Role of the broadly expressed olfactory receptor OlfCc1 in mediating amino acid detection in Zebrafish

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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 at Berkeley

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Spring 2010
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Abstract

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A family of 63 class C GPCR olfactory receptors, termed the ‘OlfC’ family, is expressed in the microvillous cell population of the zebrafish olfactory epithelium and has been predicted to be involved in amino acid detection. While the majority of the receptors are expressed in a scattered, random fashion, the receptor OlfCc1 is present in most or all microvillous cells. The overlapping expression patterns of the broadly expressed OlfCc1 with the punctuate OlfCs suggests that there may be co-expression of these populations. I was interested in investigating the function of OlfCc1 as well as the prediction that the microvillous cells expressing it are amino acid responsive. My hypothesis is that OlfCc1 plays a critical role in mediating olfactory amino acid detection. In order to test this, I first generated a peptide antibody against OlfCc1 to better characterize its distribution in adult and larval zebrafish. I then employed an antisense morpholino mediated knockdown strategy to investigate the effects of loss of OlfCc1 function. An immunohistological assessment with a panel of markers revealed that the loss of OlfCc1 did not cause gross alterations to the cellular architecture of the developing olfactory system. To explore possible affects on odorant detection, I established an in vivo assay to examine odorant-evoked activity in the olfactory bulb of morphant zebrafish expressing the genetically encoded calcium sensor GCaMP1.6 under the neuronal promoter HuC. The results of these experiments indicated that the loss of OlfCc1 severely abrogates the olfactory bulb response to a pool of 9 amino acids containing members of each of the four amino acid classes: acidic, basic, and short chain and long chain neutral. The responses to pools of other odorants thought to act through different cellular pathways, the bile acids and the biogenic amines, as well as to a complex food extract, were not significantly affected. I showed in fish in which OlfcC1 expression had not been knocked down that sub-pools of amino acids, isoleucine and leucine vs. arginine and lysine, evoked differential patterns of activity. This suggests that these responses are mediated by different populations of receptors, and that the effects of OlfCc1 knockdown are consistent across these populations. The severe effect on amino acid evoked activity in the olfactory bulb, which receives innervation from all populations of olfactory neurons, also supports the hypothesis that OlfC expressing microvillous cells are primarily mediating the response
to amino acids. Together, these results support the hypothesis that OlfCc1 is broadly critical for amino acid detection and raise the intriguing possibility that it may be acting as a heteromeric co-receptor with the other OlfC family members.
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Acknowledgements

I would like to first and foremost thank my husband Neil, who has been immensely supportive and wonderful throughout all of this. I’m also very grateful to my family for the understanding and advice they’ve given me throughout my graduate years and life, and for imparting upon me the appreciation for science that lead me here and the understanding of academia that prepared me for it.

My advisor, John Ngai, provided the invaluable advice and scientific guidance that made this project possible. I’d also like to thank him for putting up with my cranky emails when the fish weren’t cooperating (which was often). I can honestly say I’ve learned more in the past six years than I ever could have expected, and feel like I will leave the lab feeling prepared for anything I might do next. I’d also like to extend my appreciation to the members of the Ngai lab, in particular Todd Ferreira for being my mentor on all things fishy, and Team Foxy Fish (Cynthia Duggan and Ariane Badhuin) for collaboration on a fun and productive project. Finally, I’d like to thank the Wednesday crew (Jose Estrada and Orapim Tulyathan) for helping me maintain my sanity, not to mention some great discussions, scientific and otherwise.

Altogether, it has been a truly interesting, sometimes challenging, and usually unpredictable graduate career, and I’d like to take a step back and profoundly thank everyone who’s been there to offer me help, advice, or an ear to bend over the years.
Chapter one: Introduction to vertebrate olfaction

Chemoreception and beyond: diverse roles of the olfactory receptor in the function and development of the olfactory system

Introduction:

One of the principal goals of neuroscience is to understand the pathways that bridge an external stimulus to its internal representation as a percept. As reception is necessarily the first step of perception, understanding the initial relay in this system is vital to this goal. The primary sensory afferents play a unique role as the interface between the physical world and the nervous system, and it is here that the processes that will eventually result in sensation are initiated. The five sensory systems each face unique challenges with respect to how to decode and encode diverse forms of physical information, and the nature of the stimuli being detected necessarily shapes every part of the apparatus required to detect and dissect them.

Odor space – the vast universe of chemicals to which the olfactory system responds – is richly complex and highly dynamic. Individual odorants may possess strikingly different chemical structures, and most odors in the natural world comprise complex mixtures drifting through space in plumes. The olfactory system is therefore tasked with the detection of and discrimination between chemical entities across a wide range of concentrations. The first key to meeting this challenge lies with the receptors that have evolved to detect the specific features of their sensory space; in this case, chemical space. However, while it is natural to think of a sensory receptor as the primary determinant of the functional specificity of sensory neurons, the receptor turns out to be directly involved not only in tuning chemical specificity, but also in important steps in the development and organization of the olfactory system. For instance, the receptor protein is involved via feedback mechanisms in maintaining its own exclusive expression as well as in instructing the precise targeting of the neuron expressing it. This chapter will examine the various roles of the classical olfactory receptor in both chemosensation and in guiding the development of the olfactory system, with a focus on where the pathways underlying these processes may converge.

