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Dissecting neuronal specification in the Drosophila taste system

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Dissecting Neuronal Specification in the *Drosophila* Taste System

By

Lisa Marie Dennison

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Kristin Scott, Chair
Professor Gian Garriga
Professor Iswar Hariharan
Professor Daniela Kaufer

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Abstract

Dissecting Neuronal Specification in the Drosophila Taste System

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Professor Kristin Scott, Chair

Establishing a complex brain and nervous system requires a wide array of neurons with diverse forms, functions, and characteristics. Developing nervous systems employ many strategies to create this neuronal diversity, and these strategies remain the subject of ongoing investigations in developmental neuroscience.

Neuronal diversity in the taste system of Drosophila melanogaster is important for the appropriate detection of chemosensory signals in the fly’s environment. Drosophila can distinguish sweet substances, bitter compounds, pheromones, and pure water using distinct populations of taste neurons. The correct detection of such substances is critical for correct ingestion of nutritive foods, avoidance of toxins, recognition of appropriate mates, and hydration, all of which are essential for the survival and propagation of the fly. The first part of this thesis focuses on the developmental relationship between these classes of neurons and the signaling pathways used to diversify them. Mosaic analysis revealed at least two stereotyped lineages for taste neurons innervating bristles on the proboscis of the fly. Furthermore, mutant mosaic studies showed that Numb inheritance or lack thereof is necessary for correct cell fate decisions. The involvement of Numb strongly indicates that Notch signaling is the key player in establishing taste neuron diversity.

The second part of this thesis describes an expression screen for transcription factors with specific expression patterns in subsets of taste neurons. The transcription factors activated in each cell type ultimately establish that cell’s unique identity by activating expression of appropriate receptors, axon guidance cues, neurotransmitters, and more. The screen identified two transcription factors, knot and Lim3, with specific expression in bitter-sensing neurons. Results of loss-of-function and gain-of-function studies with each gene indicate that neither one alone is necessary or sufficient to specify the full bitter neuron fate, but it remains possible that each could play a role in subsets of the bitter neuron’s identity not observed by these analyses.
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CHAPTER 1:

Introduction
Overview

Understanding how neurons acquire their identities and how these identities are translated into functional differences is a long-term goal of developmental neuroscience. The *Drosophila* taste system is well suited to addressing this question because there are relatively few functionally distinct taste neuron classes. Taste perception in the fruit fly *Drosophila melanogaster* is mediated by sensory neurons that are specific to different taste modalities. These neurons innervate chemosensory bristles located on the proboscis, legs, wings, internal mouthpart organs, and ovipositor and detect taste stimuli directly via dendrites that are exposed to the environment. Each bristle contains 3 support cells, 1 mechanosensory neuron, and 4 gustatory neurons, each of which is tuned to distinct taste classes including sweet, bitter, water, and pheromones. The neurons that detect sweet and bitter substances express genes of the gustatory receptor (GR) family, while the neurons tuned to water and pheromones express genes of the *pickpocket* (*ppk*) family.

It is known from lineage tracing studies that the eight cells—5 neurons and 3 support cells—that constitute a single bristle share a common lineage, and that the 4 chemosensory neurons all derive from the PIIa cell within this lineage. PIIa divides once to produce PIVa and PIVb, and then each of these precursors divides once to produce the 4 distinct chemosensory neurons. It is still unknown which two classes of chemosensory neurons derive from PIVa and which two derive from PIVb. While the functional and molecular differences between the taste neurons occupying a bristle are apparent, the underlying mechanisms that lead to this neuronal diversity remain unclear.

This thesis proposes that taste neuron classes are specified via a stereotyped lineage and that Notch signaling is required for this process. Notch signaling plays a vital role in neuronal specification in many organisms and has been shown to be important for the specification of different classes of *Drosophila* olfactory neurons, as well as for the specification of the mechanosensory bristles that decorate the body of the fly. I propose that Notch may also be necessary to diversify the taste lineage by asymmetrically activating downstream signals that lead to the expression of distinct taste receptors in neighboring neurons. Dissecting the developmental specification of gustatory neurons will provide general insight into the problem of how neural identities are established and will provide a platform to manipulate neural identity to study connectivity and function in the taste system.

Sensory Organs in *Drosophila*

*Drosophila* possess a number of diverse sensory organs with which they sense their external and internal environments. Proprioception and audition are both perceived by chordotonal organs, a class of internal sensory organs that are linked to the cuticle by attachment cells, rendering them stretch-sensitive. Vision is achieved via photoreceptor cells located within the ommatidia of the compound eye. External sensory organs innervated by a single mechanosensory neuron confer a form of touch sensitivity, while different classes of poly-innervated external sensory organs are used for chemosensation, including gustation and olfaction, separately. While the stimuli perceived by these different sensory organs are quite disparate, the cell lineages and developmental processes utilized to create these organs are strikingly similar.
Proneural genes in sense organs

All sensory organs first express proneural genes in the founder cell of the lineage, the sensory mother cell (SMC) or sensory organ precursor (SOP). These proneural genes are Class A basic-helix-loop-helix (bHLH) transcription factors whose pattern of expression is determined by pre-pattern genes that act upon proneural gene regulatory elements. The proneural gene(s) expressed in the SMC/SOP help determine what type of sensory organ it will become. For instance, the restricted expression of the proneural gene Atonal in the developing eye disc specifies R8 photoreceptors, the founder cells of ommatidia. Atonal also plays a role in specifying both the precursors of chordotonal organs, by repressing the Hox transcription factor Cut, and the precursors of coeloconic olfactory sensilla. Trichoid and basiconic olfactory sensilla are conversely specified by Amos, another proneural gene of the same subtype as Atonal (the Ato class). Meanwhile, proneural genes of the AS-C class, which includes Achaete and Scute, are responsible for the first specification step of mechanosensory and gustatory sensory organs. The achaete-scute complex upregulates expression of Cut, which seems to act as a switch between external and internal sensory organs. Accordingly, ectopic expression of Cut transforms chordotonal organs into external sensory organs, while Cut loss-of-function mutants produce a reciprocal phenotype. SMCs expressing Cut and the paired-box gene Pox-neuro (Poxn) become poly-innervated external sensory organs, specifically, gustatory sensilla, while SMCs expressing Cut without Poxn become mono-innervated external sensory organs, or mechanosensory sensilla.

Lateral inhibition in precursor selection

Lateral inhibition is a common developmental mechanism used to create asymmetry between adjacent cells. At the start, neighboring cells express similar amounts of an activator gene, whose expression will confer a distinct cell fate once it reaches a certain level. This activator gene can also drive expression of itself and of an inhibitory molecule that reduces its expression in neighboring cells. In this manner, small differences in initial expression are quickly amplified and solidified, as the cell with the initial advantage uses positive feedback to reach the necessary threshold to adopt one cell fate while simultaneously preventing its neighbors from doing the same.

The use of lateral inhibition to select the precursors cells sensory organs results in the even spacing of such organs on the body of the fly. This can be seen in the neatly spaced microchaete (mechanosensory) bristles on the notum, as well as in the highly ordered placement of ommatidia in the compound eye. Notch signaling is employed to single out the sensory mother cell (SMC) or sensory organ precursor (SOP) from within a cluster of cells expressing specific proneural genes, which act as the activator genes driving lateral inhibition. The proneural genes have three important functions: 1) they bind to their own enhancers and drive their own expression; 2) they bind to the enhancers of genes that promote the SMC/SOP fate; 3) they drive expression of the Notch ligand Delta. Delta is expressed on the cell surface where it binds to and activates Notch receptors on neighboring cells. Activation of Notch signaling in those cells serves to inhibit the expression of proneural genes, while promoting the alternative fate of an epidermal cell.
After the first cell of a sensory organ is specified via lateral inhibition, one of two different developmental mechanisms is employed: lineage-independent recruitment or lineage-dependent asymmetric cell division.

**Case study of recruitment: Ommatidia of the Drosophila eye**

Lineage-independent recruitment is used to specify cells in developing ommatidia, wherein highly specific, short-range inductive events recruit the remaining photoreceptor neurons and non-neuronal accessory cells. R8, the founder photoreceptor cell determined by lateral inhibition, secretes Spitz, a ligand for the epidermal growth factor receptor (EGFR), which is a receptor tyrosine kinase (RTK). Neighboring cells express EGFR, which dimerizes and is activated upon Spitz binding; in cells which also express the homeobox protein Rough, this EGFR activation leads to the specification of the R2 and R5 photoreceptor neurons. R2 and R5 next recruit R3 and R4 respectively, also via Spitz/EGFR signaling, followed by R1 and R6. The final photoreceptor cell to be specified is R7, which requires activation of EGFR by Spitz in addition to activation of a second RTK, Sevenless (Sev), by its ligand Bride-of-sevenless (Boss). Importantly, Boss is a membrane-tethered ligand located in the apical region of the R8 cell, allowing it to specifically activate the neighboring R7 precursor cell, which also expresses Sev in its apical region. Finally, after the eight photoreceptors are specified, cone cells and pigment cells are recruited to the ommatidium via both Notch and EGFR signaling. The resulting compound eye is composed of highly ordered ommatidia whose cells have no clonal relationship to one another.

**Case study of lineage-dependent asymmetric cell division: Microchaetes**

In contrast to ommatidia, other sensory organ classes are composed of clonally related neurons and support cells. The lineage of the SOPs that form the microchaetes, small mechanosensory bristles on the fly notum, has been extensively studied and has become a prime model for dissecting the intricacies of asymmetric cell division. The SOP, deemed p1, first localizes the adaptor protein Numb to the anterior end of the cell in an actin-dependent manner directed by the Par/aPKC complex. Lethal-giant-larvae (Lgl) also plays a role in the recruitment of Partner-of-numb (Pon) to an anterior cortical crescent, which then further helps to localize Numb and the E3 ubiquitin ligase Neuralized (Neu) anteriorly. p1 then divides within the plane of the epithelium, along the A-P axis, and Numb, Neu, and Pon are thus unequally distributed between the two daughter cells, ending up in the anterior cell, pIIb. This asymmetry renders pIIb the Notch signal-sending cell and pIIa the signal-receiving cell, via mechanisms to be discussed shortly. This process is then reiterated in subsequent divisions. pIIa divides along the A-P axis, segregating Numb to the anterior daughter which becomes the hair cell, while the posterior daughter becomes the socket cell. pIIb rotates its spindle and divides twice, perpendicularly to the plane of the epithelium, along its apical-basal axis. The first division produces a basal glial cell that contains Numb, and an apical pIIIb cell that does not. Finally, pIIIb segregates Numb to its basal daughter, which becomes the neuron, while the apical daughter becomes the sheath cell.
Notch and Numb as cell fate determinants

There are a few different models for the mechanisms by which Numb and Neur lead to the signal-sending and signal-receiving cell fates of pIIb and pIIa, respectively. Numb negatively regulates Notch signaling cell-autonomously, and may do so by acting as an adaptor between the AP-2 endocytic complex, via the α-adaptin subunit, and Notch receptors, leading to their subsequent endocytosis. Thus, with fewer Notch receptors on the cell surface, the ability of the cell to receive Notch signals is compromised. In a second, non-mutually exclusive model, Numb and AP-2 may target the protein Sanpodo (Spdo) for endocytosis; as Spdo has been shown to positively regulate Notch signaling at the cell membrane, its removal also inhibits Notch signal reception. Consequently, the localization of Numb to pIIb represses the signal-receiving fate in that cell, while the lack of Numb in pIIa allows it to keep expressing Notch receptors on the cell surface and thus remain competent to receive signals. Furthermore, at each division in the lineage this process is repeated, wherein sister cells send reciprocal signals to activate the Notch pathway, but due to asymmetric segregation of Numb, only one cell is competent to receive the signal. This leads to two opposing cell fates, one Notch-dependent, one Notch-independent.

