primary neurons activated by this stimulus. Carbonation in the mouth evokes a gustatory response primarily through activation of the sour labeled line. Unlike the mechanism by which acids elicit sourness, this response depends on the action of Car4, a membrane tethered carbonic anhydrase at the surface of sour cells. A smaller component of the response to CO_2 is mediated by partial activation of the bitter and sweet TRCs through Car7, differentiating the representation of carbonation from that of purely acidic stimuli. A further distinction between carbonation and other sour stimuli is that low pH suppresses sweet and umami taste, while carbonation does not. This means that carbonated solutions are uniquely capable of robustly activating the sweet and sour labeled lines at the same time. Thus, carbonation perception does not define a unitary taste quality in mammals, as it does in insects, but rather reflects combinatorial engagement of several taste qualities simultaneously.

The complex pattern of activation elicited by carbonation may in part explain the elusive and controversial status of carbonation as a 'taste' stimulus. Psychophysical experiments in humans indicate that simultaneous stimulation of multiple taste qualities

suppresses the ability to perceive the component qualities, and that increasing the complexity of mixtures diminishes the ability of subjects to identify the components (Marshall et al. 2006). As CO_2 mimics some of the properties of a taste mixture, similar perceptual mechanisms may make it difficult to describe the taste it elicits in terms of the more familiar primary taste qualities.

It is interesting to note that the gustatory aspects of CO_2 have been best appreciated in the context of the 'champagne blues' phenomenon caused by carbonic anhydrase inhibitors. Under these circumstances, carbonation has been described as having a 'dirty dishwasher' taste (Graber and Kelleher, 1988). It is tempting to speculate that this might be due to differential access or inhibition of the two gustatory carbonic anhydrases by pharmacological inhibitors. For example, it could be that Car4 is inhibited to greater extent than Car7, resulting in suppression of the sour component of the mixture, allowing enhanced perception of the bitter/sweet/umami component. It would be interesting to identify carbonic anhydrase inhibitors with greater isoform selectivity, and determine their effects on carbonation perception.

5.2 Mechanisms for Detection of CO_2

Does the discovery of mechanisms for taste reception of CO_2 have implications beyond taste? There is precedent for the study of signal transduction in the gustatory system illuminating other areas; in particular, the discovery of T1Rs on the tongue has been followed by studies implicating these receptors in nutrient sensing in the gut and male germ line (Kokrashvili et al., 2009, Mosinger et al., 2013). Similarly, T2R bitter

receptors have recently been suggested to serve as important sensors for noxious compounds in the airways (Shah et al., 2009).

In the case of the studies described here, the most obvious implication of medical significance is the detection of CO_2 by central and peripheral chemoreceptors. An obvious extension of this work would be to determine whether receptor neurons in these systems express any of the molecules implicated in taste detection of carbonation. More broadly, the techniques and approach utilized here to define gustatory CO_2 receptors could be profitably employed to investigate the cellular and molecular basis of CO_2 detection elsewhere. A gene expression screen using RNA isolated from carotid body neurons would be an excellent entry point to identify candidate receptor molecules, and possibly subdivide receptor neurons into more specific populations that could be manipulated with mouse genetics. These neurons send information to the petrosal ganglia, which is structurally close to the geniculate ganglia and could be imaged in a similar manner to decode the pattern of activity in response to altered levels of CO_2 and oxygen (Jonz and Nurse, 2012).

A key theme that is supported by this work is the pervasive role of carbonic anhydrase enzymes in CO_2 sensing. There is now evidence for carbonic anhydrase dependence in each of the mammalian systems known to sense CO_2, including taste, smell, somatosensation and peripheral chemoreception (Chandrashekar et al., 2009; Hu et al., 2007; Komai and Briant, 1993; Black, 1971). Despite this commonality, there are some interesting distinctions between these mechanisms; while olfaction relies on intracellular Car2 and bicarbonate production, sour cells detect CO_2 through an extracellular Car4 and proton detection, and somatosensory detection is proposed to rely

on intracellular acidification and proton detection. It will be interesting to examine the mechanisms of additional CO_2 sensors as they are discovered, to see if logic emerges as to why evolution has generated multiple related solutions to a single sensory problem.

5.3 Methodological Implications

The work described here fits into a larger effort within neuroscience, to define the physical basis underlying sensory perceptions. The primary concerns in the gustatory system are the same as in any other sensory system: how are environmental stimuli detected and encoded into patterns of neuronal activity; how are these patterns transformed and distributed within the nervous system to influence behaviors, form associations, and generate subjective representations of the world. The approach presented here tackles these questions by working from the outside in, first identifying receptor genes and cells and then following the flow of information towards the brain. This relies primarily on two technical approaches. The first is the use of mouse genetics to manipulate taste expressed genes and sets of TRCs. Up to this point, this sort of approach has provided the strongest evidence for labeled line models of taste coding. Our previous understanding of coding *in vivo* is largely derived from recording peripheral neural responses from genetically modified mouse strains, supplemented by analysis of receptor expression patterns and functional de-orphaning of receptors in heterologous systems (Chandrashekar et al., 2006). The second approach employed here is the use of a novel calcium imaging assay to monitor activity in populations of primary gustatory neurons. Previous studies have performed serial recordings from gustatory afferent fibers

or soma, so this is not new in concept, but the parallel nature of optical imaging allows for the rapid acquisition of larger data sets from each experimental subject.

As might be expected, application of a novel methodology to the study of taste coding revealed unanticipated features. With respect to taste coding of carbonation, calcium imaging revealed a distinct non-sour pathway for CO_2 detection that was marginally detectable by whole-nerve recording. Similarly, the sensitivity of bitter neurons to acid, independent of Pkd2l1-expressing sour cells, was not suspected in previous studies of acid reception (Huang et al., 2006). There are two considerations that may potentially explain these discrepancies. Firstly, the geniculate ganglion receives information from both the chorda tympani and greater superficial petrosal nerves, while the extracellular recording data is derived from the chorda tympani nerve alone. The chorda tympani nerve receives information from the fungiform taste buds, where bitter TRCs are relatively rare as compared to the palate, foliate or circumvallate papillae (Adler et al., 2000). Consequently, taste responses to bitter in the chorda tympani nerve likely under-represent the contribution of the bitter taste receptor population. Secondly, the different populations of geniculate taste neurons may contribute differentially to the neural response to taste stimuli. This is supported by electrical recordings from individual afferent fibers, showing that both basal firing rates and evoked activity are significantly higher in acid sensitive fibers than in the other classes of afferents (Breza et al., 2010). Taken together, these factors appear sufficient to account for the apparent qualitative and quantitative discrepancies between data obtained by these two methodologies.

An additional, novel finding was the convergence of sweet and umami information. This was surprising, particularly given evidence for narrowly-tuned umami

sensing neurons at higher stations in taste processing in the mouse (Chen et al., 2011). Further work is needed to determine whether this pattern of activation represents convergence, or broad tuning of umami TRC at the periphery. In the future, the population imaging approach should be transferrable to each of the relay stations for taste processing, from primary taste receptor cells to cortical taste areas, providing tremendous potential to uncover the logic by which taste information is distributed and transformed in the brain to evoke behavior and perception.

5.3 Future Directions

There are a number of gaps in our understanding of the sequence of events by which the stimulus of carbonation is encoded as neural activity. At the molecular level, the most interesting piece currently missing is the identity of the receptor or receptors responsible for detection of Car4 generated protons by Pkd2l1-expressing sour cells. The gene expression screen identifying Car4 and Car7 as taste specific CO_2 transducers also provides a rich source of candidate genes differentially expressed in populations of taste receptor cells (see Appendix A). If an abundantly expressed transcript encodes the acid sensor, this dataset is likely to contain the relevant gene. While the key test of necessity for any candidate acid sensor is measurement of taste responses in the mutant mouse, it would be highly beneficial to develop a heterologous assay to screen candidate receptors for appropriate biophysical properties. In parallel, physiological analysis of currents

associated with acid reception in isolated Pkd2l1 expressing TRC will help to narrow down the properties of the acid sensor.