I) Chemosensory transduction and olfactory coding: canonical roles of the olfactory receptor

i) Anatomical organization of the olfactory system

The classical olfactory receptors (OR) represent the largest single gene family in mammals/vertebrates. The identification of this family of GPCRs in 1991 (L Buck & R Axel 1991) has proven so critical to the advancement of the field that it garnered the Nobel prize in Physiology and Medicine in 2004. In mice, there are over 1000 ORs. (Xiaohong Zhang & Stuart Firestein 2009) (Young et al. 2003) Humans, in contrast, express ~300 Ors (Niimura & Nei 2005) (Selbie et al. 1992), while zebrafish express on the order of 150 (Tyler S Alioto & John Ngai 2005), revealing a large degree of variability in this gene family across vertebrates. However, the fundamental principles of
the system appear to be similar among vertebrates, even as the number of receptors varies across an order of magnitude.

The ORs are expressed in a population of neurons residing in the olfactory epithelium within the nasal cavity. The olfactory receptor neurons (ORNs) project a single dendritic knob to the surface of the olfactory epithelium, extending receptor-rich cilia into the nasal cavity to catch inhaled odorants. Their unbranched axons in turn project to the adjacent olfactory bulb, a central nervous system structure rostral to the telencephalon. Here they coalesce into discrete neuropil structures dense with synapses known as glomeruli. There are approximately 2000 such glomeruli in each half of the bilaterally symmetric olfactory bulb in mice. (Zou et al. 2009)

The OR itself is a rhodopsin-family G-protein-coupled receptor that triggers a signal transduction cascade upon odorant binding. G-alpha(olf) activates adenylyl cyclase 3 (AC3). (Fig 1.1) This enzyme in turn catalyzes the production of cAMP, activating the cyclic nucleotide-gated channel A2 (CNGA2) (Belluscio et al. 1998) (Wong et al. 2000) (Lisa J Brunet et al. 1996). The CNGs are non-specific cation channels that allow the influx of sodium and calcium, depolarizing the neuron. This depolarization is further amplified by the subsequent activation of calcium-gated chloride channels (Stephan et al. 2009); in contrast to the situation in most neural populations, the gradient of this ion is such that chloride effluxes rather than influxes the cell. Depolarization results in the opening of voltage-sensitive ion channels and the firing of action potentials. Receptor activation is rapidly terminated, resulting in desensitization to continuously present odors. The mechanism by which signaling is terminated is thought to involve the calcium-calmodulin dependent desensitization of the CNG channel. (Kurahashi & Menini 1997) (Song et al. 2008)

While the number of genes devoted to the detection of odorants is large, the number of potential odorants that terrestrial vertebrates can detect is far vaster. How then to organize the system such that it can both detect and discriminate between the greatest number of potential novel ligands given a limited, if large, number of receptors? The first step to answering this question lies in one of the fundamental organizational principles of the olfactory system, the so-called ‘one receptor, one neuron’ rule. It has been shown in mice that out of the entire repertoire of genes, a given olfactory neuron selects only a single allele of a single OR to express. (Chess et al. 1994) (Shou Serizawa et al. 2003) (Lewcock & Randall R. Reed 2004) In turn, each neuron expressing a given receptor typically innervates two glomeruli in the olfactory bulb, one located medially and one laterally; a given glomerulus receives innervation only from neurons expressing the same receptor. Hence, each of the 2000 glomeruli represents only one OR, and each OR is represented by 1-2 glomeruli. (Mombaerts et al. 1996) (Belluscio et al. 2002) While the distribution of olfactory neurons expressing a particular receptor in the olfactory epithelium is random, the location to which those neurons project in the olfactory bulb is largely invariant from animal to animal. (F Wang et al. 1998) (However, see also (Yuki Oka et al. 2006)).
Although there is no fixed receptor-to-neuron map in the olfactory epithelium, there is a broader degree of anatomical organization with respect to the distribution of ORs. The expression of given receptors is restricted to broad zones of the OE distributed along the dorsoventral axis. This is mirrored by a dorsal-ventral distribution of the respective target glomeruli at the olfactory bulb. (Vassar et al. 1993) (Ressler et al. 1993) (Miyamichi et al. 2005) Zone 1, the most dorsal region of the olfactory organ, is the most strictly delineated, while the other expression domains are less molecularly distinct and broadly overlapping. The importance of one aspect of this dorsal-ventral patterning of receptor expression has been revealed in recent research and suggests that certain innate unlearned behaviors, such as murine fear responses to predator odors, are specifically mediated by ORNs located in the dorsal region of the olfactory epithelium, whereas learned olfactory responses are dependent on the more ventral ORNs (K. Kobayakawa et al. 2007). The anatomical segregation of receptors responding to odorants that give rise to innate behaviors from behavior-neutral ones that can in turn give rise to associations through classical conditioning are likely the first features of complex differences in the higher order circuitry into which these ORNs feed.