In parallel, Neur works in a non-cell-autonomous manner to help establish the fate of the signal-receiving cell. Evidence suggests that Neur ubiquitinates Delta, the membrane-tethered ligand of Notch, which leads to the endocytosis of Delta bound by the extracellular domain of Notch from a neighboring cell. This removal of the Notch extracellular domain is thought to facilitate activation of the Notch intracellular domain (NICD), which goes on to act as a transcriptional regulator. Thus, the localization of Neur in pIIb helps activate Notch signaling in pIIa by ubiquitinating Delta in pIIb and facilitating the release of NICD in pIIa.

Recent elegant work by the Posakony group has shed light on the long-standing question of how further asymmetric segregation of numb is achieved in cells that do not receive Numb in earlier rounds of cell division. For instance, since pIIa does not inherit Numb from pI, how does it then asymmetrically segregate Numb to its own anterior daughter so that it can adopt the fate of the hair cell? Computational work uncovered a Notch-responsive cis-regulatory module within the second intron of the numb gene that activates its transcription. Indeed, precisely because pIIa did not inherit Numb, it can be activated by Notch signaling and subsequently turn on transcription of numb. This allows other downstream targets of Notch to be activated as well before a sufficient amount of Numb protein is produced to pass on to the anterior daughter cell.

Variations on a theme: Notch in chordotonal and olfactory bristle development

The lineages of other clonally related sense organs appear to be variations on the basic microchaete lineage. Individual units of chordotonal organs, known as scolopedia, have one neuron and four specialized support cells that are developmentally analogous to the socket, shaft, sheath, and glial cell of the microchaete lineage. The use of Notch signaling and Numb segregation in the scolopedia lineage also mirrors that of the developing microchaete. While many other factors, including Cut, as previously mentioned, make the internal chordotonal organ very different from the external microchaete, the underlying frameworks of the two lineages echo each other.
Olfactory bristles are structurally much more similar to microchaetes than are chordotonal organs, and as such it stands to reason that Notch and Numb would be used in this lineage as well. Indeed, work in the olfactory system has shown that Notch signaling is important for cell fate at multiple steps within the lineage. Unlike mechanosensory bristles, most olfactory bristles house at least two neurons, one with a Notch-independent cell fate, and one with a Notch-dependent cell fate. Genetic mosaic analysis using MARCM demonstrated that both neurons of a sensillum would adopt the fate of the Notch-dependent neuron in *numb* mutant clones, while both neurons would adopt the fate of the Notch-independent neuron in *mastermind* mutant clones, where Notch activation is disrupted. This cell fate switch affected both axon targeting and olfactory receptor (OR) expression of the neuron.

**Role of Notch in gustatory bristle development**

This thesis investigates the roles that Notch activation and Numb segregation play in the development of gustatory bristles. Gustatory bristles are most structurally similar to olfactory bristles, and previous lineage analysis by BrdU labeling indicated a clonal relationship among the cells of gustatory bristles. These similarities to olfactory bristles made Notch signaling an excellent candidate for setting up the taste neuron lineage. By using the MARCM technique to analyze wild-type development, I found that the specification of the taste neuron classes may follow more than one stereotyped pattern within the same proboscis. Based on analysis of *numb* mutant clones, I found that Notch signaling differentiates daughters of pIVa and pIVb and may also play a roll in the division of earlier precursors. Sugar- and bitter-sensing neurons likely inherit Numb and exhibit Notch-independent cell fates, while the water-sensing does not inherit Numb and is thus dependent on Notch signaling to determine its identity.
References


CHAPTER 2:

Gustatory cell fate decisions are controlled by Notch signaling
Summary

This chapter comprises a detailed investigation of the gustatory neuron lineage in *Drosophila*. Mosaic analysis shows that at least two separate stereotyped lineages exist within the taste bristles on the labellum of the fly. In one lineage, sugar- and water-sensing neurons are derived from a common precursor, and in another lineage, sugar- and bitter-sensing neurons are derived from a common precursor. What signaling pathways are involved in determining the fates of the cells within these lineages? Analysis of clonal populations of taste neurons lacking *numb*, an inhibitor of Notch signaling, indicate that Notch likely plays a prominent role in the differentiation of individual taste neuron classes. Without *numb*, sugar- and bitter-sensing neurons failed to be specified, indicating that Numb inheritance is critical to their normal specification. However, removal of *numb* also leads to the duplication of the water-sensing neuron, suggesting that high Notch signaling likely drives its fate.
Introduction

Understanding how neurons acquire their identities and how these identities are translated into functional differences is a long-term goal of developmental neuroscience. The *Drosophila* taste system is well suited to addressing this question because there are relatively few functionally distinct taste neuron classes. Taste recognition in the fruit fly *Drosophila melanogaster* is mediated by sensory neurons that are specific to different taste modalities. These neurons innervate chemosensory bristles located on the proboscis, legs, wings, internal mouthpart organs, and ovipositor and detect taste stimuli directly via dendrites that are exposed to the environment. Each bristle contains 3 support cells, 1 mechanosensory neuron, and 4 gustatory neurons tuned to distinct taste classes including sweet, bitter, water, and pheromones. The neurons that detect sweet and bitter substances express genes of the gustatory receptor (GR) family, while the neurons tuned to water and pheromones express genes of the pickpocket (ppk) ion channel family.

It is known from lineage tracing studies that the eight cells—5 neurons and 3 support cells—that constitute a single bristle share a common lineage, and that the 4 chemosensory neurons all derive from the pIIIa cell within this lineage. pIIIa divides once to produce pIVa and pIVb, and then each of these precursors divides once to produce the 4 distinct chemosensory neurons. Here, we show that the specification of the taste neuron classes may follow more than one stereotyped pattern within the same proboscis. Furthermore, Notch signaling differentiates daughters of pIVa and pIVb.
Results

There are at least two potential lineages for gustatory neurons

Each labellum of the Drosophila proboscis contains an average of 31 taste bristles, which are organized into three categories: S-type (12-13 bristles), L-type (9 bristles), and I-type (9-10 bristles)\(^6\). Four gustatory neurons innervate S-type and L-type bristles, while only two gustatory neurons innervate I-type bristles. I examined the neurons in two-cell sensilla by mosaic analysis with a repressible cell marker (MARCM)\(^7\). All neurons were labeled with an anti-elav antibody, and all sensilla with 3 elav-positive cells were classified as I-type, as these three cells represent two gustatory neurons and a single mechanosensory neuron. Two-cell clones of sibling neurons were marked while different pairs of taste neurons were labeled in the background. The frequency with which each given pair of taste neurons were marked as sibling cells was analyzed. No sensilla were observed in which the mechanosensory neuron was the sibling of a gustatory neuron. This lineage analysis showed that I-type bristles contain one sugar-sensing neuron and one bitter-sensing neuron, which are sister cells within the I-type lineage (Fig 2.1a).

Using the same mosaic approach to lineage tracing, I also evaluated the lineage of and relationship between the 4 classes of gustatory neurons in S- and L-type bristles, which contain one neuron of each class. Is the relationship between the 4 taste neurons stereotyped or stochastic? If it is stereotyped, which pairs of cells are most closely related to each other in the lineage?

S- and L-type sensilla were identified by the presence of at least four elav-positive cells. Again, two-cell clones of sibling neurons were marked while labeling different pairs of taste neurons in the background. If the pair of taste neurons was located within a two-cell clone, or if both neurons were in the same sensilla but outside of a two-cell clone, they were classified as “together” (sibling cells). If one taste neuron was within a two-cell clone and the other was in the same sensilla but outside of the two-cell clone, they were classified as “apart” (non-sibling cells).

Sugar- and bitter-sensing neurons were marked as sibling cells in ~40% of observed clones and non-sibling cells in ~60% of observed clones (Fig. 2.1a’ & a’’, 2.2b). Conversely, sugar- and water-sensing neurons were marked as siblings in ~80% of observed clones and non-siblings in ~20% of observed clones (Fig. 2.1b & b’, 2.2b). Bitter- and water-sensing neurons were only observed as non-sibling cells (Fig. 2.1c, 2.2b).

From these data, I conclude that bitter- and water-sensing neurons never arise from a common precursor cell, and thus that there is some degree of stereotypy to the gustatory neuron lineage. The observation that sugar-sensing neurons can pair with either water- or bitter-sensing neurons means that there are at least two distinct lineage possibilities (Fig 2.2c,d). It is interesting to note that the ratio of cases in which sugar- and bitter-sensing neurons are together or apart roughly mirrors the ratio of L-type to S-type sensilla respectively. These two classes of bristles may have distinct gustatory neuron lineages, but this possibility remains untested, as it is not currently possible to discern the bristle type of a given sensilla in MARCM analyses. L- and S-type bristles are intercalated on the surface of the labellum, so the locations of their respective sensilla are not spatially separable when dissected proboscises are examined. Furthermore, the repertoire of gustatory bristles may be more complex than we have assumed. Our present hypothesis that one taste neuron of each class innervates L- and S-type bristles may be incorrect or oversimplified, as we typically experimentally label only one to two neuron classes at once. A detailed and complete analysis of the classes of neurons innervating each of the 31 bristles on the
labellum has not been performed, to our knowledge. A greater diversity in the make-up of the neuron classes within each bristle would render the current 2-cell MARCM clone data uninterpretable until these bristle types can be experimentally distinguished.

**Asymmetric inheritance of Numb leads to distinct gustatory neuron fates**

What is the involvement of Notch signaling in establishing the distinct taste neuron fates? Notch signaling plays a vital role in neuronal specification in many organisms and has been shown to be important for the specification of different classes of *Drosophila* olfactory neurons, as well as for the specification of the mechanosensory bristles that decorate the body of the fly. Clones comprising all four taste neurons of a single bristle were selectively marked and mutated with MARCM and the fate of each neuron class was individually assessed within these clones. A null mutation of *numb* (*nb*), a negative regulator of Notch, was employed to investigate the consequences of high levels of Notch signaling on taste neuron fates. Most 4-cell *nb* clones do not contain a sugar-sensing neuron, whereas all 4-cell wild-type clones contain at least one sugar neuron (Fig 2.3a,d). The same phenotype was observed for the bitter sensing neuron: most 4-cell *nb* clones lose this fate entirely, while most 4-cell wild-type clones contain a single bitter neuron (Fig 2.3b,d). Conversely, the water-sensing neuron was duplicated in most 4-cell *nb* clones, as most *nb* clones contain two water-sensing neurons, compared to the single water neuron seen in most wild-type clones (Fig 2.3c,d).