Some ambiguities remain in interpreting exactly which populations of TRC are being activated by CO_2. For example, the sensitivity of umami TRC to CO_2 is unclear, as we do not know the pattern of convergence that produces doubly tuned sweet-umami neurons. It would be highly advantageous to be able to monitor the activity of the TRCs themselves in response to CO_2 and to prototypical taste stimuli, for example by calcium imaging. Some co-workers and I have made efforts in this direction. We are able to target genetically encoded calcium indicators to TRCs and observe putative taste evoked responses in an *ex vivo* preparation. Considerable effort will be needed to generate a physiologically relevant assay, but this approach could provide a strong test for the model of carbonation sensing I propose. More generally, direct monitoring of activity in TRCs has the potential to resolve many of the ambiguities in our current understanding of taste coding.

Finally**,** carbonation is an example of a true multi-modal taste stimulus. Is it more useful to think of our perception of carbonation as a single sensory “cue”, or a collection of disparate reactions to a stimulus? This depends on whether these multiple streams of information are integrated in the brain to evoke perception and behavior. In the mammalian olfactory system, complex chemical stimuli are not represented by a simple sum of the component responses. Neural responses to mixes exhibit both suppressive and synergistic interactions (Stettler and Axel, 2009). The representation of taste mixtures at the level of the taste ganglia, as described in this thesis, provides a starting point for

future studies of how the representation of complex taste stimuli are transformed as gustatory information is distributed within the brain.

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Appendices

Appendix A: Screening for Novel Taste Receptor Molecules and Cells

A.1 Motivation

Our current understanding of peripheral taste transduction stems from the discovery of genes encoding receptors and transducers for taste, and the use of these genes to identify and manipulate distinct populations of receptor cells on the tongue. With the aim of uncovering novel aspects of taste reception, I developed a screen to identify three classes of molecules: 1) Genes transcribed in the sweet, bitter and umami TRCs. 2) Genes specifically transcribed in the sour TRC populations, (e.g. Car4) 3) Genes expressed in taste buds, but not in any of the known classes of TRC. Such genes could serve as molecular markers for novel classes of TRC.

A.2 Screening Strategy

Prior to initiating this screen, we had developed transgenic mouse lines expressing Cre recombinase under the control of regulatory elements for an array of taste-specific genes, including Pkd2l1 and T1R3 (Huang et al., 2006, Chandrashekar et al., 2009). Pkd2l1 is specifically expressed in sour cells, while T1r3 is expressed by sweet and umami cells. T1r3 is also expressed transiently during the development of bitter cells, such that Cre induced recombination of a reporter labels in sweet, umami and bitter cells in T1r3-Cre mice. By crossing to appropriate reporter lines, these transgenics could be used to label or to ablate the respective populations of TRCs.

I developed a microarray based screening strategy consisting of three approachea. I first isolated RNA from taste tissue and compared it to the surrounding non-sensory lingual epithelium to identify taste specific genes in an unbiased manner. To examine region-specific differences in gene expression, I profiled isolated fungiform taste buds, isolated circumvallate taste buds, and whole circumvallate papillae. I then collected the same taste tissues from mice in which Pkd2l1-Cre, T1r3-Cre, or a combination of the two were used to drive expression of diphtheria toxin fragment A (DTA), thus ablating selected populations of taste receptor cells. Finally, ass a complementary screen, I picked individual GFP labeled cells marked by crossing Cre lines to a fluorescent reporter and pooled cells of the same genotype to generate a selective population for gene expression profiling (Figure A.1).

Analysis of data generated by this screen required integration of information obtained from each type of experiment. In addition to performing multiple comparisons, I utilized a number of different microarray platforms to profile differential gene expression. Each microarray consists of thousands of individual gene expression assays. To integrate data across platforms and experiments, I normalized differences in gene expression according to the variance of intensities observed across biological replicates for each platform. This provided scores that could be meaningfully compared regardless of platform or experiment type. I then consolidated this information by mapping each probeset to a single gene identifier, and calculating an average score across platforms for each comparison performed (e.g. Pkd2l1-DTA versus wild type taste buds, T1R3-GFP vs Pkd2l1-GFP TRCs, fungiform buds versus lingual epithelium, etc.)

Figure A.1

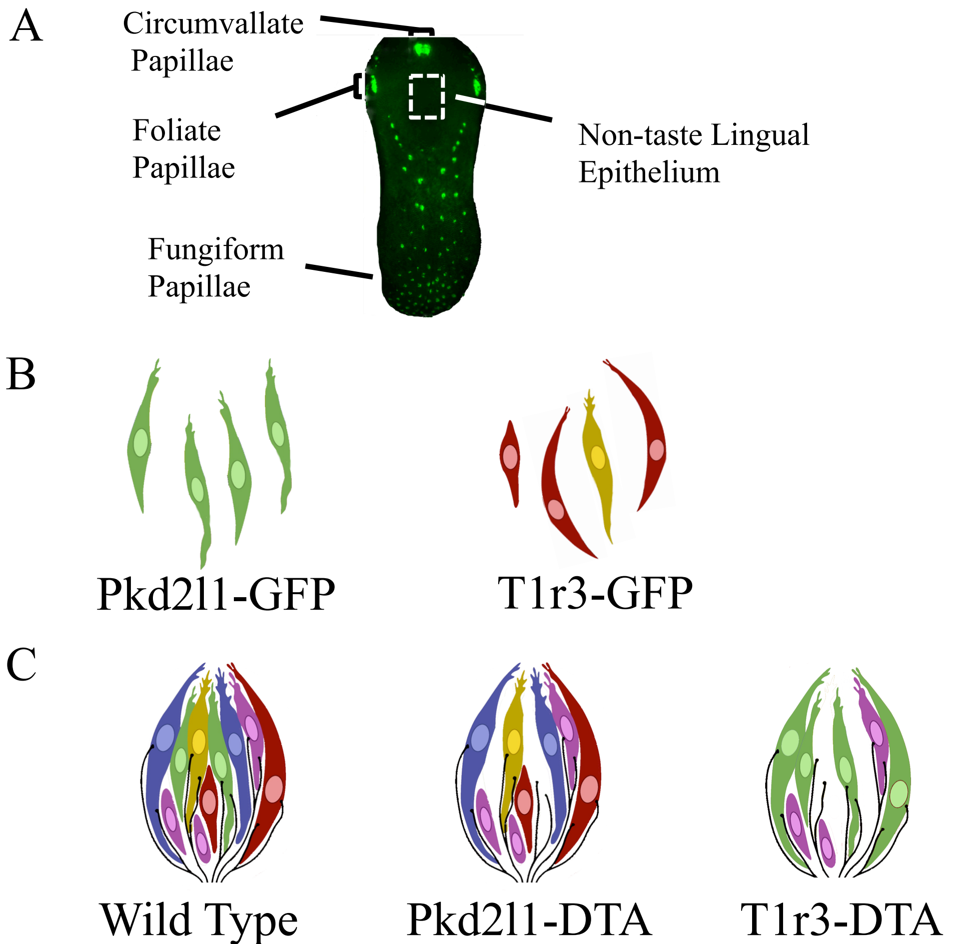


Figure A.1
Screening strategy for novel taste cell expressed transcripts. Microarray based gene expression profiling was performed on RNA from taste and non-taste lingual tissue to identify taste specific genes (A). I also profiled populations of dissociated taste receptor cells (TRCs), (B), marked by expression of Pkd2l1 (sour TRC) or T1R3 (sweet and umami TRC). As a complementary strategy to classify taste expressed transcripts, I profiled taste tissue from mice in which either sour cells (Pkd2l1-DTA) or bitter/sweet/ umami TRC (T1R3-DTA) had been ablated. Notably, the population of sour cells expanded in T1R3-DTA taste tissue.

of these scores to select for gene expression profiles of interest. I proceeded to sort genes into three categories: 1) Enriched in sour TRC; 2) Enriched in bitter/sweet/umami TRC; and 3) Taste specific, but not specifically enriched in any of the known TRC categories. This third category conceivably contains genes expressed in novel subpopulations of TRC, but also genes expressed uniformly in all TRC.