Receptor-specific convergence at the olfactory bulb allows for the formation of what can be considered a chemotopic map, wherein odor-evoked activity is represented as a spatial pattern rendered across an anatomical surface. The utilization of topographic representations of stimulus information is common in the nervous system, where there are also somatotopic, retinotopic, and tonotopic maps representing other sensory modalities. Understanding the logic of this chemotopic map requires first understanding the response properties of the individual ORNs represented in it, and then the principles by which different ORN populations are organized across it.

iii) Olfactory receptor tuning and combinatorial coding

Despite the identification of vertebrate OR genes and the availability of extensive genome sequence information for various vertebrate species, determining the chemical specificities of individual receptors in a high-throughput fashion has proven to be challenging. This is in large part due to difficulties in expressing functional ORs in heterologous cell systems. Approaches taken to circumvent these difficulties have involved making modifications to the receptor sequence that make them more amenable to heterologous expression, co-expression of ORs with putative chaperones, electrophysiological characterization of a virally overexpressed OR in the OE, and RT-PCR based retrospective identification of the receptor expressed in dissociated ORNs that respond to a certain odorant as demonstrated by a calcium-indicator assay. (T Sato et al. 1994) (Malnic et al. 1999) (K Touhara et al. 1999) (Krautwurst et al. 1998) (H Zhao et al. 1998) (Araneda et al. 2000) (Kajiya et al. 2001) (Katada et al. 2003) (Araneda et al. 2004) (Zhuang & Matsunami 2007) The results of these experiments have provided valuable insights into the nature of olfactory coding. Given that the number of potentially perceived odors is non-discrete and nearly limitless even in humans, who possess fewer ORs than their murine counterparts, it is unsurprising that some sort of combinatorial activation of receptor
populations must underlie olfactory coding. Accordingly, studies into OR and ORN specificity have elucidated the basic principles of olfactory combinatorial coding: a given receptor may respond to many odorants and a single odorant may activate many receptors. (Fig 1.3) Additionally, there is a broad range of fidelity among receptors, with some being highly specific in their responses and others being broadly activated by many different compounds. (Araneda et al. 2000) (Malnic et al. 1999) In this way, an odor is represented by the activity of ensembles of neurons detecting different molecular features and responding with varying levels of activity.

It is also clear that the precise receptor-to-bulb organization is critical for this code to function. This was recently demonstrated in a strikingly direct fashion in which this patterning in a mouse was disrupted by the forced expression of a single OR in the majority of ORNs. This manipulation resulted in atypical exuberant targeting of these ectopic receptor-expressing ORNs to all glomeruli. The resultant mutant mice could no longer behaviorally detect the odorant known to activate that receptor, in spite of showing a robust electrophysiological response to it in their olfactory epithelium. (Araneda et al. 2004) Despite the ability of the odorant to evoke an electrical response within ORNs, without being restricted in some way in its representation at the bulb, the animal had no way to discriminate it.

iv) From receptor to perception; features of the combinatorial code

The presence of receptor populations that possess different affinities for a given odorant allows for greater receptive range across concentrations and provides an explanation for the fact that certain odorants possess qualitative features that change drastically with concentration. For example, the molecule indole is described as having a sweet or floral quality at extremely low concentrations, but a putrid and foul one at higher concentrations. One simple explanation for this shift might be that as odor concentration increases, populations of neurons with lower affinities and different perceptual associations are respectively recruited. Yet not every odor changes in quality along with intensity, and the extensive activation of overlapping receptor populations might be predicted to confound the ability to distinguish between different compounds. However, examples for which there are both behavioral data and characterization of large populations of ORs imply that there are complexities within the combinatorial code beyond what could be thought of as simple additive properties. (Johnson & Leon 2007)

A striking example of the ability of the olfactory system to detect subtle differences in chemical structure is the ability of both humans and rodents to discriminate between different chiral enantiomers of the same molecule. (Laska & Shepherd 2007) One such example is carvone. Humans perceive (S+)-carvone as possessing an odor like the spice caraway, while (R-)carvone is said to have a spearmint-like scent. (Leitereg et al. 1971) An examination of the activity of isolated mouse olfactory neurons induced by these compounds found that 80% of the responding neurons were activated by both enantiomers at elevated concentration, and that most of the carvone-responsive neurons also responded to other odorants. (Hamana et al. 2003) However, the neurons that were selectively responsive to one enantiomer or the other responded at 10-fold lower
concentrations of the compounds than the non-selective ones. As the perceptual qualities of each enantiomer intensify but do not otherwise change with increasing concentrations, these findings imply that there are sensitivity-based hierarchies within the combinatorial code that allow distinguishing odor qualities to be preserved even as large numbers of non-enantiomer specific olfactory receptor neurons are recruited to respond.