In order to determine the effect of Notch signaling at each round of cell division within the taste cell lineage, two-cell and one-cell *nb* MARCM clones were induced in order to observe the resulting fate of each neuron class. By random chance, 2-cell and 1-cell clones will have zero of any given cell type at least half of the time, so it was important to compare *nb* clones directly to clones of the same size generated in wild-type flies. As in 4-cell clones, most 2-cell clones observed lacked both sugar- and bitter-sensing neurons in much higher proportions than wild-type control clones (Fig 2.3a',b',c'). A significant increase was also observed in the proportion of 2-cell clones containing two water-sensing neurons and a decrease in the proportion containing zero water-sensing neurons, as compared to wild-type control clones (Fig 2.3c',d').

In 1-cell clones, no significant differences were observed between wild-type and *nb* clones (Fig 2.3f). This result makes sense given that any single mutant cell could still inherit Numb from its mother, and thus would still be capable of blocking Notch signaling, were that its normal fate.

Taken together with the wild-type lineage proposed in Fig 2.2c in which sugar and water-sensing neurons are siblings, the 2-cell *nb* clone data indicate that Notch signaling and asymmetric segregation of Numb are required in the last round of cell division, when pIVa and pIVb each divide once to produce the 4 distinct gustatory neurons (Fig 2.7c). The duplication of the water-sensing neuron in *nb* clones indicates that it is specified in response to high levels of Notch signaling (Fig 2.7a). The lack of a sugar-sensing neuron in *nb* clones indicates that it normally inherits Numb, and that the prevention of Notch signaling is necessary for its correct fate specification (Fig 2.7a). The respective phenotypes of sugar- and water-sensing neurons in 2-cell *nb* clones fit well with the lineage model in which they arise from a common pIV precursor cell. In the same lineage model, bitter- and pheromone-sensing neurons arise from the opposing pIV precursor cell. The bitter-sensing neuron likely also inherits Numb to prevent Notch signaling, since its fate specification is prevented in *nb* clones. I would expect the pheromone-sensing neuron to be duplicated at the expense of the bitter-sensing neuron in *nb*
clones (Fig 2.7b), but this has not been tested because we do not yet have a GAL4-independent marker for this cell.

The \( nb^2 \) 4-cell clone data largely follows the trend of the \( nb^2 \) 2-cell clone data, meaning that Notch may not play a clear role when pIIIb divides to produce pIVa and pIVb. If, for instance, high Notch signaling were sufficient to produce the pIVa fate, then we would expect the pIVa lineage to be duplicated in \( nb^2 \) 4-cell clones. And since Notch plays a role in the division of pIVa, we would thus expect the fully formed bristle to contain either 4 water-sensing neurons or 4 pheromone-sensing neurons, instead of only two, as observed (for water). One explanation may be a perdurance of Numb protein in pIIIb, sufficient to produce asymmetric distribution between its daughters upon division, but insufficient to influence the subsequent division. Alternatively, the division of pIIIb could require Notch signaling but not Numb inhibition. In \( C. \ elegans \), Notch signaling is required for the specification and differentiation of the AC and VU cell fates in the gonad, but Numb is not involved. Instead, Notch-mediated lateral specification between the two cells drives stochastic assignment of their cell fates\(^{15} \).

While Notch signaling may still play a role in the pIIIb division, its suppression by Numb is not sufficient for the differentiation of pIVa and pIVb in this lineage model (Fig 2.7a-c).

It is more difficult to interpret the \( nb^2 \) MARCM results in the context of the second lineage model, in which sugar and bitter neurons arise from a common precursor (Fig 2.2d), as they both appear to be specified in low Notch environments. In the context of this model, the 4-cell \( nb^2 \) clone data could indicate that Notch does indeed play a role in the division of pIIIb to produce the pIV precursors. pIVa, which later gives rise to the sugar and bitter sensing neurons, would inherit Numb from pIIIa and inhibit Notch, while pIVb, which later gives rise to the water and pheromone neurons, would have high levels of Notch signaling (Fig 2.7d). In 4-cell \( nb^2 \) clones, the pIVb lineage would be duplicated, resulting in 2 water- and 2 pheromone-sensing neurons within the clone (Fig 2.7e). In this scenario, Notch would not play a role at the next division as well, or we would observe 4 water-sensing neurons instead of two. However, the 2-cell \( nb^2 \) clone data do not fit this model, as the observation of 2-cell \( nb^2 \) clones containing 2 water-sensing neurons indicates that Notch is crucial for the last division. One possible explanation for this discrepancy would be that Notch acts differently upon lineages of different bristle classes, acting only at the pIIIb division in one class and only at the pIV divisions in the opposing class. We cannot currently test this hypothesis without a way to distinguish bristle classes from one another in dissected proboscises.

**Alternate manipulations of the Notch pathway**

In order to better understand the role of Notch in the taste lineage, three additional approaches were employed to manipulate its activity. First, to evaluate the effect of low levels of Notch signaling on taste neuron specification, I utilized a null mutation of \textit{mastermind} (\textit{mam} \textit{\( k^{1514} \))}, a nuclear protein that mediates Notch signaling. As with \( nb^2 \), because \textit{mam} \textit{\( k^{1514} \)} is homozygous lethal, 4-cell, 2-cell, and 1-cell marked clones homozygous for \textit{mam} \textit{\( k^{1514} \)} were generated with MARCM, and the fates of sugar-, bitter-, and water-sensing neurons were individually assessed within these clones. Because these MARCM clones were marked with UAS::CD8-GFP instead of UAS::HUNtdTOMATO, separate wild-type controls were performed. It should be noted that the incidence of wild-type clones lacking any given cell type is higher when the clones are marked with GFP, which may be due to a difference in strength between the green and red signals. Unlike \( nb^2 \) clones, there were no dramatic fate changes within \textit{mam} \textit{\( k^{1514} \)} MARCM clones. There was a slight increase in the proportion of 4-cell...
mam\(^{k1514}\) clones with a single sugar-sensing neuron (65%), as compared to wild-type clones of the same size (58%) (Fig. 2.4a,d). The same slight increase was true for the proportion of 2-cell mam\(^{k1514}\) clones with a single sugar neuron: 58% of mam\(^{k1514}\) clones compared to 43% of wild-type (Fig 2.4a’,e). There was also a slight increase in the proportion of 4-cell mam\(^{k1514}\) clones with zero bitter-sensing neurons (55%), as compared to wild-type clones of the same size with zero bitter sensing neurons (38%) (Fig 2.4b,d). The same increase was observed in the proportion of mam\(^{k1514}\) to wild-type 2-cell clones with zero bitter sensing neurons (88% to 59% respectively) (Fig 2.4b’,e). There were no observed changes in the proportions of water-sensing neurons in clones of any size (Fig 2.4c,c’,d-f). 63% of wild-type and mam\(^{k1514}\) 4-cell clones contain a single water-sensing neuron. 61% of mam\(^{k1514}\) 2-cell clones contain zero water-sensing neurons, as compared to 70% of wild-type, but this difference is not statistically significant. The observation that the fate of water-sensing neurons was unchanged in mam\(^{k1514}\) clones was particularly surprising, since the nb\(^2\) clone data indicate that Notch signaling is important for the specification of the water-sensing neuron, and mam\(^{k1514}\) clones should lack Notch signaling. In the study of the role of Notch signaling in the olfactory lineage, the same allele of mam was used with success; however, results were only presented for clones that encompassed the entire olfactory bristle, including the sensory mother cell (pl).

Perhaps the omission of smaller 4-cell nb\(^2\) clones indicates that no phenotypes were observed in such clones. One explanation for this phenomenon may be a perdurance of Mastermind protein, inherited from mother cells with a functional copy of the gene. Conceivably, a small amount of Mastermind may be sufficient to complex with Notch and activate key fate determination genes. An antibody against Mastermind was recently published\(^{16}\), so I could test this hypothesis by looking for perdurance of the protein in mam\(^{k1514}\) clones.

In a second experiment to evaluate the effects of low Notch levels on taste neuron specification, a Numb-GFP fusion protein was expressed under the control of the OSeg-GAL4 driver, which is expressed in all taste neurons. Overexpression of Numb should suppress the activation of Notch signaling in all taste neurons. When I performed this experiment, I lacked the tools to label any of the taste neurons with a non-GFP marker, with the exception of the bitter-sensing neuron. Since my nb\(^2\) clone data indicate that the bitter-sensing neuron normally inherits Numb, I would not expect to lose the bitter neuron fate upon Numb overexpression. However, I might expect to see extra bitter neurons if Notch repression is sufficient for the bitter fate. But with overexpression of Numb-GFP in all taste neurons, each sensillum contains a single bitter-sensing neuron (Fig. 2.5a), as seen in wild-type flies. One explanation for this result would be that Notch repression is necessary but not sufficient to specify the bitter neuron, and that other signals are required. It is also possible that OSeg-GAL4 drives expression after fate specification occurs. Expression from OSeg-GAL4 can be seen approximately half a day after puparium formation (APF), but not in labial discs. Because all of the taste neuron divisions take place between 0-16 APF, OSeg-GAL4 may miss the window in which it would be effective.

In a converse experiment, the activated form of Notch, its untethered intracellular domain (Notch\(^{\text{intra}}\)), was expressed under the control of OSeg-GAL4, as an alternate way to achieve high levels of Notch in all taste neurons. If OSeg-GAL4 drives expression in the correct developmental window, I would expect to see the same phenotypes observed in nb\(^2\) clones. However, each sensillum contains a single bitter neuron, as observed in wild-type flies, rather than the typical lack of bitter-sensing neurons in nb\(^2\) clones (Fig. 2.5b). Each sensilla also contains a single bitter neuron when UAS-Notch\(^{\text{intra}}\) is expressed under the control of Gr66a-GAL4, a driver specific to the bitter neuron (Fig. 2.5c). This indicates that while suppression of
Notch signaling is necessary to specify the bitter neuron, such suppression is not required to maintain the bitter neuron fate.

**Notch-mediated lateral inhibition controls taste bristle precursor selection**

Notch, a temperature-sensitive mutant of Notch, is another available tool for manipulation of Notch signaling. Because Notch is inactivated when the flies are heated to 30°C, pupae were heat-shocked for pulses ranging from 10 minutes to 6 hours during the window in which taste bristle precursors are dividing (0-16APF). While this technique did not prove to be a fine enough manipulation to study the effect on individual bristles, a substantial increase in bristle number as compared to wild-type was observed when flies were heat-shocked at 0APF (Fig. 2.6a,b). These extraneous bristles appear to be innervated normally, as observed by a pan-neuronal marker and a marker for the bitter-sensing neuron. These results indicate that Notch-mediated lateral inhibition controls the selection of taste bristle SMCs in the labial imaginal disc (Fig 2.6c). Removal of Notch relieves this inhibition and results in extra SMCs and fewer cuticle cells. This finding is not surprising, given that SOP/SMC precursor cells in the eye disc and the notum are selected in the same manner.
Conclusions

The studies described in this chapter represent a detailed analysis of the gustatory neuron lineage and its utilization of Notch signaling to delineate distinct cell fates. Based on the current data, I conclude that there is some degree of stereotypy in the development of this lineage, but that there is probably more than one way in which it can proceed. My data fit two different lineage models, which may represent two different classes of bristles. It would be interesting to see if these two classes are indeed L-type and S-type bristles. Since gustatory bristles are specified in three overlapping waves, the timing of my heat shock may bias the results toward one type of bristle over others, which might explain why sugar- and water-sensing neurons are found together in 2-cell clones so frequently.