A.3 Validation and Results

To validate the effectiveness of the screen, I needed a set of genes known to be specific to each population. At the time I initiated the screen, a relatively small number of genes had been identified as specifically expressed in the various TRC populations. The best characterized were the T1R and T2R taste receptors, so I examined the ability of my screening methodology to enrich for known taste receptors in the bitter/sweet/umami dataset.

I ranked the database of gene identifiers according to the criteria of enrichment in T1R3 cells over Pkd2l1 TRC, enrichment in wild type taste tissue over T1R3-DTA tissue, and enrichment in taste tissue over lingual epithelium. This exercise indeed rank-ordered all T1R genes to within the top 30 of all 44,293 molecular identifiers in the database. T2R bitter receptors were also highly enriched, with 29 T2Rs within the top 50 genes encoding integral membrane proteins. Similarly, the corresponding experiment for sour cells identified Pkd2l1 as highly specific (Figure A.2).

In order to validate expression of new candidate genes I generated RNA probes for *in situ* hybridization analysis of gene expression patterns in taste tissue. Figure A.3

Figure A.2

Known Genes Expressed in T1r3$^+$ Cells

Gene Symbol	Function	Rank Order (of 44293)
Trpm5	Ion channel, transduces bitter/sweet/umami	1
Tas1r2	GPCR, sweet receptor subunit	8
Tas1r1	GPCR, umami receptor subunit	14
Tas1r3	Sweet, umami receptor subunit	26

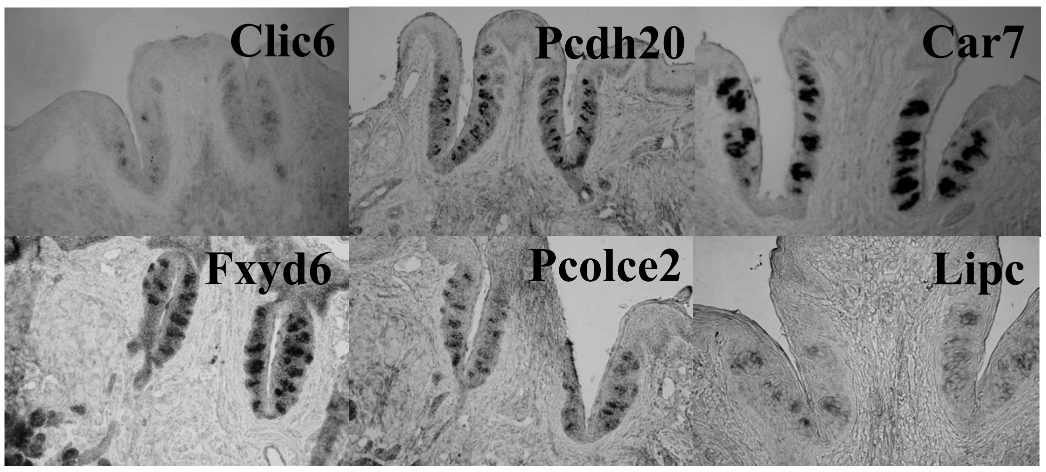
Known Genes Expressed in Pkd2l1$^+$ Cells

Gene Symbol	Function	Rank Order (of 44293)
Pkd2l1	Putative acid sensor channel	8
Pkd1l3	Putative acid sensor channel	785
Car4	Enzyme, CO_2 transduction	6
Snap25	Vesicular fusion, synaptic transmission	17

Figure A.2
Validation of screening strategy. Results from microarray experiments was integrated to create lists of genes ranked by evidence for taste specific and cell type (sour or bitter/sweet/umami) specific expression. Known taste specific transcripts were highly enriched at the top of these ranking. Pkd1l3 is ranked lower because it is not expressed in fungiform taste buds; when only data from vallate taste buds is included, Pkd1l3 appears highly specific to sour TRCs.

Figure A.3

A



B

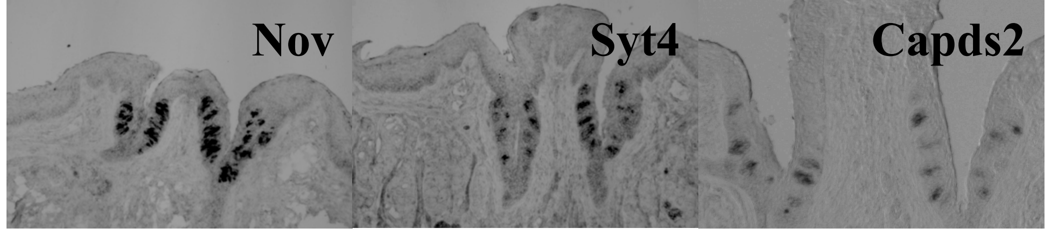


Figure A.3
Validation of genes predicted to be expressed in known populations. RNA *in situ* hybridization experiments were performed on sections from circumvallate taste papillae, to examine genes predicted by a gene expression screen to be expressed by bitter/sweet/umami TRCs (A) or sour TRCs (B). Pictured are a subset of *in situ* results scored as positive and taste specific, spanning the range of signal intensities from weak (Clic6) to intense (Nov, Car7).

shows examples of these patterns. To determine the specificity of expression for probes exhibiting positive signal, I then tested expression in mice engineered to lack specific populations of TRC. For all genes tested, ablation of the predicted cell type resulted in greatly diminished or absent *in situ* signal.

Car4 and Car7, as described in this thesis, were identified as selective to the sour and bitter/sweet/umami populations, respectively. A range of other genes were identified and validated as taste specific by *in situ* hybridization (Figure A.3). Interesting examples include neural adhesion molecules potentially involved in mediating contact between TRCs and afferent fibers, such as the protocadherin Pcdh20, putative ion channels such as transmembrane channel-like 4 (Tmc4), and potential mediators of synaptic transmission such as synaptotagmin 4 (Syt4). Intriguingly, transcripts for dopa decarboxylase (Ddc) and glutamic acid decarboxylase (Gad1), both enzymes involved in neurotransmitter biosynthesis, were identified as specific to sour cells.

A.4 Markers for Candidate "Novel" Taste Cell Populations

A primary motivation for this project was the identification of genes to mark and manipulate novel and uncharacterized classes of TRC. To prioritize candidate markers, I scored each gene with a value weighted positively for taste specific expression, and negatively for enrichment in either the sour or bitter/sweet/umami populations. As these criteria enrich for genes expressed evenly across all TRC, as well as markers of novel subsets, I then conducted an *in situ* screen with these candidates to identify those that

exhibited subset-selective expression patterns. Some examples of the gene expression patterns are shown in Figure A.4.

Two candidate markers genes were validated as markers of novel cell classes and used as drivers to label these TRC: Ectonucleoside triphosphate diphosphorylase 2 (Enptd2) and lysozyme 2 (Lyzs). Entpd2 encodes an integral membrane protein that degrades extracellular ATP, and was recently proposed to contribute to regulation of purinergic signaling in TRCs (Vandenbeuch et al., 2013). Lyzs encodes an enzyme with antibacterial properties that is a component of the innate immune system, and is expressed in myeloid cells (Clausen et al., 1999). Immunohistochemical staining suggested that Entpd2 marks a large population of cells within taste buds that is distinct from the known TRC classes (Figure A.5a,b). I developed a BAC transgenic mouse line to mark these cells by expression of Cre. Crossing this line to a fluorescent reporter recapitulated this pattern of expression in a novel set of taste cells (Figure A.5c). By using a previously generated Lyzs-Cre knock-in mouse (Clausen et al., 1999), I determined that Lyzs expression also defines a subset of novel cells within the Entpd2 population (Figure A.6). Thus, Entpd2 defines at least two populations of uncharacterized cells, one positive for Lyzs and one negative. The availability of these Cre lines provides tools to assign function to these cell types through genetic labeling, activation and ablation, in combination with nerve recording and calcium imaging of taste responses.