Finally, while broad populations of receptors might be recruited during the response to a single odor, the sequence of a single receptor has also been shown to be critical to the eventual perceptual features of an odorant. When presented to different people, the molecule androstenone curiously evokes qualitative descriptions that fall broadly into three extremely distinct categories: pleasant and sweet, unpleasant and sweat or urine-like, and completely odorless. Using population genetics, Keller et al., demonstrated that a variant of the human odorant receptor OR7D4 bearing two single nucleotide polymorphisms was the basis of these differences. (Keller et al. 2007) The SNP-bearing variant receptor was non-responsive to androstenone; people possessing two copies of the variant receptor were unable to detect the molecule, while those with a single SNP-free copy found it to be pleasant and those with two functional copies found it foul. Similarly, human hypersensitivity to the “sweaty” smelling compound isovaleric acid was found to be associated with the absence of nonsense SNPs in the receptor OR11H7P. (Menashe et al. 2007)

Given both the spatial map of receptor identity in the olfactory bulb and the complex response profiles of individual receptors, what is the spatial logic of odor coding at the olfactory bulb? Various lines of evidence suggest that the bulb is at least roughly organized around molecular features. Highly related families of ORs are present as physical clusters scattered through the genome, tend to respond to related odorants, and project to neighboring regions on the OB. (Tsuboi et al. 1999) (Conzelmann et al. 2001) Examinations of the activity evoked by a broad range of odorants in the mouse olfactory bulb reveals that different spatially restricted clusters of glomeruli tend to respond to distinct molecular features. (Matsumoto et al. 2010) (Kensaku Mori et al. 2009)

II) Olfactory receptor gene selection and stabilization

In order for the coding schema described above to be possible, the olfactory system must first assemble the necessary anatomical framework. This process entails each nascent olfactory receptor neuron selecting and expressing a single receptor, extending its axon to the olfactory bulb in a bundle of other axons comprising the olfactory nerve, and then defasciculating from the bundle and converging to its proper glomerular target along with every other neuron expressing a like receptor. To discuss the mechanisms underlying the initial step in this process, the unique features of OR gene expression should first be touched upon.

i) Feedback enforced monoallelic expression of ORs
During the initial characterizations of the expression of the OR gene family, in situ hybridizations revealed that ORs were expressed in a scattered and random fashion in the OE, with different receptors restricted to continuous and overlapping dorsal to ventral
zones. (Vassar et al. 1993) (Ressler et al. 1993) (Miyamichi et al. 2005) The expression of multiple OR transcripts within a single cell was never observed, laying the foundation for the one-neuron, one-receptor rule. In fact, the specificity of OR expression was subsequently found to be even further refined, with only a single allele of an OR locus being transcribed in each neuron. This phenomenon is revealed by the exclusive expression of ORs bearing either a maternal or paternal polymorphism in a given ORN (Chess et al. 1994) as well as in experiments with genetically altered receptors in heterozygous mice, in which the manipulated and unmanipulated alleles are restricted to discrete populations of neurons. (Lewcock & Randall R. Reed 2004).

Subsequent work revealed that when a receptor locus is deleted or replaced with a non-receptor, another OR is selected and expressed in its place. (T Imai & H Sakano 2009) Thus, in the absence of an OR, monoallelic expression of the initially selected locus is no longer enforced. (Shou Serizawa et al. 2003) This implies that there is some feedback-mediated mechanism by which the expression of multiple OR alleles is prevented. In light of this, the previous finding that ORs expressing LacZ under the P2 promoter projected diffusely could be re-interpreted as the diverse targeting of neurons that had gone on to choose many different receptors, as opposed to the meanderings of receptorless neurons. In the endogenous system, such switching occurs when an ORN selects non-functional pseudogenes but is otherwise extremely rare. (Shykind et al. 2004)

Receptor switching is also observed when an endogenous receptor gene is substituted for one encoding a start-codon lacking mRNA, indicating that it is the translated receptor protein that is critical for the feedback process. (Lewcock & Randall R. Reed 2004) (Shou Serizawa et al. 2003) This raises the question as to what the determinants of a receptor protein that convey upon it the ability to repress switching are. ORs in which the G-protein interacting tripeptid motif DRY was scrambled to RDY or ALE, presumably rendering the receptor non-functional while leaving its structure largely intact, could be stably expressed, repress switching, and instruct convergence. (Takeshi Imai et al. 2006) (Nguyen et al. 2007) Thus, while an intact receptor is required, it does not necessarily have to be functional.