However, a second interpretation of the data is that our tools are imperfect for addressing this problem, precluding a clear and accurate answer. Promoter-GAL4 and LexA transgenes do not always faithfully recapitulate the expression pattern of the target gene, and any mislabeling by the GAL4 and LexA lines marking each taste neuron class could significantly confuse the results. While cell-specific antibodies for each taste neuron class would provide much cleaner results, currently only an antibody for the bitter neuron exists, and our attempts to generate antibodies for the other three neurons were unsuccessful. The MARCM experiments to study the wild-type lineage require at least 9 different transgenes in a single fly, and each line takes many months to build or rebuild upon incorporation of improved drivers or reporters. While including a third driver-reporter system (Q), in order to concurrently label two classes of taste neurons in different colors might provide additionally clarity, it is unfortunately not genetically feasible. We also currently lack a GAL4-independent reporter for the pheromone-sensing neuron, and the two drivers I made were unsuccessful. I am currently trying whole mount in situ for ppk23 on MARCM-labeled proboscises in order to definitively determine how the pheromone-sensing neuron fits into the wild-type lineage.

By manipulating Notch signaling in a number of different ways, I showed that Notch plays an important role at multiple levels of gustatory bristle development and specification. At the very beginning of its development, Notch is responsible for selecting the cell that will become the bristle precursor, or SMC. And at the final round of cell division within the gustatory neuron branch of the lineage, Notch likely differentiates the daughters of pIVa and of pIVb, which asymmetrically inherit Numb. However, if Numb has Notch-independent functions, it is also possible that asymmetric Numb inheritance is required for the differentiation of pIVa and pIVb, while Notch signaling is not. It is currently still unclear how and if Notch functions at the steps in between, but our current tools may hinder further dissection of its roles at this time. It would be very useful to have drivers specific to the various precursors within the bristle lineage, in order to precisely add or remove Notch at each stage. Also, a GAL4-independent reporter for the pheromone-sensing neuron would again fill in crucial gaps in our current knowledge. If the model I propose in Figure 2.7b is correct, I would expect to see a duplication of the pheromone neuron fate in 4-cell nb^2 clones, as well as in 2-cell nb^2 clones. Whole mount in situ are still currently our best bet for testing this hypothesis.

Finally, it is also clear that more than just Notch is at work in the specification of the gustatory neurons. An interesting possibility is that Numb has a Notch-independent function. Given that only weak phenotypes were observed when Notch was manipulated more directly in mam^ki514 clones, perhaps Numb inheritance regulates cell fate in another manner. My nb^2 MARCM experiments indicate that sugar- and bitter-sensing neurons both inherit Numb and
presumably inhibit Notch signaling in order to specify their fates. But another factor must be inherited or expressed so that sugar and bitter neurons adopt distinct fates from one another. It would be useful to know the set of transcription factors specific to each cell type to further understand what drives their fate decisions, and this work is the focus of Chapter 3.
Materials and Methods

Clonal analysis

All wild-type and mutant clones were generated using the MARCM technique, as previously described. To label GRNs, one of the following Gal4 drivers was used: elav-Gal4, OSeg-Gal4. Clones were marked with one of the following UAS-reporters: UAS-HUNT::tdTOMATO (a gift from Steven Stowers, Montana State University), UAS-mCD8::GFP. Individual taste neurons were labeled with the following LexA drivers and/or direct promoter-GFP fusions: ppk28-LexA on X or III, Gr66a-LexA, Gr64fLexA (a generous gift from Hubert Amrein prior to publication), Gr66a-IRES-GFP. The following LexAop-reporters were used: LexAop-rCD2::GFP, LexAop-myr::cherry. Bitter-sensing neurons were sometimes labeled with an antibody against Gr33a (described in Immunostaining methods section).

MARCM clones were induced by a 1-hr heat shock at 37°C or 37.5°C. Larger (4+ cell) clones were induced by heat-shocking at 0 h APF, while smaller (1-2 cell) clones were induced by heat-shocking slightly later in development, between 2 to 6 h APF.

Fly strains

The genotypes of the flies used for various MARCM experiments are as follows:
1. wild-type clones with bitter and sugar neurons labeled: y w hsFLP122/Gr66a-LexA; FRT40A/tubP-Gal80 FRT40A; OSeg-Gal4, UAS-HUNT::tdTOMATO/Gr64fLexA, LexAop-rCD2::GFP
2. wild-type clones with bitter and water neurons labeled: y w hsFLP122/Gr66a-LexA; FRT40A/tubP-Gal80 FRT40A; OSeg-Gal4, UAS-HUNT::tdTOMATO/ppk28-LexA, LexAop-rCD2::GFP
3. wild-type clones with sugar and water neurons labeled: y w hsFLP122/ppk28-LexA; FRT40A/tubP-Gal80 FRT40A; OSeg-Gal4, UAS-HUNT::tdTOMATO/ppk28-LexA, LexAop-rCD2::GFP
4. Red wild-type clones with only bitter neurons labeled: y w hsFLP122; FRT40A/tubP-Gal80 FRT40A; elav-Gal4, UAS-HUNT::tdTOMATO, Gr66a-IRES-GFP/ elav-Gal4, UAS-HUNT::tdTOMATO
5. Green wild-type clones with only bitter neurons labeled: y w hsFLP122/ w or Y; FRT G13/FRT G13 tubP-Gal80; elav-Gal4/UAS-mCD8::GFP
6. Red wild-type clones with only sugar neurons labeled: y w hsFLP122/ w or Y; FRT40A/tubP-Gal80 FRT40A; OSeg-Gal4, UAS-HUNT::tdTOMATO/Gr64fLexA, LexAop-rCD2::GFP
7. Green wild-type clones with only sugar neurons labeled: y w hsFLP122/ w or Y; FRT G13 UAS-mCD8::GFP/FRT G13 tubP-Gal80; elav-Gal4 /Gr64fLexA, LexAop-myr::cherry
8. Red wild-type clones with only water neurons labeled: y w hsFLP122/ w or Y; FRT40A/tubP-Gal80 FRT40A; OSeg-Gal4, UAS-HUNT::tdTOMATO/ppk28-LexA, LexAop-rCD2::GFP
9. Green wild-type clones with only water neurons labeled: y w hsFLP122/ w or Y; FRT G13 UAS-mCD8::GFP/FRT G13 tubP-Gal80; elav-Gal4 /ppk28-LexA, LexAop-myr::cherry
10. nb2 clones with bitter neurons labeled: y w hsFLP122/ w or Y; nb2 FRT40A/tubP-Gal80 FRT40A; elav-Gal4, UAS-HUNT::tdTOMATO, Gr66a-IRES-GFP/ elav-Gal4, UAS-HUNT::tdTOMATO
11. nb2 clones with sugar neurons labeled: y w hsFLP122/ w or Y; nb2 FRT40A/tubP-Gal80 FRT40A; OSeg-Gal4, UAS-HUNT::tdTOMATO /Gr64fLexA, LexAop-rCD2::GFP
(12) $nb^2$ clones with water neurons labeled: $y\ w\ hsFLP122/w\ or\ Y;\ nb^2\ FRT40A/tubP-Gal80\ FRT40A;\ OSeg-Gal4,\ UAS-HUNT::tdTOMATO/ppk28-LexA,\ LexAop-rCD2::GFP$

(13) $mam^{kl514}$ clones with sugar neurons labeled: $y\ w\ hsflp122/w\ or\ Y;\ FRTG13\ mam^{kl514}\ UAS-mCD8::GFP/FRTG13\ tubP-Gal80;\ elav-Gal4/Gr64f,\ LexAop-myr::cherry$

(14) $mam^{kl514}$ clones with water neurons labeled: $y\ w\ hsflp122/w\ or\ Y;\ FRTG13\ mam^{kl514}\ UAS-mCD8::GFP/FRTG13\ tubP-Gal80;\ elav-Gal4/\ ppk28-LexA,\ LexAop-myr::cherry$

(15) $mam^{kl514}$ clones with bitter neurons labeled: $y\ w\ hsflp122/w\ or\ Y;\ FRTG13\ mam^{kl514}\ UAS-mCD8::GFP/FRTG13\ tubP-Gal80;\ elav-Gal4/TM6B$

The fly strains used for non-MARCM experiments are as follows:

(1) $y\ N\ts^g\ f^j/Y;\ Gr66a-IRES-GFP/+$

(2) $UAS-numbGFP\ 2.2/\ OSeg-GAL4$

(3) $UAS-Notch^{B2A2 (intra)}/\ OSeg-GAL4;\ Gr66a-IRES-GFP/TM6$

**Immunostaining**

Labeling of the proboscis was performed as previously described. Primary antibodies used were: mouse anti-GFP (1:100, Sigma), rabbit anti-GFP (1:100, Invitrogen), rabbit anti-RFP (1:500, Clontech, or 1:200, BioVision) rabbit anti-Gr33a (1:200, 20), rat anti-elav (concentrated form, 1:50, DSHB).


Figure Legends

Figure 2.1. Mosaic analysis of the wild-type gustatory neuron lineage
Clonally-related cells were marked with UAS::HUNTtdTOMATO driven by OSeg-GAL4, using the MARCM technique, and the signal was amplified with rabbit anti-RFP (Clontech) and anti-rabbit Alexa 568, shown in magenta. Subsets of taste neurons were marked with LexAop::rCD2-GFP, amplified with mouse anti-GFP and anti-mouse Alexa 488, shown in green. Rat anti-elav and anti-rat Alexa 647 were used to label all neurons, shown in blue. Asterisks designate 2-cell clones and dashed white lines encompass a single sensillum, except in anti-elav panels, where they also outline each visible cell. All scale bars = 5µm.

Analysis of L-type sensilla, as determined by presence of only 3 elav-positive neurons (2 gustatory + 1 mechanosensory neuron).
a) Gr64f^{ΔexA} and Gr66a-LexA drive expression of rCD2-GFP in sugar and bitter neurons, respectively. Two-cell MARCM clones were analyzed. If the 2-cell clone contained two GFP-positive neurons, the neurons were classified as “together.” n=18.