Figure A.4

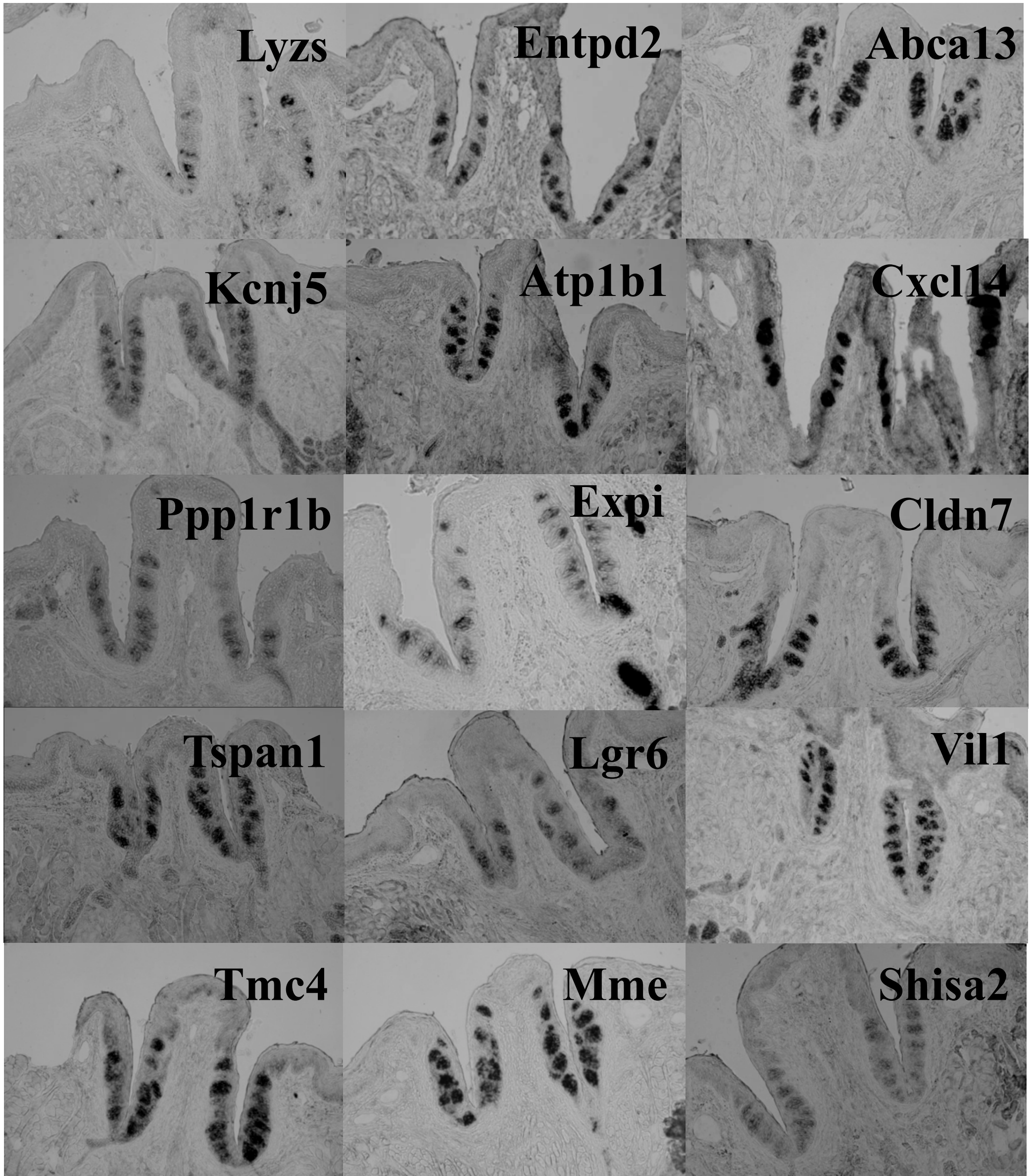


Figure A.4
Candidate markers for novel taste cells. RNA *in situ* hybridization experiments were performed on sections from circumvallate taste papillae, demonstrating patterns of taste bud specific expression for a number of genes potentially useful as markers of novel TRC classes.

Figure A.5

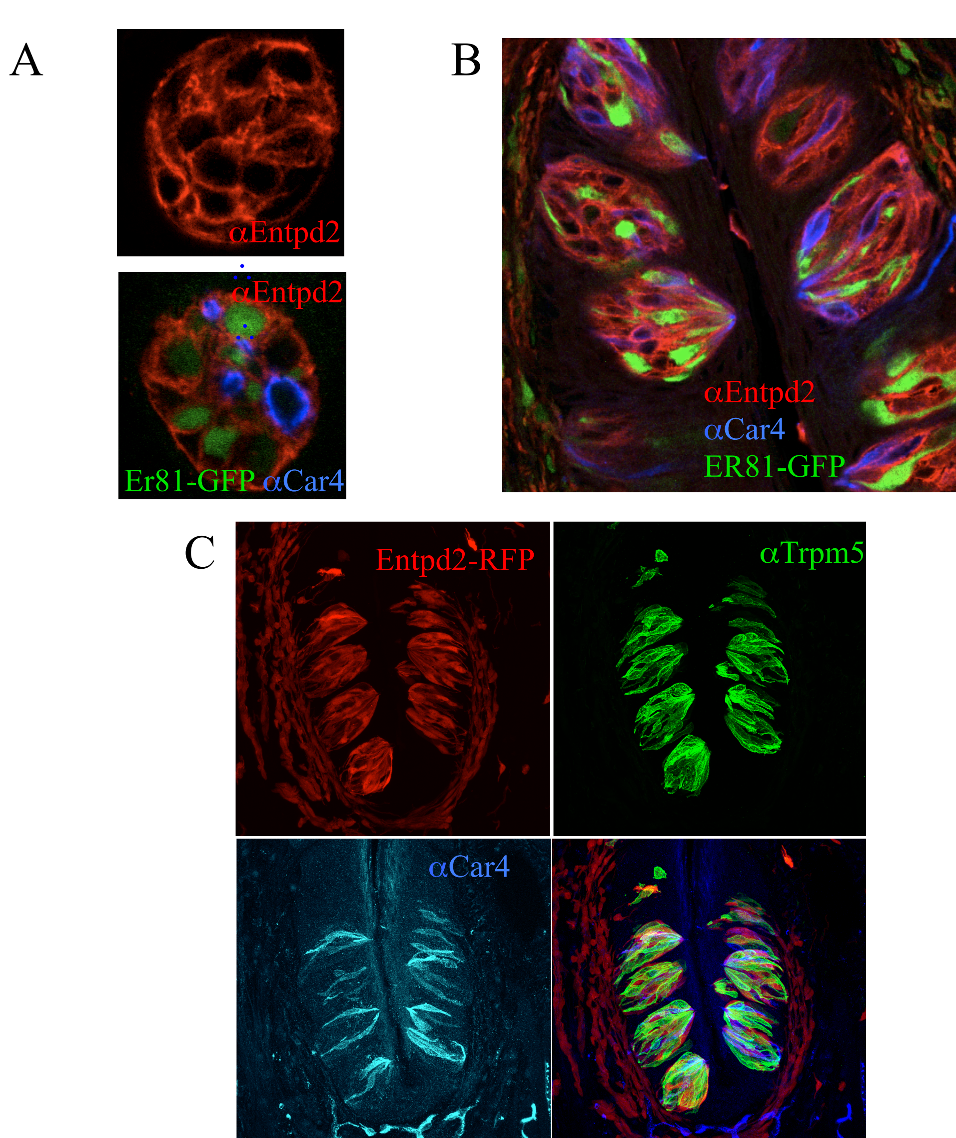


Figure A.5
Entpd2 marks an uncharacterized set of cells in taste buds. Fluorescent labeling of fungiform (A) and circumvallate (B) taste buds with an Entpd2 antibody uncovers a population of cells intercalating between sour (Car4 expressing) and sweet/umami (ER81-GFP) TRCs. A BAC transgenic line expressing Cre under Entpd2 regulatory elements labels these cells in red when crossed with a Cre-dependent red fluorescent reporter.