The necessity of the receptor coding sequence raises intriguing and as yet unanswered questions about how the translated receptor is acting to maintain its own stable expression. The molecular links between receptor and expression have yet to be identified; while it is possible that there are novel factors interacting with the receptor to transmit information about its presence back to the transcriptional machinery, other attractive candidates for such messengers are the already known interacting molecules. As a GPCR, the OR is necessarily capable of interacting with many signal transduction components, including the heterotrimeric G-protein. While odor-induced activity may be abolished in the RDY/ALE mutant receptors, it is not clear that the levels of intrinsic activity of these receptors are likewise affected. There may therefore be alternative signaling pathways the receptor is still capable of activating through interactions with its downstream signaling partners that are capable of instructing the neuron that an odorant receptor has been successfully expressed.
As for clues into how an OR protein might repress the expression of other receptors, there is at least some evidence that it is the receptor coding sequence, rather than the transcriptional machinery proximal to it, that serves as the target. Surprisingly, OR genes placed under the control of a non-OR pan-olfactory promoter driven by the trans-activational TetO system failed for the most part to be expressed in ORNs that had already undergone receptor selection, in spite of being located on a transgene that lacked the normal transcriptional regulators of an OR. (Nguyen et al. 2007) Conversely, when the ORs were driven by a TetO associated pan-olfactory promoter that was active prior to endogenous receptor selection, the transgene-based receptors were successfully expressed in the majority of ORNs. These data may point towards a model in which the OR protein suppresses expression of other ORs by acting directly on their coding sequences. However, it is important to note that OR expression could only be achieved in these experiments through the use of the TetO system, which physically separates the receptor from the promoter. ORs placed directly under control of the promoters failed to express in either case. Hence, the expression system employed here may already be bypassing some mechanisms of endogenous gene expression, confounding interpretation of the results. Also see (Fleischmann et al. 2008), where an OR was successfully driven in the majority of olfactory receptor neurons using an olfactory promoter element known as the ‘H region.’

ii) Mechanisms of OR gene selection

While these experiments demonstrate that the receptor protein itself is critical in maintaining its exclusive expression, they do not explain how it was chosen in the first place. Monoallelic gene expression is relatively rare and requires a more complex system of regulation than simple transcription factor/promoter-driven expression, as each allele of the gene should share the same promoter region and any factor that would transcriptionally activate one allele should do so to the other.

Among the competing models first put forth to explain how OR genes might be selected for expression were the notion of a network of hierarchical regulatory elements, a single genome-wide limiting locus control region that could stochastically associate with only a single OR allele, and finally, an irreversible recombinase-mediated gene selection, similar to the V(D)J recombination strategy employed during generation of the antibody repertoire. (Hsu 2009) The latter hypothesis was ruled out in an ambitious series of experiments that used somatic cell nuclear transfer to clone a mouse line from a single mature ORN. It could be predicted that if some non-epigenetic alteration had occurred, for instance a rearrangement that placed the selected OR gene in proximity to a promoter element, that this change would be preserved in the mice cloned from a post-recombination neuron. However, the resultant mice expressed the full repertoire of ORs, indicating that no irreversible genetic change had occurred at these loci during the gene selection process in the originating ORN. (Eggan et al. 2004) (Li et al. 2004)

More recent advancements in studying this problem have provided tantalizing glimpses at the underlying machinery. A ~2kb region was identified upstream of the mouse MOR28 gene cluster by Serizawa et al., (2003) (Shou Serizawa et al. 2003) that exhibited high
homology to a region upstream of the human HOR28 gene. This sequence was thus termed the ‘H’ region. Initially, it was shown that deleting the H region in a yeast artificial chromosome containing fluorescently tagged variants of the genes in the MOR28 gene cluster, expression of the tagged receptors was lost. Conversely, moving the H region closer to MOR28 resulted in a dramatic increase in the number of MOR28 expressing neurons. Based on these findings, the H-region has the features of a cis-acting regulatory sequence that governs the selection of genes from the MOR28 cluster. Closer proximity would increase the chance of H associating with the promoter of MOR28, explaining the increased representation of this gene. In fact, the proximity effect is mirrored in wild-type animals, where the MOR28 gene that is closest to H has the greatest representation of all of the MORs in the cluster. (Tsuboi et al. 1999) The proximity effect and the ability of H to activate only a single receptor suggests that there may be a stable intrachromosomal interaction between H and the promoter region of its chosen receptor, similar to the mechanism employed in exclusive expression of the red or green opsins. (Cook & Desplan 2001)

For a time, it was speculated that H might serve as both a cis- and trans-acting regulatory element, possible functioning as a global locus control region that would stably interact with and allow expression of a single receptor. While there was some intriguing evidence to support this model (Lomvardas et al. 2006), subsequent studies that genetically ablated the H region showed that it was acting as a local rather than global transcriptional regulator. Deleting the H region resulted only in the loss of expression of the proximal MOR28 cluster, but left unperturbed the expression of more distal gene families and those on different chromosomes. (Nishizumi et al. 2007) (Fuss et al. 2007)