Analysis of L- and S-type sensilla, as determined by presence of at least 4 elav-positive neurons.
a’-a”) Gr64f^{ΔexA} and Gr66a-LexA drive expression of rCD2-GFP in sugar and bitter neurons, respectively. Two-cell MARCM clones were analyzed. If the 2-cell clone contained two GFP-positive neurons, or if two GFP-positive neurons were in the same sensilla as but outside of a two-cell clone, they were classified as “together” (a). If one taste neuron was within a two-cell clone and the other was in the same sensilla but outside of the two-cell clone, they were classified as “apart” (a’).

b-b’) Gr64f^{ΔexA} and ppk28-LexA drive expression of rCD2-GFP in sugar and water neurons, respectively. 2-cell MARCM clones were analyzed and classified as in (a’-a’). In (b), the 5th elav-positive cell within the sensillum is the mechanosensory neuron.
c) Gr66a-LexA and ppk28-LexA drive expression of rCD2-GFP in bitter and water neurons, respectively. MARCM clones were analyzed and classified as in (a’-a’). Bitter and water neurons were only observed “apart.”

Figure 2.2. Two potential lineages for gustatory neurons
a) The lineage of a single gustatory bristle. Boxed area enlarged in (c).
b) Graphical representation of all two-cell wild-type MARCM clones, examples of which were shown in Figure 2.1. Sugar and water neurons “together” n=35, “apart” n=8. Sugar and bitter neurons “together” n=6, “apart” n=9. Water and bitter neurons “together” n=0, “apart” n=8.
c) Two proposed lineages of the gustatory bristles, one in which sugar and water neurons are siblings (left), and one in which sugar and bitter neurons are siblings (right).

Figure 2.3. Asymmetric inheritance of Numb leads to distinct gustatory neuron fates
nb^2 clones were marked with UAS::HUNTtdTOMATO driven by elav-GAL4, using the MARCM technique, and the signal was amplified with rabbit anti-RFP (Clontech) and anti-rabbit Alexa 568, shown in magenta. Subsets of taste neurons were either marked with LexAop::rCD2-GFP or with a promoter-fusion-GFP, and then amplified with mouse anti-GFP and anti-mouse Alexa 488, shown in green. Dashed white lines encompass a single sensillum. All scale bars = 5µm. Fisher’s exact test used for all statistical analyses.
a) Gr64f^{ΔexA} drives expression of rCD2-GFP in sugar neurons. The outlined MARCM clone encompasses all of the taste neuron within the sensillum. The merge shows a marked nb^2
sensillum with no GFP-positive cells, and unmarked neighboring sensilla each with a single cell expressing GFP. In (a’), zero GFP-positive cells are observed within a 2-cell clone.
b) Gr66a-IRES-GFP marks bitter neurons. The outlined MARCM clone encompasses all of the taste neuron within the sensillum. The merge shows a marked nb2 sensillum with no GFP-positive cells, and unmarked neighboring sensilla each with a single cell expressing GFP. In (b’), zero GFP-positive cells are observed within a 2-cell clone.
c) ppk28-LexA drives expression of rCD2-GFP in water neurons. The outlined MARCM clone encompasses all of the taste neuron within the sensillum. The merge shows a marked nb2 sensillum with two GFP-positive cells, and unmarked neighboring sensilla each with a single cell expressing GFP. In (c’), two GFP-positive cells are observed within a 2-cell clone.
d) Graphical representation of the frequency with which 0-4 neurons of each taste neuron class are found within wild-type and nb2 4-cell clones. For sugar, WT n=68, nb2 n=173, p=<.001. For bitter, WT n=58, nb2 n=143, p=<.001. For water, WT n=39, nb2 n=151, p=<.001.
e) Graphical representation of the frequency with which 0-2 neurons of each taste neuron class are found within wild-type and nb2 2-cell clones. For sugar, WT n=50, nb2 n=135, p=<.001. For bitter, WT n=47, nb2 n=78, p=<.001. For water, WT n=51, nb2 n=103, p=<.001.
f) Graphical representation of the frequency with which 0-1 neurons of each taste neuron class are found within wild-type and nb2 1-cell clones. For sugar, WT n=7, nb2 n=86, p=<.001. For bitter, WT n=24, nb2 n=25, p=<.001. For water, WT n=17, nb2 n=46, p=<.001.

Figure 2.4. mastermind does not have a clear effect on gustatory neuron fates

mam<sup>k1514</sup> clones were marked with UAS::CD8-GFP driven by elav-GAL4, using the MARCM technique, and the signal was amplified with mouse anti-GFP and anti-mouse Alexa 488, shown in green. Subsets of taste neurons were either marked with LexAop::myr-cherry or with a cell-specific antibody (anti-Gr33a), and then amplified with rabbit anti-RFP or rabbit anti-Gr33a and anti-rabbit Alexa 568, shown in magenta. Dashed white lines encompass a single sensillum. All scale bars = 5µm. Fisher’s exact test used for all statistical analyses.
a) Gr64A<sup>lexA</sup> drives expression of myr-cherry in sugar neurons. The outlined MARCM clone encompasses all of the taste neuron within the sensillum. The merge shows a marked mam<sup>k1514</sup> sensillum with one myr-cherry-positive cell, and unmarked neighboring sensilla each also with a single cell expressing myr-cherry. In (a’), one myr-cherry-positive cell is observed within a 2-cell clone.
b) anti-Gr33a marks bitter neurons. The outlined MARCM clone encompasses all of the taste neuron within the sensillum. The merge shows a marked mam<sup>k1514</sup> sensillum with no Gr33a-positive cells, and unmarked neighboring sensilla each with a single cell expressing Gr33a. In (b’), zero Gr33a-positive cells are observed within a 2-cell clone.
c) ppk28-LexA drives expression of myr-cherry in water neurons. The outlined MARCM clone encompasses all of the taste neuron within the sensillum. The merge shows a marked mam<sup>k1514</sup> sensillum with one myr-cherry-positive cell. In (c’), zero myr-cherry-positive cells are observed within a 2-cell clone.
d) Graphical representation of the frequency with which 0-4 neurons of each taste neuron class are found within wild-type and mam<sup>k1514</sup> 4-cell clones. For sugar, WT n=15, mam<sup>k1514</sup> n=48, p=<.05. For bitter, WT n=24, mam<sup>k1514</sup> n=67, p=<.01. For water, WT n=22, mam<sup>k1514</sup> n=11, p=0.3.
e) Graphical representation of the frequency with which 0-2 neurons of each taste neuron class are found within wild-type and mam<sup>k1514</sup> 2-cell clones. For sugar, WT n=28, mam<sup>k1514</sup> n=76,
p=<.05. For bitter, WT n=59, mam\textsuperscript{kl1514} n=64, p=<.001. For water, WT n=40, mam\textsuperscript{kl1514} n=23, p=0.17.

f) Graphical representation of the frequency with which 0-1 neurons of each taste neuron class are found within wild-type and mam\textsuperscript{kl1514} 1-cell clones. For sugar, WT n=8, mam\textsuperscript{kl1514} n=64, p=<.13. For bitter, WT n=33, mam\textsuperscript{kl1514} n=34, p=<.37. For water, WT n=32, mam\textsuperscript{kl1514} n=25, p=<.13.

Figure 2.5. Transgenic manipulations of Notch signaling
a) Expression of UAS::Numb-GFP was driven in all taste neurons by OSeg-GAL4, amplified by mouse anti-GFP and anti-mouse Alexa 488, shown in green. Bitter neurons were labeled with rabbit anti-Gr33a and anti-rabbit Alexa 568, shown in magenta. Overexpression of Numb-GFP in all taste neurons does not change the number of bitter neurons per sensillum. 75% of OSeg::Numb-GFP sensilla contain one bitter neuron, n=63. Scale bar = 10\mu m.

b) Expression of UAS::\textit{Notch}\textsuperscript{intra} was driven in all taste neurons by OSeg-GAL4. Bitter neurons were labeled by Gr66a-IRES-GFP, amplified by rabbit anti-GFP and anti-rabbit Alexa 488, shown in white. Overexpression of \textit{Notch}\textsuperscript{intra} in all taste neurons does not change the number of bitter neurons per sensillum (30 bitter neurons observed/labellum). Scale bar = 10\mu m.

c) Expression of UAS::\textit{Notch}\textsuperscript{intra} was driven in bitter neurons by Gr66a-GAL4. Bitter neurons were labeled by Gr66a-IRES-GFP, amplified by rabbit anti-GFP and anti-rabbit Alexa 488, shown in white. Overexpression of \textit{Notch}\textsuperscript{intra} in bitter neurons does not change the number of bitter neurons per sensillum (an average of 28 bitter neurons observed/labellum). Scale bar = 10\mu m.

Figure 2.6. Notch-mediated lateral inhibition controls taste bristle precursor selection
a) Wild-type proboscises have 31 bristles per labellum. Gr66a-IRES-GFP marks bitter neurons, and elav-GAL4 driving UAS::HUNT\textit{tdTOM} marks all neurons. Scale bar = 20\mu m.

b) \textit{Notch}\textsuperscript{ts} was inactivated at 0APF with heat-shock. The number of bristles increases to ~51 per labellum. These bristles are each innervated with multiple neurons, marked with anti-elav, and include a single bitter neuron, marked with Gr66a-IRES-GFP. Scale bar = 20\mu m.

c) A graphic representation of Notch-mediated lateral inhibition in a wild-type labial disc. Cells with the highest levels of Notch inhibit Notch activity in their neighbors. Cells with high levels of Notch activity become SMCs and then develop into normal taste bristles, while cells with low levels of Notch activity become cuticle cells.

d) When Notch is inactivated with N\textsuperscript{ts}, all cells become cuticle cells.