Figure A.6

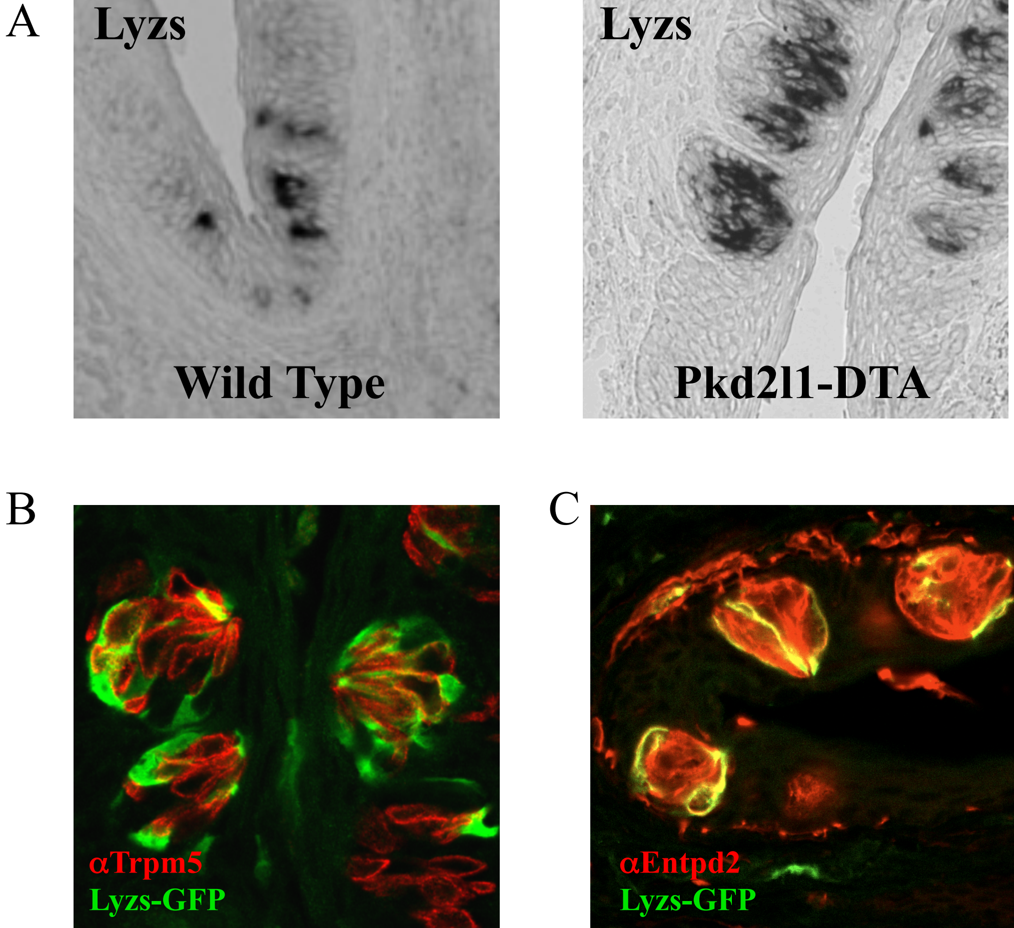


Figure A.6
Lyzs marks a subset of Entpd2^{+}, Trpm5^{-} Pkd2l1^{-} cells. Lyzs marks a subset of TRCs in both wild type and mice lacking sour cells (A). A Cre knock-in into the Lyzs locus drives expression in a subset of cells that does not overlap with sweet/bitter/umami cells marked by Trpm5 immunoreactivity (B), but does comprise a subpopulation of Entpd2 positive cells (C).

A.5 Intersectional Ablation Strategy to Characterize Novel Populations

One obstacle to the functional characterization of novel cell types on the tongue is that the novel marker molecules are often likely to be expressed elsewhere in the body. Therefore, genetic ablation using these genes as drivers would likely have deleterious or lethal effects on the organism. To circumvent this problem, I generated a transgenic mouse line in which conditional toxin expression is restricted to taste receptor cells. I constructed a bacterial artificial chromosome in which the genomic sequence of the pan-TRC expressed gene cytokeratin 19 (Krt19) was modified to contain a loxp flanked GFP followed by the coding sequence of DTA (Figure A.7a). Prior to Cre mediated recombination, transcriptional termination sequences and frameshift prevent transcription of the toxin, while after recombination the full length toxin sequence is in frame with the start codon of Krt19.

Mice carrying this construct expressed GFP within taste buds (Figure A.7b). However, the Krt19 regulatory sequence did not drive expression across all TRC as intended, resulting in only partial labeling of cells within each taste bud. Nevertheless, I tested the ability of this construct to mediate ablation of TRC by crossing it to a T1R3-Cre line. Consistent with the pattern of GFP expression, these mice exhibited a clear, albeit incomplete loss of sweet taste responses, with responses to sour and salt unaffected (Figure A.7c). This suggests that this mouse may be useful for intersectional ablation of novel classes of TRC marked genes expressed outside the gustatory system. Crossing this line to Lyzs-Cre and Entpd2-Cre mice may produce interesting and informative, though partial phenotypes. Still, it would be worthwhile to generate mice where the conditional