While the question of how an OR gene is selected is far from resolved, the H region findings may offer a glimpse at a larger regulatory strategy that could involve many such cis-acting transcriptional activators. It is possible to envision a system in which clusters of genes on a particular chromosome are locally selected from by a local cis-acting activator, with many activators that could potentially be called upon. The mechanism underlying the choice of a single functional cis-activator would then present a similar set of questions as those regarding the selection of a single receptor. However, certain features of olfactory receptor expression, such as zonal restriction, would be better explained by a hierarchical mechanism of gene choice than by a simpler one. In such a model, the logic of receptor-mediated repression of the expression of other ORs also becomes more clear; if the initial selection involves a transitory process rather than a stable and limiting interchromosomal interaction, a mechanism would need to be in place to ensure that once that transitory phase had passed, every other ‘H-like’ region didn’t select its own receptor in parallel. Identifying the mechanism by which receptors ensure their own exclusivity may provide key insights into how such a choice is made in the first place. The potential cast of characters such a regulatory process could call upon range from the receptor itself, which could physically interact with some part of the repressive molecular machinery, to the various downstream signaling cascade components the receptor influences once expressed.

III) Establishing the olfactory map
Having stably selected a receptor, a nascent ORN must next target its axon to the olfactory bulb and coalesce into discrete receptor-specific glomeruli. This process can be divided into two intertwined processes, pathfinding and convergence. A fascinating observation underscoring this division is that a large part of the ORN convergence machinery seems to operate independent of instruction from the target tissue. It has been shown using mice in which the olfactory bulb has been either physically or genetically ablated that the axons of ORNs expressing a given receptor still roughly converge to a localized region. (Ardiles et al. 2007) (Chehrehasa et al. 2006) (St John et al. 2003) (Bulfone et al. 1998) However, the dorsal to ventral, rostral to caudal, and medial to lateral organization of the projections to the olfactory bulb also employ more canonical strategies of guidance cue-mediated neuron-to-target mapping. For instance, the familiar axon guidance cues Slit and its receptor Robo are involved in zonal segregation and dorsal-ventral patterning, while the ephrins and their receptors are involved in anterior-posterior positioning. (Nguyen-Ba-Charvet et al. 2008) (Cho et al. 2007) (Cutforth et al. 2003) The process of olfactory map formation thus likely involves both target-dependent mechanisms of pathfinding that guide extending axons to the roughly correct region of the bulb and ORN intrinsic mechanisms that underlie the formation of receptor-specific glomeruli.

i) Activity dependence of map formation

A common paradigm in neural development is that complementary gradients of guidance cues in the target tissue and receptors in the projecting neurites set up the crude organization of a region and neuronal activity subsequently acts to refine the delicate features of the circuitry by strengthening correct connections and causing ectopic ones to be pruned away. The formation of the retinotopic map in the visual system is perhaps the best-understood example of such principles at work, (Schmidt 1985). Neuronal activity is well-known to be important in guiding and refining the organization of many compartments of the nervous system, and the olfactory system is no exception.

There is evidence that refinement occurs during the development of the olfactory system, as some glomeruli transiently receive innervation from multiple populations of ORN. (Zou et al. 2004) However, there is not evidence of exuberant targeting of all ORNs to all regions of the bulb, suggesting that this may represent a subpopulation of mis-targeted neurons that are eliminated rather than a general developmental strategy. The elimination process is influenced by evoked activity, as when the nostril is sealed to occlude odor stimulation, these ectopic projections persist.

The precise mechanisms by which neuronal activity shapes and refines the developing olfactory system have been investigated by many approaches. The genetic ablation of the CNGA2 channel results in the loss of odorant-evoked activity, and the mutant mice are incapable of smelling odorants detected by the main olfactory epithelium. However, the basic olfactory map in these animals is grossly unperturbed; ORNs still project to the bulb and converge to form glomeruli in the expected region of the bulb. (Lin et al. 2000) (Wong et al. 2000) (Belluscio et al. 1998) However, this manipulation affects the entire
population of neurons equally and does not silence spontaneous activity or the resultant release of neurotransmitters from synaptic sites at the axon terminal.

Inhibiting neurotransmitter release in the global ORN population by expressing the tetrodotoxin light chain (which cleaves the molecular machinery that allows vesicle fusion at the synapses) similarly did not disrupt formation of the map. However, suppressing spontaneous neuronal activity of ORNs was found to delay and disrupt formation of receptor-exclusive glomeruli. (Yu et al. 2004) In mice engineered to express a hyperpolarizing potassium channel in their olfactory neurons, which results in the silencing of spontaneous action potentials, glomerulus formation was delayed and ORNs expressing receptor P2 were widely targeted to many glomeruli, while those expressing receptor MOR28 projected to multiple targets instead of one. Performing both of these manipulations in only a small subset of neurons instead of the entire population, however, revealed much more profound effects; when synaptic release was inhibited only in the P2 neurons, these neurons were unable to form a stable glomerulus. When spontaneous activity was inhibited in this subset of neurons alone, their axons failed for the most part to even enter the olfactory bulb. Relative levels of neuronal activity thus play a greater role than the activity of the entire system. Examining the molecular substrates of neural activity and the role these play in pathfinding and convergence will shed further light onto why this is so.