Figure 2.7. A model for Notch regulation of gustatory neuron specification
Legend indicates cells with high levels of Notch activity in wild-type diagrams (N), cells with high levels of Notch activity in \textit{nb}\textsuperscript{2} clone diagrams (-/-), and cells which inherit Numb protein in wild-type diagrams (orange border).

a) The model for the majority of 4-cell bristles, in which sugar- and water-sensing neurons are sibling cells. The water- and pheromone-sensing neurons have high levels of Notch signaling.

b) In \textit{nb}\textsuperscript{2} 4-cell clones, pIVa and pIVb lack Numb protein, so each of their two daughters adopt the high Notch fate (water- or pheromone-sensing neurons, respectively).

c) In a pIVa \textit{nb}\textsuperscript{2} 2-cell clone, pIVa lacks the Numb protein, so both of its daughters adopt the high Notch fate and become water-sensing neurons.
d) The model for a second, less frequently observed class of bristles, in which sugar- and bitter-sensing neurons are sibling cells. The pIVa precursor inherits Numb and has low levels of Notch signaling, while the pIVb cell lacks Numb and has high levels of Notch signaling.

e) In nb2 4-cell clones, pIIIb lacks Numb protein, so both of its daughters adopt the pIVb high Notch fate. The pIVb lineage is thus duplicated, resulting in two water-sensing neurons and two pheromone-sensing neurons. As indicated, 2-cell nb2 clone data do not fit this model.
Figures

Figure 2.1. Mosaic analysis of the wild-type gustatory neuron lineage

(Images of cellular analysis and lineage tracing with various fluorescence markers and annotations for different conditions.)
Figure 2.2. Two potential lineages for gustatory neurons

a. Two potential lineages for gustatory neurons

b. Bar chart showing the percentage of neurons responding to different stimuli:
- Sugar + Water: Approximately 70%
- Sugar + Bitter: Approximately 85%
- Bitter + Water: Approximately 95%

C. Proposed lineages for 60-80% of bristles:
- Sugar and Water
- Bitter and Pheromone

Proposed lineage for 20-40% of bristles:
- Bitter and Water
Figure 2.3. Asymmetric inheritance of Numb leads to distinct gustatory neuron fates
Figure 2.4. *mastermind* does not have a clear effect on gustatory neuron fates.
Figure 2.5. Transgenic manipulations of Notch signaling

a. OSeg::Numb-GFP    anti-Gr33a    Merged

b. Gr66a-IRES-GFP

OSeg-GAL4, UAS-Notch

intra

c. Gr66a-IRES-GFP

Gr66a-GAL4, UAS-Notch

intra
Figure 2.6. Notch-mediated lateral inhibition controls taste bristle precursor selection
Figure 2.7.
Proposed lineage for 60-80% of bristles

a. pIVa  
   \[\text{Sugar} \rightarrow \text{Water} \rightarrow \text{Pheromone}\]  
   pIIlb

b. \(\text{numb}^{-/-}\)  
   4-cell clone  
   \(\text{pIVa} \rightarrow \text{Water} \rightarrow \text{Water} \rightarrow \text{Pheromone?}\)  
   pIIlb

\(\text{pIVb} \rightarrow \text{Pheromone?}\)

c. \(\text{numb}^{-/-}\)  
   2-cell clone  
   \(\text{pIVa} \rightarrow \text{Water} \rightarrow \text{Water}\)  
   pIIlb

\(\text{pIVb} \rightarrow \text{Water}\)

Proposed lineage for 20-40% of bristles

d. pIVa  
   \[\text{Sugar} \rightarrow \text{Bitter} \rightarrow \text{Water} \rightarrow \text{Pheromone}\]  
   pIIlb

e. \(\text{numb}^{-/-}\)  
   4-cell clone  
   \(\text{pIVa} \rightarrow \text{Bitter} \rightarrow \text{Water} \rightarrow \text{Pheromone?}\)  
   pIIlb

\(\text{pIVb} \rightarrow \text{Pheromone?}\)

2-cell clone data do not fit this model

\(\text{N}\) High Notch activity

\(\text{numb}^{-/-}\) High Notch activity

Numb protein Low Notch activity
CHAPTER 3:

A screen for transcription factor expression in gustatory neurons
Summary

A cell’s identity is determined in part by its expressed profile of transcription factors, which in turn activate the genes that make the cell unique. Knowing which transcription factors are specific to each taste neuron class could uncover the key players in the cell fate determination of each neuron. To address this, I screened for specific transcription factor expression in gustatory neurons using in situ hybridization and immunohistochemistry. I found that two transcription factors, knot and lim3, are selectively expressed in bitter-sensing taste neurons. Further analysis of these genes, through loss-of-function and gain-of function experiments, showed no discernable phenotypes in taste neuron fate decisions.
Introduction

How does a neuron determine its unique identity and defining characteristics during development? Signaling pathways like Notch play a significant role in guiding a neuron’s fate determination down the correct path. However, it is the specific set of transcription factors activated downstream of pathways like Notch that dictate expression of the full complement of genes that give a neuron its identity. These genes may include specific receptors, enzymes to produce specific neurotransmitters, and axon path finding cues. There is significant precedent for direct control of transcription factors on each of these aspects of neural identity. For instance, in Drosophila olfactory receptor neurons, a recent study showed that seven transcription factors are continuously and differentially required, in a combinatorial code, for the specific expression of at least 32 odorant receptors. Similarly, a combinatorial code of Lim-homeodomain (LIM-HD) and POU domain transcription factors are required for axonal target specificity between various motoneuron subclasses in the embryonic ventral nerve cord (vNC). In a separate cell lineage within the vNC, which produces serotonergic neurons, the zinc finger transcription factors Eagle and Huckebein are necessary to determine serotonergic cell fate and are required for serotonin expression.

The four gustatory neurons that comprise a single bristle express non-overlapping sets of receptors: sweet- and bitter-sensing neurons express different members of the gustatory receptor (GR) family of genes, while water- and pheromone-sensing neurons express different members of the pickpocket (ppk) ion channel family. The four classes of neurons also project to different regions of the subesophageal ganglion (SOG), the primary relay for taste information in the brain, and drive different behaviors when activated. There may also be differential neurotransmitter expression between the four neurons, but promiscuous neurotransmitter-GAL4 lines and weak antibody staining in GRN cell bodies preclude careful investigation into this question. This work aims to discover transcription factors unique to each class of taste neuron, and to assess what role such genes may play in determining each taste neuron’s identity.

In order to address these questions, I began by taking a candidate gene approach using a microarray data set of genes found to be upregulated in taste tissue. A number of excellent candidates emerged, including 7 transcription factors that fell into three different classes with prominent roles in neural fate specification: bHLH domain proteins, POU-domain proteins, and LIM-HD proteins. I performed an expression screen on all transcription factors in the data set, followed by loss-of-function and gain-of-function experiments on those with specific expression patterns.

A systematic and unbiased RNAi knock-down screen of all of the transcription factors in the Drosophila genome would likely result in a more complete set of genes specific to and required for the determination of each taste neuron class. However, I found that RNAi knockdown of select candidates did not significantly affect protein expression, as assessed by immunohistochemistry, thus precluding a large-scale RNAi screen.
Results

An expression screen to identify taste neuron-specific transcription factors

A microarray-based screen was performed in the Scott lab to identify transcripts enriched in taste tissue by comparing proboscises with and without gustatory neurons. Flies without gustatory neurons were generated by utilizing a homozygous mutation in the gene Pax-neuro (poxn), in which gustatory neurons are converted to mechanosensory neurons. Proboscis RNA from flies homozygous for the poxn mutation was compared to proboscis RNA from poxn heterozygote control flies, which still possess functional gustatory neurons. Genes enriched >2 fold in poxn heterozygotes comprise the microarray data set of genes upregulated in taste neurons.

I used this microarray data set to generate a list of 16 candidate transcription factors that might play a role in the fate specification of individual taste neurons. This list included 5 bHLH-domain proteins (clock, Fer-1, knot, spineless, tap), one POU-domain protein (nubbin), and one LIM-HD protein (Lim3). Six additional potential candidates were selected that also contain a bHLH, LIM-HD, or POU-domain and have previously been shown to play a role in neural fate specification. The expression of these 19 genes was evaluated with immunohistochemistry when specific antibodies were available (15 genes), or by in situ hybridization with gene-specific RNA probes (9 genes). The results of this screen are described in Table 3.1. The majority of the transcription factors screened either showed expression not specific to taste neurons, or no expression at all. However, two genes, knot and lim3, showed specific expression in one taste neuron per sensillum.

Knot and Lim3 are expressed in bitter-sensing gustatory neurons

Two transcription factors from the expression screen, knot and lim3, showed specific expression in a subset of taste neurons as determined by antibodies specific to each protein. Knot is a member of the conserved COE family of bHLH transcription factors, which are found in mouse, Xenopus, and zebrafish, among other species. In the nervous system, COE transcription factors are restricted to post-mitotic neurons, where they serve to regulate neuron differentiation. In Drosophila, knot plays many roles in development, and it is involved in processes including neural fate specification in the ventral nerve chord, lineage specific embryonic muscle specification, and embryonic head patterning. It is also negatively regulated by Notch signaling, and thus its lineage-specific restriction depends on asymmetric segregation of Numb.

Lim3 is a member of the LIM-HD family of transcription factors, whose members control various aspects of neural fate identity including neurotransmitter selection and axon pathfinding. Many of the studies to date on members of this gene family have focused on the embryonic vNC. Lim3 is required for the proper axonal pathway selection of a specific set of islet-expressing motorneurons. Islet, also a member of the LIM-HD family, is required for dopamine and serotonon synthesis, as well as axon pathfinding, in a subset of vNC interneurons and motorneurons. Yet another LIM-HD gene, apterus, controls axon pathway selection for a different subset of interneurons in the vNC.

In which specific taste neuron class are knot and Lim3 expressed? Double-labeling experiments showed that both Knot and Lim3 are expressed in only the bitter-sensing GRNs, as determined by co-expression with Gr66a-IRES-GFP (Fig 3.1 A-B). Because of their respective
roles in neural fate specification and their specific expression pattern in taste neurons, knot and Lim3 appeared to be excellent candidates for determinants of the bitter neuron fate.

**numb clones lack expression of Knot and Lim3**

numb	extsuperscript{2} MARCM clones encompassing the four taste neurons lack a bitter-sensing taste neuron, as discussed in Chapter 2 of this thesis. The fate of the bitter neuron was assessed using an antibody specific to Gr33a, a bitter gustatory receptor, and by expression of GFP driven by the promoter of Gr66a, another bitter gustatory receptor. Since Knot and Lim3 are both expressed in the bitter-sensing neuron and may be involved in its fate specification, I tested whether their expression was also altered in nb	extsuperscript{2} MARCM clones. 4-cell nb	extsuperscript{2} MARCM clones were generated in flies expressing Gr66a-IRES-GFP. The proboscises of these flies were dissected and labeled with either anti-Knot or anti-Lim3. I found that Knot and Lim3 both co-localize with Gr66a-IRES-GFP in unmarked sensilla, but that marked nb	extsuperscript{2} sensilla lack expression of Knot, Lim3, and Gr66a-IRES-GFP (fig. 2 A-B). These data indicate that numb not only affects the expression of gustatory receptors in the bitter neuron, but also affects the transcription factor expression profile, which may be the key to determining the bitter cell fate. The lack of Knot expression in nb	extsuperscript{2} clones is also consistent with previous studies in embryonic muscle specification showing that Notch signaling negatively regulates transcription of knot.

**Knot and Lim3 loss-of-function mutant clones do not have altered taste neuron fates**

Does the fate of the bitter neuron change in the absence of knot or Lim3? Because kn	extsuperscript{col-1} and Lim3	extsuperscript{2}, the mutant alleles selected for each gene, are homozygous lethal, mutant MARCM clones were generated in the proboscis and the fate of the bitter neuron was assessed within such clones. kn	extsuperscript{col-1} MARCM clones did indeed lack a Knot-positive neuron (Fig 3.3A) but still contained a bitter-sensing neuron, as identified by Gr66a-IRES-GFP. This result indicates that knot is not necessary for determining the bitter neuron fate.

MARCM clones homozygous for Lim3	extsuperscript{2} still contained a cell recognized by the Lim3 antibody (Fig 3.3B). However, Lim3	extsuperscript{2}, a very strong allele of Lim3, contains a 2300bp intragenic insertion into the Lim3 locus [Stathakis et al 1995], so perhaps the Lim3 antibody is capable of recognizing the mutated portion of the protein that is still expressed. Lim3	extsuperscript{2} MARCM clones still contain a neuron that expresses Gr66a-IRES-GFP, indicating that Lim3 is also not necessary for fate specification of the bitter neuron.