Figure A.7

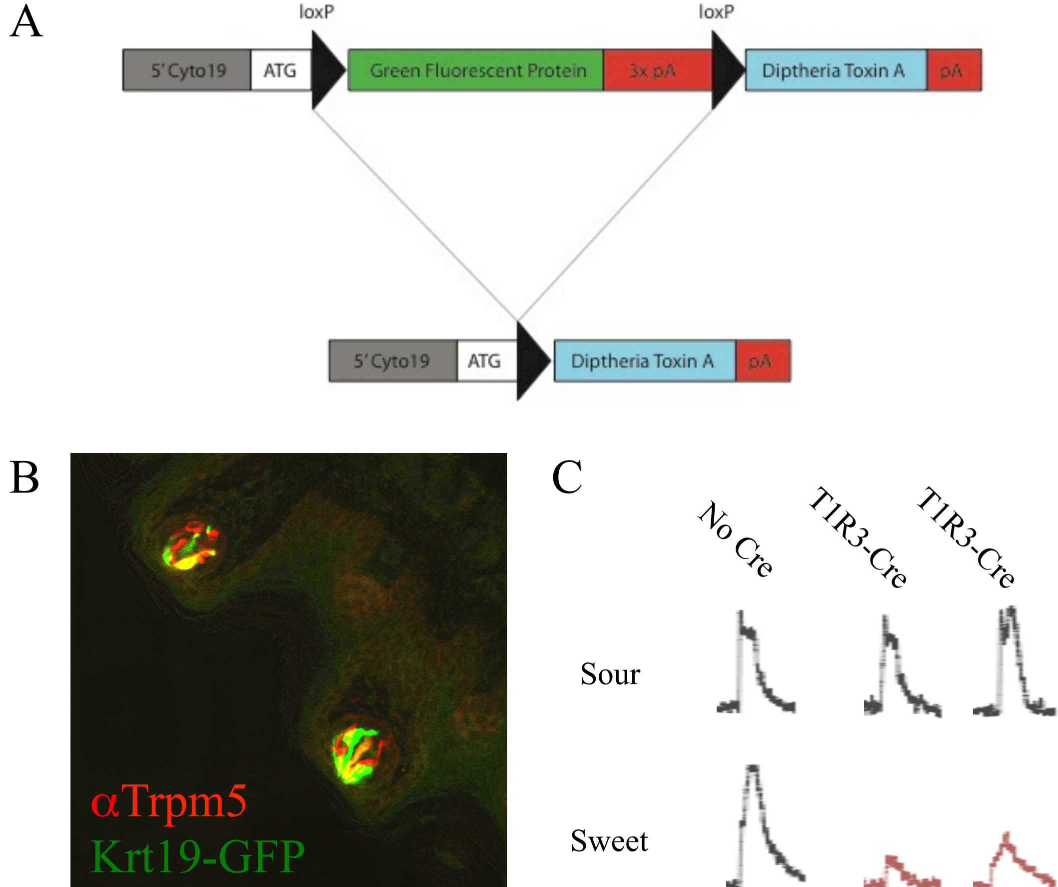


Figure A.7
Intersectional strategy for ablating taste cells. I constructed a bacterial artificial chromosome in which the ATG of cytokeratin-19 is replaced with sequence coding for a green fluorescent protein (GFP) flanked by loxP sites (A). Cre induced recombination disrupts GFP and places the sequence of Diphtheria toxin A subunit into frame. The GFP sequence is followed by a triple polyadenylation sequence to suppress read-through. Analysis of transgenic mice generated with this construct shows expression of GFP in a subset of TRC, including Trpm5+ and Trpm5- TRC(B). Crossing this line to a T1R3-Cre strain resulted in mice exhibiting a partial loss of sweet taste sensitivity as assayed by recording from the chorda tympani nerve (C)

toxin construct is driven in a more robust fashion within taste buds in order to kill all relevant cells; some of the genes identified by this screen as expressed throughout the taste bud might be useful for this purpose.

A.7 Summary

The screen described in this appendix succeeded in identifying known and novel transcripts expressed in the subsets of TRCs. The discovery of specific carbonic anhydrases in taste tissue provided important clues as to the existence of gustatory CO_2 sensing. Other molecules identified may play interesting roles in taste biology, including connectivity, signaling and differentiation. Given the discovery of Gad1 in sour cells, it would be particularly interesting to determine the role, if any, of the neurotransmitter gamma-aminobutyric acid in taste transduction.

What functions could the Entpd2 and Lyzs expressing cells serve in taste biology? One possibility is that these cells serve a general supportive function within the taste bud, in which case ablation of this population might generate a broad deficit in taste signaling. Alternatively, these cells might in fact be receptors for as yet unappreciated taste modalities. Combined with physiological or imaging studies, manipulation of these uncharacterized populations has the potential to reveal novel aspects of taste function.

A.8 Experimental Procedures

Microarray experiments

Taste tissue was isolated from peeled lingual epithelium (Lyall et al., 2001). Total RNA from whole taste papillae and lingual epithelium were prepared using TRIZOL extraction and RNAeasy micro kits (Qiagen). Fungiform and vallate papillae and lingual epithelium were assayed using Mouse WGA 430 2.0 arrays (Affymetrix), while some vallate and lingual epithial control samples were also hybridized to Mouse Gene 1.0 ST microarrays (Affymetrix). Isolated fluorescently labeled TRC were hand picked after papain digestion of lingual epithelium and RNA prepared using the PicoPure RNA isolation kit (Life Technologies). These samples were linearly amplified (Ovation RNA Amplification, Nugen), and assayed using Mouse WGA 430 2.0 microarrays.

Histology

Single label *in situ* hybridizations were performed as described previously (Hoon et al., 1999). Trpm5, Car4 and Entpd2 antibodies were as described (Zhang et al., 2003; Chandrashekar et al., 2009, Dranoff et al., 2002)

Mouse lines

The cytokeratin-19-lox-GFP-lox-DTA construct was constructed by modification of sequence obtained from ROSA-loxp-lacz-loxp-DTA mice (Brockshnieder et al., 2004). LacZ was replaced with EGFP sequence, and a triplex polyadenylation sequence inserted between EGFP and the second loxp site. This cassette was recombined into a bacterial

artificial chromosome (BAC) carrying genomic sequence for Krt19 and flanking regions, such that the start codon of EGFP replaced the start codon of the Krt19 gene. This BAC was purified and used to generate transgenic mice by pronuclear injection. All other mice were as described previously (Chandrashekar et al., 2009).

Appendix B: Somatosensory Encoding of CO_2

B.1 Introduction

This section is not integral to this thesis, bus is presented here as a reference for future stuides of peripheral somatic sensation. The somatosensory system also responds to a limited repertoire of chemical stimuli capable of eliciting a variety of sensations; some familiar examples are the cooling sensation of menthol, the pungency of wasabi, and the burning heat of capsaicin in chili peppers. This recruitment of somatosensory signals by chemicals on the skin is referred to as 'chemesthesis,' and is sometimes referred to as a third chemical sense, supplementing taste and smell (Viana, 2011). The prickly, irritating component of the sensation of carbonation is another familiar example of a chemesthetic stimulus. Recently, *in vitro* characterization of CO_2 responses in dissociated neurons suggests that TrpA1, the wasabi receptor, mediates at least some component of this somatosensory response to carbonation (Wang et al., 2010).

Given the complexity of taste responses to carbonation as revealed by imaging of primary gustatory neurons, I wondered whether a similar methodology might reveal the logic of CO_2 reception when applied to the somatosensory system. To this end, I developed an imaging preparation to directly visualize the activity of neurons within the trigeminal ganglion.

B.2 Visualization of Somatosensory Responses *In Vivo*

First, I confirmed that Thy1-GCaMP transgenic mice express calcium indicator in the trigeminal ganglia. Double staining for GCaMP and a Nissl stain to highlight all neurons demonstrated essentially complete overlap (Figure B.1a). To image cell bodies of GCaMP expressing somatosensory neurons in the live animal, I adapted a surgical approach previously used for imaging of voltage sensitive dyes in the trigeminal ganglion of the rat (Rothermel et al., 2011). After craniotomy and partial decerebration, the entire dorsal surface of the trigeminal ganglia is accessible for imaging with a long working distance objective (Figure B.1b,c).

As the primary stimuli carried by the trigeminal ganglia are mechanical and thermal in nature, I began by examining responses to such stimuli. I observed robust and reproducible activation of neurons in response to cooling of the oral cavity (Figure B.2c,d), and in response to mechanical stimulus (nitrogen puff) applied to either the whisker pad or the oral cavity (Figure B2.a,b). As somatosensory coding is quite complex, comprising a large number of sensory neuron types, a more comprehensive effort would necessary to determine whether all classes of receptor neuron are amenable to characterization by calcium imaging. Nevertheless, this assay provides a powerful platform to elucidate the coding logic for somatosensory stimuli in the trigeminal ganglia.