An important distinction here is the difference between neural activity being instructive or permissive. Globally knocking down activity does not prevent the formation of the olfactory map; only in the most severe manipulation (inhibition of spontaneous activity) did it begin to be disorganized. This suggests that activity is not strictly necessary for the neurons to reach their targets; however the relative activity within subsets of neurons seems to play an instructive role in guiding each population to their correct targets. This also suggests that activity must feed into downstream signaling pathways that can alter a neurons course relative to its surrounding neighbors. Interesting research on this front has shown that activity levels affects cAMP production, which in turn affect levels of Eph receptor expression in pathfinding neurons. (Shou Serizawa et al. 2006)

ii) Mechanisms of axon guidance

It is unsurprising that many of the factors known to play a role in guiding the olfactory neuron axons to the bulb are familiar in neuronal pathfinding. This includes the guidance molecules Slits and their receptors, the Robos. Slit1 is expressed in the ventral periphery of the olfactory bulb, and its receptor Robo2 is expressed in ORNs in a high dorsomedial to low ventrolateral gradient across the OE. (Cho et al. 2007) Mice in which one or the other is mutated show defects in dorsal-ventral segregation of ORNs; axons that should target the dorsal bulb instead project to ventral regions. Double Slit1/2 and Robo1/2 mutants display an even more severe phenotype wherein their axons fail to penetrate the olfactory bulb entirely. (Nguyen-Ba-Charvet et al. 2008) A more novel guidance pathway occurs through IGFR1 signaling. (Scolnick et al. 2008) While IGF is traditionally regarded as a growth factor, it appears to also have a role in instructing medial versus
lateral positioning of axons in the olfactory bulb by directly acting as a chemoattractant to direct some ORNs medially.

The cognate receptor pair Neuropilin-1 (Nrp1) and Sema3A, which act as repulsive cues, are also both expressed by the projecting ORNs (Takeshi Imai et al. 2009) where they are proposed to be involved in pre-target sorting within the olfactory nerve. Nrp1/Sema are involved in the anterior-posterior positioning of ORNs, and the expression of Nrp1 is related to levels of the second messenger cAMP, setting up a model in which axons within the olfactory nerve are potentially sorted based on levels of intrinsic activity. Neurons with higher levels of cAMP would sort away from those with lower activity.

In fact, cAMP-mediated signaling may be a general mechanism that links intrinsic activation of ORs to activity-dependent aspects of pathfinding. A series of studies have indicated that cAMP levels are critical in axonal convergence (Alexander T Chesler et al. 2007), and that cAMP signaling may be involved in sorting the axons of projecting neurons within the olfactory nerve by affecting the expression of the ephrin guidance molecules and the homotypic adhesion molecules kirrel2 and kirrel3. (Shou Serizawa et al. 2006) Olfactory map formation is also disrupted in an AC3 mutant mouse (Zou et al. 2007), which displays persistent ectopic targeting of ORNs to incorrect glomeruli.

**ii) OR instruction of convergence**

Underscoring the role of the OR in precise convergence to the olfactory bulb, it has been shown that substituting the coding sequence of one receptor with that of another, such as expressing the mouse ORs M71 or M72 under the natural promoter of the receptor P2, results in the convergence of the neurons expressing the ectopic receptor on a location close to but still distinct from the target glomerulus of the substituted receptor. (F Wang et al. 1998) Hence, the location of the convergence of the population is more dependent on the identity of the expressed receptor than the locus it is expressed from. Similarly, expressing a rat odorant receptor, I7, in the place of the mouse M71 receptor results in axonal convergence and formation of a novel glomerulus (Bozza et al. 2002) as does expressing the non-olfactory B-adrenergic receptor, and making changes to a given receptor structure results in slight shifts in the position of the resultant glomerulus. (Feinstein et al. 2004) These findings suggest that the OR plays a critical role in determining the point on the olfactory bulb on which to converge, but that it is not the sole determinant. The graded shifts in glomerular position and the ability of ectopic GPCRs to mediate convergence also imply that there are not a series of fixed targets in the olfactory bulb that the axons are guided to, but rather that the coalescence of the axons itself is determining the location of a resultant glomerulus.