It is possible that knot and Lim3 have redundant functions in specifying the fate of the bitter neuron, and that removal of both genes is necessary to eliminate or alter the identity of the bitter neuron. However, because of their positions on opposite arms of the second chromosome, MARCM experiments to make double-mutant clones were not possible. Thus, the possibility of knot and Lim3 redundancy remains untested.

**Knot and Lim3 loss-of-function mutant clones do not have altered axonal projections**

While knot and Lim3 are not necessary for the expression of bitter-specific GRs, it is possible that one or both genes are involved in the correct targeting of bitter GRN axons in the SOG, especially since Lim3 is required for axonal targeting in the vNC. To test this possibility, mutant MARCM clones were generated in which only the bitter neuron could be visualized by using Gr22e-GAL4, a bitter-specific driver. While each mutant clone may encompass 1-4 gustatory neurons, reporter expression will only occur in the bitter neuron so that its axonal projections can be easily discerned in the SOG. The projections of the bitter-sensing GRNs form
a recognizable ring shape in the SOG, so *kn*<sup>col-1</sup> and *Lim3*<sup>2</sup> projections were evaluated for any noticeable deviations from this shape. While minor targeting defects are still a possibility, no gross deviations from the canonical ring projection pattern were observed in either *kn*<sup>col-1</sup> or *Lim3*<sup>2</sup> bitter neurons (Fig 3.4 A,B), leading to the conclusion that neither gene is necessary for the axonal path finding process of bitter neurons.

**Mis-expression of Knot and Lim3 does not alter taste neuron fates**

While *knot* and *Lim3* are not individually necessary for the specification of the bitter neuron, their expression may be sufficient to induce the bitter neuron fate. Alternatively, their expression may repress the specification of other taste neuron fates, in order to prevent the bitter neuron from expressing ppk ion channels or sugar-specific GRs. To test these hypotheses, *knot* and *Lim3* transcripts, individually or together, were expressed via the GAL4/UAS system in all taste neurons (using OSeg-GAL4).

If *knot* and/or *Lim3* are sufficient to specify the bitter neuron, I would expect to see four bitter neurons per sensillum when driving expression of UAS-*knot* and/or UAS-*Lim3* with OSeg-GAL4. However, although the mis-expression of Knot and Lim3 in all taste neurons was confirmed by immunostaining with their respective antibodies, neither gene alone nor the combination of the two leads to expression of Gr66a-ires-GFP in extra neurons (Fig 3.5 A,B,D). Thus, *knot*, *Lim3*, and both genes together are not sufficient to specify the bitter neuron fate.

If *knot* and/or *Lim3* serve to suppress the expression of other taste receptors, we would expect to lose other neuron fates when expressing *knot* and *Lim3* transcripts in all taste neurons. However, the sugar-sensing neuron is still able to drive expression from the Gr5a and Gr64f promoters when either or both genes are mis-expressed in this cell (Fig 3.5 C,D). Thus, neither *knot* nor *Lim3* suppress the fate of the sugar-sensing neuron.

**An RNAi screen to identify transcription factors involved in taste neuron fate decisions**

While *knot* and *Lim3* were the only genes from my screen with clear, specific expression in taste neurons, many of the other transcription factors had no specific antibody, and thus their expression pattern was unsatisfactorily assessed. Because specific sets of transcription factors undoubtedly control the fate of each taste neuron class, a thorough and unbiased loss-of-function screen might unveil key players in taste neuron specification. Despite the power of MARCM to investigate individual genes, it is not an efficient means to screen a large set of transcription factors. Thus, I tested whether an RNAi screen would allow us to assess the role of different transcription factors in determining taste neuron fate.

Initiation of expression in taste neurons by various GAL4 driver lines was determined in order to better understand and control the timing of the RNAi expression. The expression of UAS-GFP was driven with seven different GAL4 lines. Labial discs from late third instar larvae and pupal proboscises from various developmental stages were subsequently dissected and assessed to determine the initial stage containing GFP expression (Fig 3.6A-E). Since OSeg-GAL4 turns on after the taste neurons are finished dividing, but before the GR-GAL4s start to be expressed, it was determined to be the best driver of RNAi constructs.

In order to assess the efficacy of gene knock-down, RNAi knock-down was tested on genes whose expression levels could be tracked with specific antibodies, *knot* and *Lim3*. I found that no matter which GAL4 line was used to drive expression of a number of different UAS-*knot*-RNAi and UAS-*Lim3*-RNAi constructs, there was never an observable difference in protein
levels of either Knot or Lim3 (Fig 3.7 A,B). Even if moderate knock-down was achieved, the perdurance of any protein in the cell would likely preserve any fate-determining function it might have. While there has been some success with gene knockdown via transgenic RNAi in *Drosophila*, there is a wide variance in effectiveness among the available RNAi transgenes. Within a sample of 64 RNAi lines tested from the Vienna Drosophila RNAi Center, less than 40% showed >75% knock-down of RNA levels, while ~25% showed 0-25% knock-down \(^{19}\). RNAi knock-down success may also vary between cell-types. The finding that RNAi did not sufficiently reduce protein expression in gustatory neurons argues that a large scale RNAi screen is not a viable option.
Conclusions

Uncovering the full set of transcription factors that are specific to each taste neuron class remains a key component in determining how each cell type achieves its unique identity. The expression screen detailed in this chapter identified two transcription factors with specific expression in the bitter-sensing neuron. While neither knot nor Lim3 appear to be necessary or sufficient for the determining the complete identity of the bitter neuron, each gene may still be important for aspects of the bitter fate unrelated to expression driven by the Gr66a or Gr33a promoters. There also may be functional redundancy between knot and Lim3, in which case a double mutant would be necessary to observe a loss-of-function phenotype. It is also possible that the MARCM technique produced mutant cells after each gene had already played its critical role in the determination of the bitter neuron fate. Further investigation into other windows of mutant clone induction may provide insight into the timing of each gene’s relevant activity.

The Drosophila genome encodes over 500 transcription factors, and although only 16 of these appeared on the microarray, a thorough immunohistochemistry expression screen with all available transcription factor antibodies might elucidate other genes with specific expression in taste neurons. Perhaps no single transcription factor is sufficient to induce a given taste neuron fate, and rather a combinatorial code of factors is necessary. One way to identify potential genes in such a combinatorial code would be to determine the distinct transcriptomes of each taste neuron class and identify the unique set of transcription factors expressed by each. To elucidate these transcriptomes, we could use GR-GAL4 lines to drive cell-specific expression of poly-A binding protein, followed by pull-downs and RNA-seq. A similar approach was used successfully to identify additional GRs expressed in the sugar neuron. Cell-type specific transcription factors identified by RNA-seq would be verified by immunohistochemistry and tested for loss-of-function and gain-of-function effects on taste neuron fate. Combinations of multiple or all transcription factors specific to a given cell type may need to be knocked out or overexpressed in concert to elucidate an underlying combinatorial code for cell fate.
Materials and Methods

Clonal analysis

All mutant clones were generated using the MARCM technique, as previously described. 22 GRNs were labeled with one of the following GAL4 driver lines: elav-Gal4, Gr22e-GAL4. Clones were marked with UAS-HUNT::tdTOMATO or UAS-tdTOMATO (a gift from Steven Stowers, Montana State University). Bitter neurons were labeled with Gr66a-IRES-GFP. MARCM clones were induced by a 1-hr heat shock at 0 h APF at 37°C or 37.5°C.

The genotypes of the flies used for MARCM experiments are as follows:
(1) nb2 clones with bitter neurons labeled: y w hsFLP122/w or Y; nb2 FRT40A/tubP-Gal80 FRT40A; elav-Gal4, UAS-HUNT::tdTOMATO, Gr66a-IRES-GFP/ elav-Gal4, UAS-HUNT::tdTOMATO
(2) kn col-1 clones with bitter neurons labeled, all neurons visible: y w hsflp122/w or Y; FRTG13 kn col-1 / FRTG13 tubP-Gal80; elav-Gal4, UAS-HUNT::tdTOMATO, Gr66a-IRES-GFP/ elav-Gal4, UAS-HUNT::tdTOMATO
(3) kn col-1 clones with bitter neurons labeled, only bitter neurons visible: y w hsFLP122/w or Y; FRTG13 kn col-1 / FRTG13 tubP-Gal80; UAS-tdTOMATO/ Gr22e-Gal4
(4) Lim3 clones with bitter neurons labeled, all neurons visible: y w hsFLP122/w or Y; Lim3 FRT40A/tubP-Gal80 FRT40A; elav-Gal4, UAS-HUNT::tdTOMATO, Gr66a-IRES-GFP/ elav-Gal4, UAS-HUNT::tdTOMATO
(5) Lim3 clones with bitter neurons labeled, only bitter neurons visible: y w hsFLP122/w or Y; Lim3 FRT40A/tubP-Gal80 FRT40A; UAS-tdTOMATO/ Gr22e-Gal4

Overexpression and RNAi

OSeg-GAL4 23 was used to drive RNAi and over-expression constructs. The following UAS lines were used: UAS-knot, UAS-Lim3, UAS-knot-RNAi (VDRC), UAS-Lim3-RNAi (Transgenic RNAi Project). Gr5a-IRES-GFP and Gr64f lexA >> LexAop-rCD2::GFP were used to label the sugar neuron.

Immunostaining

Labeling of the proboscis was performed as previously described. 18 Primary antibodies used were: mouse anti-GFP (1:100, Sigma), rabbit anti-GFP (1:100, Invitrogen), rabbit anti-RFP (1:200, BioVision) rabbit anti-Gr33a 24(1:200), guinea pig anti-knot 25(1:1000), guinea pig anti-Lim3 26(1:1000). Antibodies used in the screen (pictures not shown) include: mouse anti-prospero (1:40, DSHB), mouse anti-cut (1:100, DSHB), rabbit anti-ribbon (1:1000), goat anticlock (1:200, Santa Cruz Biotechnology), rabbit anti-DHR3 (aka Hr46, 1:1000, Carl Thummel), mouse anti-islet (DSHB), mouse anti-Acj6 (DSHB), rat anti-Drifter (Sarah Certel), guinea pig anti-apterous (1:1000, Juan Botas), mouse anti-svp (Yasushi Hiromi), rabbit anti-BarH1 (Kaoru Saigo), anti-nubbin, guinea pig anti-Hairless-A (1:500 Anette Preiss), rat anti-Hairless-B (1:250, Anette Preiss), guinea pig anti-senseless (1:1000, Hugo Bellen), anti-even skipped (DSHB), rabbit anti-poxn (1:100, Werner Boll).
In situ hybridization

Double label *in situ* hybridization experiments were performed as previously described\textsuperscript{27}. Probes were labeled with Digoxigenin (Roche).
References


Table 3.1.
All screened transcription factors are listed. 16 TFs appeared in the microarray data set from Cameron, et al. Immunohistochemistry and in situ hybridization results are given, where applicable. Red text indicates expression specific to a single taste neuron class. In the antibody column, N/A indicates that a specific antibody does not exist. In the in situ column, N/A indicates that RNA probe-making for the target gene was unsuccessful.