B.3 Somatosensory Response to Carbonation

I presented anesthetized mice with a carbonation stimulus by perfusing a saturated CO_2 solution into the oral cavity. I alternated CO_2 stimulation with constant perfusion of the oral cavity with an isothermal, un-carbonated solution to avoid introducing

Figure B.1

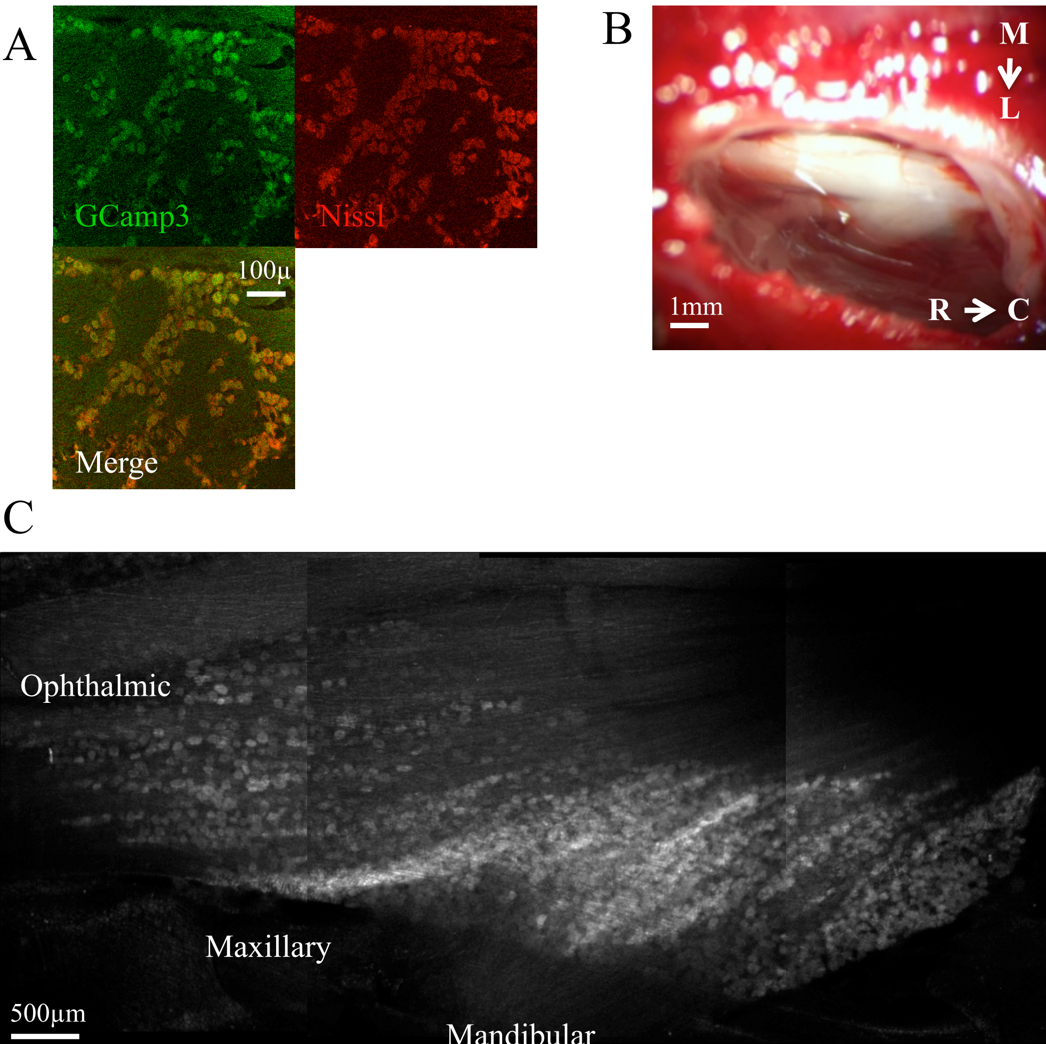
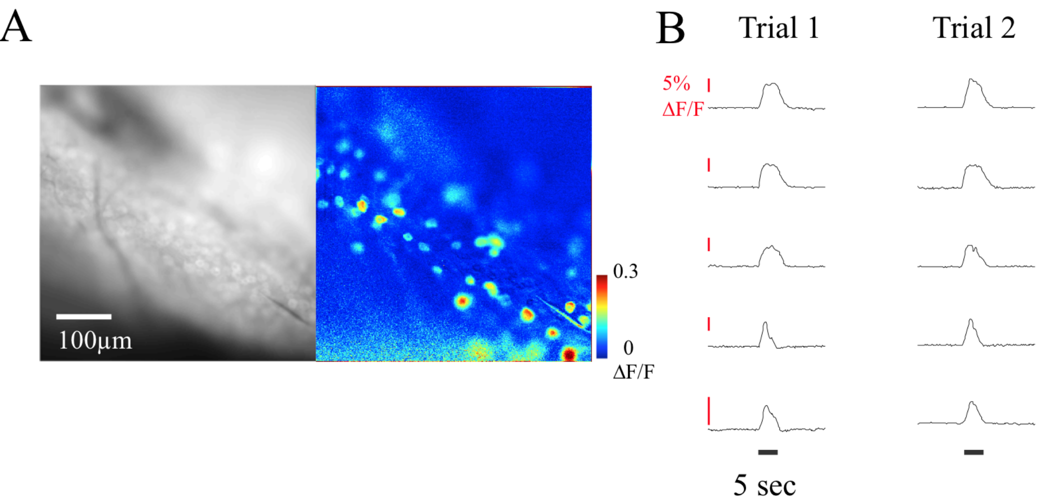


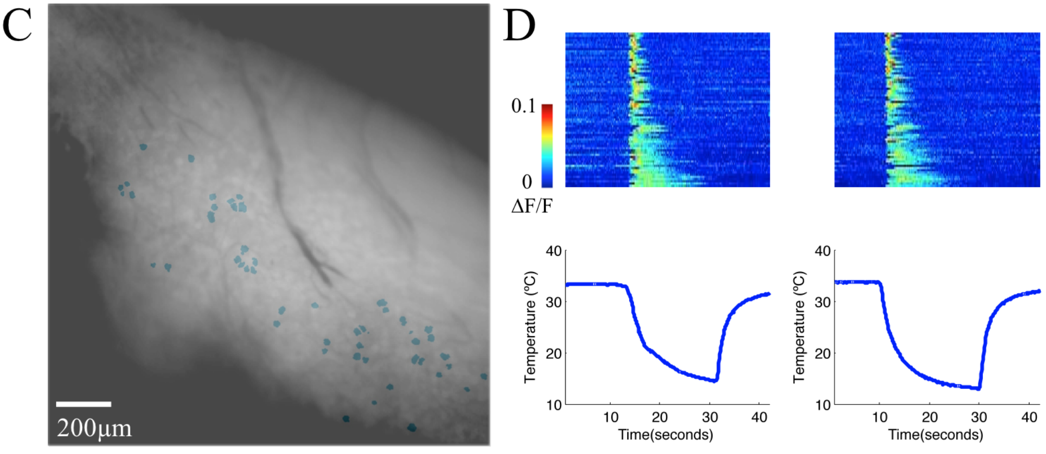
Figure B.1
Calcium imaging preparation for the trigeminal ganglia. Histology of the trigeminal ganglion in Thy1-GCaMP3 mice confirm expression of Gcamp throughout the neuron population, as visualized by fluorescent Nissl staining. (B) shows the trigeminal ganglia *in situ* in an anesthetized mouse after aspiration of the overlying cerebral hemisphere. Two-photon microscopy (C) allows for *in situ* visualization of neuronal soma labeled with GCaMP3. The image is a composite of flattened Z-stacks through the ganglion; entry points of the three nerves transmitting information to the ganglia are labeled.

Figure B.2

Figure B.2
Robust and reliable responses to thermal and mechanical stimuli. GCaMP fluorescence in trigeminal ganglia was imaged during presentation of somatosensory stimuli. Average fluorescence (left) and maximum projection of $\Delta F/F$ (right) within a trigeminal imaging field during application of an intraoral air puff is shown in (A) Fluorescence traces from five of these mechanosensory neurons in consecutive trials are shown in (B). Lines at bottom indicate stimulus window. In (C), an imaging field from another mouse is shown, with cold sensitive neurons highlighted in cyan. Fluorescence responses from all cold sensitive neurons in this field are visualized as a heat map in (D). Each row represents the fluorescence time course for a single neuron in two consecutive trials. The time course of temperature inside the mouth is plotted in the blue trace at the bottom of (D).

Figure B.2





confounding mechanical or thermal stimuli. Stimulation with CO_2 evoked robust activation of a subset of trigeminal neurons. To examine if these represented mechanical responses to bubbles, I transiently introduced nitrogen gas into the oral cavity, generating a vigorous mechanical bubble stimulus, but without a chemical active component. While this treatment evoked widespread and robust response in trigeminal neurons, the population responding to the mechanical stimulus was for the most part distinct from the population responding to CO_2 (Figure B.3). A subset of the N_2 sensitive neurons also responded to CO_2, but in most cases with much lower amplitudes. This likely reflects the less vigorous bubbling produced in the mouth by degassing of CO_2 versus the strong bubbling stimulus of directly injecting N_2 into the oral cavity.

What is the molecular identity of the neuronal population sensitive to CO_2 (but not N_2)? Given the evidence for TrpA1 channels in sensing CO_2 (Wang et al., 2010), the Trpa1 expressing population was a strong candidate. I identified TrpA1 expressing neurons by stimulating the oral cavity with allyl isothiocyanate, a strong TrpA1 agonist (Jordt et al., 2004). In agreement with results obtained *in vitro* (Wang et al., 2010), CO_2 responsive neurons were essentially contained within the population of AITC sensitive neurons, suggesting that TrpA1 expressing neurons are indeed the primary sensors for the chemesthetic detection of carbonation (Figure B.4).

B.4 Summary

Figure B.3

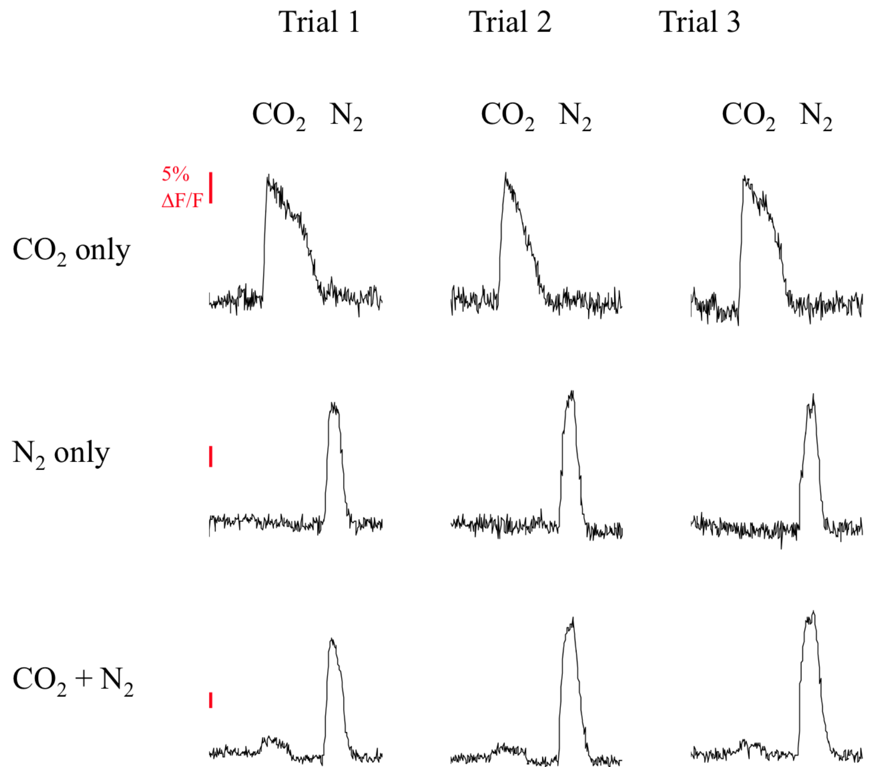


Figure B.3
Responses to CO_2 in mechanically sensitive and insensitive neurons.
Trigeminal neurons were imaged while the mouse was presented with an oral carbonation stimulus followed by a nitrogen puff, a purely mechanical stimulus. Examples of response profiles are shown. A population of CO_2 sensitive neurons (top) respond only to carbonation, while most mechanosensitive neurons only responded to N_2 (middle). A minority of mechanosensitive neurons also exhibited smaller responses to carbonation (bottom trace). Data are representative of observations in 5 animals.

Figure B.4

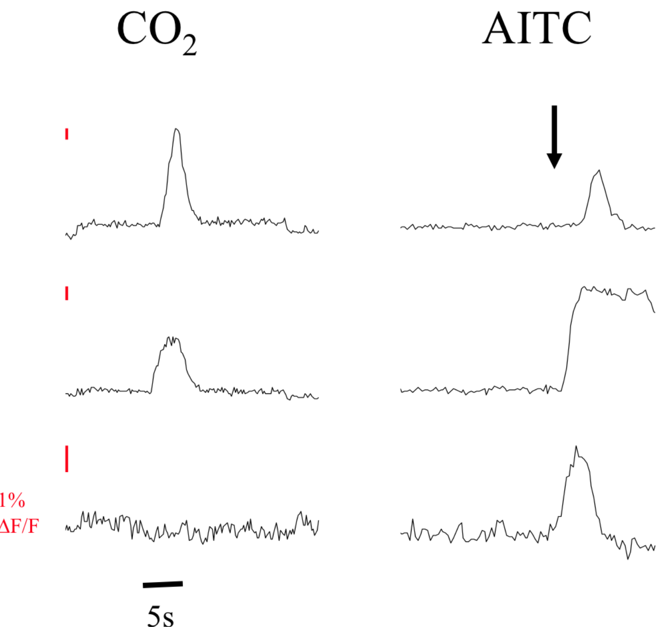


Figure B.4
Allyl isothiocyanate sensitive neurons detect CO_2. Fluorescence traces from three trigeminal neurons presented with carbonation and a chemical irritant. A trigeminal field was imaged as mice were stimulated with CO_2 (traces on left), or allyl isothiocyanate (AITC), a TrpA1 agonist (traces on right). A subset of AITC responsive neurons (5/12 total) were also CO_2 sensitive, while 4/5 CO_2 responders were AITC sensitive.

The preliminary results described here are consistent with a role for TrpA1 expressing neurons in detecting carbonation *in vivo*, which together with the mechanosensory neurons might constitute distinct substrates producing the 'fizzy' mechanical percept of carbonation, and the painful or 'prickly' sensation mediated by nociceptors. The general approach of imaging somatosensory responses should be useful for future studies examining the nature of peripheral coding of diverse somatosensory stimuli, including temperature, touch and pain. The ability to monitor activity across an entire population of sensory neurons may be of particular utility in characterizing peripheral changes in stimulus coding associated with pathologic hyperalgesia and allodynia.

B.5 Experimental Procedures

Transgenic Animals and Staining

Thy1-GCaMP3 animals were as described in the experimental procedures of Chapter 3. To confirm expression in trigeminal neurons, GCaMP3 fluorescence was visualized in fresh frozen sections stained with NeuroTrace fluorescent Nissl stain (Life Technologies).

Surgical Procedures

Mice were anesthetized by intraperitoneal injection of 100mg/Ketamine and 10mg/Kg xylazine, with booster injections applied as needed to maintain surgical depth of anesthesia. Body temperature was monitored and maintained at 37° centigrade by a closed loop system coupled to a heating pad (FHC). The mouse was mounted on a non-traumatic mandibular clamp. A tracheotomy was then performed if intraoral stimulation was to be presented, followed by fixation with dental acrylic of the dorsal skull opposite the ganglia to be imaged. Following curing of acrylic, the mandibular clamp was removed.

Under a stereoscope, a craniotomy was performed of the right cranial hemisphere (if targeting the right trigeminal ganglia). The dura was retracted and cerebral surface maintained moist with phosphate buffered saline. The cerebral hemisphere overlying the trigeminal ganglia was gently aspirated, taking care to avoid major arteries.

Calcium Imaging

Imaging was performed using an Evolve EMCCD camera (Photometrics), with excitation from a X-Cite illuminator passed through GFP filters. Images were obtained at 5 Hz at 512 x 512 resolution, using either 5x or 10x long working distance air objectives. Oral stimuli were applied with the same pressurized perfusion system used for taste stimulation (see Chapter 3). Thermal stimuli were controlled by peltier elements coupled to tubing (TETech), and monitored using an intraoral thermometer (Vernier). Image analysis was essentially as described for two-photon calcium imaging (Chapter 3).