Figure 3.1. Knot and Lim3 are expressed in bitter-sensing gustatory neurons
a) Bitter-sensing neurons are labeled with Gr66a-IRES-GFP, stained with rabbit anti-GFP and anti-rabbit Alexa 488, shown in green. Knot was stained with guinea pig anti-knot and anti-guinea pig 568, shown in magenta. The merge shows co-localization of the two signals, with example cells indicated by white arrowheads. Scale bar = 5 \( \mu m \).
b) Bitter-sensing neurons are labeled with Gr66a-IRES-GFP, stained with rabbit anti-GFP and anti-rabbit Alexa 488, shown in green. Lim3 was stained with guinea pig anti-Lim3 and anti-guinea pig 568, shown in magenta. The merge shows co-localization of the two signals, with example cells indicated by white arrowheads. There are additional Lim3-expressing support cells, but all Gr66a-positive neurons also express Lim3. Scale bar = 5 \( \mu m \).

Figure 3.2. numb clones lack expression of Knot and Lim3
a) nb\textsuperscript{2} clones were marked with UAS::HUNtdTOMATO driven by elav-GAL4, shown in magenta. Bitter neurons were marked with Gr66a-IRES-GFP and stained with rabbit anti-GFP and anti-rabbit Alexa 488, shown in green. Knot was stained with guinea pig anti-knot and anti-guinea pig Alexa 647, shown in blue. The merge shows a marked nb\textsuperscript{2} sensillum with no cells expressing either Gr66a-IRES-GFP or Knot, and an unmarked sensillum with a cell expressing both Gr66a-IRES-GFP and Knot. Scale bar = 5 \( \mu m \).
b) nb\textsuperscript{2} clones were marked with UAS::HUNtdTOMATO driven by elav-GAL4, shown in magenta. Bitter neurons were marked with Gr66a-IRES-GFP and stained with rabbit anti-GFP and anti-rabbit Alexa 488, shown in green. Lim3 was stained with guinea pig anti-Lim3 and anti-guinea pig Alexa 647, shown in blue. The merge shows a marked nb\textsuperscript{2} sensillum with no cells expressing either Gr66a-IRES-GFP or Lim3, and an unmarked sensillum with a cell expressing both Gr66a-IRES-GFP and Lim3. Scale bar = 5 \( \mu m \).

Figure 3.3. Knot and Lim3 loss-of-function mutant clones do not have altered taste neuron fates
a) \textit{kn}\textsuperscript{col-1} clones were marked with UAS::HUNtdTOMATO driven by elav-GAL4, shown in magenta. Bitter neurons were marked with Gr66a-IRES-GFP and stained with rabbit anti-GFP and anti-rabbit Alexa 488, shown in green. Knot was stained with guinea pig anti-knot and anti-guinea pig Alexa 647, shown in blue. The merge shows a marked \textit{kn}\textsuperscript{col-1} sensillum with a cell expressing Gr66a-IRES-GFP, but not Knot (white arrowhead). Unmarked sensilla each contain a single cell expressing both Gr66a-IRES-GFP and Knot. Scale bar = 5 \( \mu m \).
b) \textit{Lim3}\textsuperscript{2} clones were marked with UAS::HUNtdTOMATO driven by elav-GAL4, shown in magenta. Bitter neurons were marked with Gr66a-IRES-GFP and stained with rabbit anti-GFP and anti-rabbit Alexa 488, shown in green. Lim3 was stained with guinea pig anti-Lim3 and anti-guinea pig Alexa 647, shown in blue. The merge shows a marked \textit{Lim3}\textsuperscript{2} sensillum with one
cell expressing Gr66a-IRES-GFP, which is also stained by anti-Lim3 (white arrowhead). However, the antibody is probably recognizing the truncated Lim3 protein and does not necessarily indicate the presence of functional Lim3. Scale bar = 5µm.

**Figure 3.4. Knot and Lim3 loss-of-function mutant clones do not have altered taste neuron projections**

*a* *kn* \(^{col-1}\) clones were marked with UAS:tdTOMATO driven by Gr22e-GAL4, shown in red, so that only bitter neuron projections were visible. Brains were stained with rabbit anti-dsRED and anti-rabbit Alexa 568. Bitter neurons within *kn* \(^{col-1}\) clones still project to the canonical bitter region of the SOG in the brain. Scale bar = 10µm.

*b* *Lim3* \(^2\) clones were marked with UAS:tdTOMATO driven by Gr22e-GAL4, shown in red, so that only bitter neuron projections were visible. Brains were stained with rabbit anti-dsRED and anti-rabbit Alexa 568. Bitter neurons within *Lim3* \(^2\) clones still project to the canonical bitter region of the SOG in the brain. Scale bar = 10µm.

**Figure 3.5. Mis-expression of Knot and Lim3 does not alter taste neuron fates**

*a* Expression of UAS-knot was driven in all taste neurons by OSeg-GAL4. Bitter neurons are labeled with Gr66a-IRES-GFP, stained with rabbit anti-GFP and anti-rabbit Alexa 488, shown in green. Knot was stained with guinea pig anti-knot and anti-guinea pig 568, shown in magenta. The merge shows that although Knot is indeed expressed in all taste neurons, there is still only one bitter neuron per sensilla. Scale bar = 5µm.

*b* Expression of UAS-Lim3 was driven in all taste neurons by OSeg-GAL4. Bitter neurons are labeled with Gr66a-IRES-GFP, stained with rabbit anti-GFP and anti-rabbit Alexa 488, shown in green. Lim3 was stained with guinea pig anti-Lim3 and anti-guinea pig 568, shown in magenta. The merge shows that although Lim3 is indeed expressed in all taste neurons, there is still only one bitter neuron per sensilla. Scale bar = 5µm.

*c* Expression of both UAS-knot and UAS-Lim3 together was driven in all taste neurons by OSeg-GAL4. Sugar neurons are labeled with Gr64f-LexA driving LexAop-CD2GFP, then stained with rabbit anti-GFP and anti-rabbit Alexa 488, shown in green. Lim3 was stained with guinea pig anti-Lim3 and anti-guinea pig 568, shown in magenta. The merge shows that Lim3 (and presumably Knot), are indeed expressed in all taste neurons, including sugar-sensing neurons, but that their expression in these cells does not suppress the sugar neuron fate. Scale bar = 5µm.

*d* Expression of both UAS-knot and UAS-Lim3 was driven in all taste neurons by OSeg-GAL4. Sugar neurons are labeled with Gr5a-IRES-GFP, then stained with mouse anti-GFP and antimouse Alexa 488, shown in green. Bitter neurons were stained with rabbit anti-Gr33a and anti-rabbit Alexa 568, shown in magenta. Sugar cell fate is better assessed by Gr64f-LexA, in panel C, as the Gr5a reporter is expressed in too many cells. However, we can see here that expression from the Gr5a promoter is unhindered by expression of Knot and Lim3 in these cells. Furthermore, there is still only one bitter neuron per sensillum, so co-expression of Knot and Lim3 in other neurons is not sufficient to induce the bitter neuron fate. Scale bar = 5µm.

**Figure 3.6. Timing of expression from various GAL4 drivers during development**

*a* Timeline of development, from the late 3\(^{rd}\) instar larval stage through 5 days after puparium formation (APF). Red arrows indicate the time at which expression of a given GAL4 line was first noted.
b) Expression of UAS-GFP driven by elav-GAL4 in the labial disc of a late 3rd instar larva.
c) Expression of UAS-GFP driven by poxn-GAL4 in the labial disc of fly just entering puparium formation.
d’) Expression of UAS-GFP driven by OSeg-GAL4 begins in the middle of the first day APF, and is completely turned on (d’’) by the middle of the second day APF.
e) Expression of UAS-GFP driven by pebbled-GAL4 begins during day 3 APF, around the same time that the GR-GAL4s begin to express (data not shown).

Figure 3.7. RNAi against knot and Lim3 does not noticeably knock-down protein expression.
a) OSeg-GAL4 drives expression of UAS-knot-RNAi in all taste neurons, yet Knot can still be robustly detected by guinea pig anti-knot, stained with anti-guinea pig Alexa 647, shown in blue. Scale bar = 10µm.
b) OSeg-GAL4 drives expression of UAS-Lim3-RNAi in all taste neurons, yet Lim3 can still be robustly detected by guinea pig anti-Lim3, stained with anti-guinea pig Alexa 647, shown in blue. Scale bar = 10µm.
<table>
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<th>Transcription Factor</th>
<th>Antibody staining results</th>
<th>In situ results: co-expression with Poxn::GFP?</th>
<th>Candidate from microarray?</th>
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Figure 3.1. Knot and Lim3 are expressed in bitter-sensing gustatory neurons

a. Gr66a-IRES-GFP  anti-knot  merge

b. Gr66a-IRES-GFP  anti-Lim3  merge
Figure 3.2. *numb* clones lack expression of Knot and Lim3

a. Gr66a-IRES-GFP elav::HUNtdTOM  anti-knot  merge

b. Gr66a-IRES-GFP elav::HUNtdTOM  anti-Lim3  merge
Figure 3.3. Knot and Lim3 loss-of-function mutant clones do not have altered taste neuron fates

a. Gr66a-IRES-GFP elav::HUNTtdTOM anti-knot merge

b. Gr66a-IRES-GFP elav::HUNTtdTOM anti-Lim3 merge
Figure 3.4. Knot and Lim3 loss-of-function mutant clones do not have altered taste neuron projections

a. knot^{−/−} bitter neuron projections

b. Lim3^{−/−} bitter neuron projections
Figure 3.5. Mis-expression of Knot and Lim3 does not alter taste neuron fates

a. Gr66a-IRES-GFP anti-knot merge
OSeg-GAL4 UAS-knot

b. Gr66a-IRES-GFP anti-Lim3 merge
OSeg-GAL4 UAS-Lim3

c. Gr64fLexA::CD2-GFP anti-Lim3 merge
OSeg-GAL4 UAS-knot UAS-Lim3

d. Gr5a-IRES-GFP anti-Gr33a merge
OSeg-GAL4 UAS-knot UAS-Lim3
Figure 3.6. Timing of expression from various GAL4 drivers during development

a.

<table>
<thead>
<tr>
<th>Puparium Formation</th>
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<tr>
<td>Late 3rd instar</td>
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<tr>
<td>Day 4 APF</td>
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<tr>
<td>Day 5 APF</td>
</tr>
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</table>

elav & cha  
O Seg starts  
O Seg fully on  
pbl & GRs

b. 
elav-GAL4

c. 
poxn-GAL4

d. 
O Seg-GAL4

e. 
Pebbled-GAL4

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Figure 3.7. RNAi against *knot* and *Lim3* does not noticeably knock-down protein expression.

a. anti-knot  

b. anti-Lim3  

OSeg-GAL4, UAS-knot-RNAi  

OSeg-GAL4, UAS-Lim3-RNAi