The question then is how the OR is acting to instruct this process. Of note when considering this issue is the fact that OR proteins are present in the axon terminals (Joerg Strotmann et al. 2004) (G. Barnea et al. 2004) where they are coupled to transient changes in calcium and cAMP concentration. (Maritan et al. 2009) cAMP in fact seems well positioned to be a central mediator of activity dependent pathfinding, having been
shown (as mentioned above) to be involved in many aspects pathfinding and convergence. (Takeshi Imai & Hitoshi Sakano 2007)

Conclusions

Central to the understanding the organization of the olfactory system is the notion that the identity of each neuron is defined primarily by the OR that it expresses. Each ORN in a given region of the OE shares a common developmental lineage and until the initiation of the process of receptor selection, they comprise a largely homogenous population. It is the stochastic choice of receptor that grants the nascent ORN an identity different from its immediate neighbors, and it is this choice that will dictate the precise location to which the neuron targets and to what compounds it responds to. While the links between the receptor identity and the chemical specificity of the neuron are clear, flowing through well-established signal transduction cascades, much remains to be elucidated about those that link the receptor to mechanisms of repression of gene choice and axon convergence. The OR and the signaling molecules it interacts with represent promising candidates to further explore these questions.
1.1) The canonical olfactory signal transduction cascade. Binding of an odorant to an OR causes ATP exchange at G_olf, dissociating the heterotrimeric G protein complex. GTP-G_olf activates the enzyme adenyl cyclase 3 (AC3), which catalyzes the formation of cyclic AMP and results in the opening of the cyclic-nucleotide gated channel CNGA2. The positively charged ions sodium (Na\(^+\)) and calcium (Ca\(^{++}\)) flow into the neuron, depolarizing it. Depolarization is further amplified by the calcium gated chloride channel, which opens in the presence of Ca\(^{++}\) to allow a rush of chloride ions (Cl\(^-\)) out of the cell.
1.2) Anatomical organization of the mouse olfactory system. ORNs in the olfactory epithelium expressing a given OR project their axons to the olfactory bulb, with a dorsal-ventral gradient of receptor identity existing in both regions. Neurons of the accessory olfactory system reside in the vomeronasal epithelium and project to the accessory olfactory bulb; notably, these neurons express distinct receptor classes from the ORNs in the main olfactory system, the V1Rs and V2Rs.
Figure 1.3
1.3) Schematic of the principles of combinatorial coding. Different odorants can bind to different receptors with varying degrees of affinity based on the chemical features they possess. The identity of an odorant is represented by both the subset of receptors to which it binds and the degree to which it activates each. Receptors in turn can bind multiple odorants, with different degrees of chemical promiscuity.
Introduction to Zebrafish olfaction

Understanding olfactory mediated behaviors in zebrafish

Zebrafish are shoaling fish, native to India, which live in a natural habitat of brackish water. They maintain a loose social structure that involves spending some time in school-like groups and some time dispersed and foraging, depending on various environmental, chemical, and visual cues. Their characterized behaviors include feeding, mating, shoaling, and alarm responses. Some aspects of these behaviors, such as prey capture and mate stripe/strain preference, are known to be largely or entirely visually mediated. (Gahtan et al. 2005) (Engeszer et al. 2008) However there is evidence that chemosensation plays a critical role in mediating responses to feeding cues and an alarm substance released from crushed skin. (T Valentincic et al. 2000) (Tine Valentincic et al. 2005) (Speedie & Gerlai 2008) For instance, zebrafish have been shown to be attracted to amino acids in a tank side choice assay (Koide et al. 2009).

Another approach to understanding how fish detect and process odors is to look directly at ORN responses to putative ligands. Investigations at this level give insight into the process on a cellular level, as opposed to the integrated output that is behavior. Electrophysiological studies in adult catfish have identified a number of classes of physiologically relevant odorants to which fish olfactory sensory neurons respond. These include amino acids, bile salts, and nucleotides. (Kang & J. Caprio 1995) (Rolen & J. Caprio 2007) Experiments with fluorescent calcium indicator dyes have confirmed that these odorants also elicit ORN activation in both adult and developing zebrafish. ORN inhibition has also been observed. (Rainer W Friedrich & Laurent 2004) Fish ORNs have an intrinsic basal firing rate of between 0-20Hz,

Amino acids are a powerful feeding cue in fish, evoking behaviors such as increased turning, search-swimming, and biting/snapping when added to the water at micromolar concentrations. (T. B. Valentincic & J. Caprio 1994) The principal amino acids to which catfish respond are methionine, leucine, and alanine.(Nikonov & J. Caprio 2007) Some amino acid responsive ORNs are broadly tuned while others are highly specific in their responses, suggesting that fish can not only detect but distinguish between different amino acids. This prospect is supported by behavioral experiments in which catfish can be conditioned to differentiate in their response to paired amino acids. (T Valentincic et al. 2000) Functional imaging of the zebrafish olfactory bulb also shows divergent but stereotyped patterns of activity for nine tested amino acids. (T Valentincic et al. 2000) (R. W. Friedrich & S. I. Korsching 1998)

Nucleotides may likewise serve as appetitive stimulants while bile salts are secreted in fish urine as a waste product and may serve as a social cue. Additionally, there are both ligands which have been shown to evoke activity in the olfactory bulb without characterized behavioral correlates, as well as olfactory mediated behaviors for which neither the ligands nor the identity of the receptor they signal through is known. Into the latter category falls a behavior referred to as the alarm response, in which teleosts of various species react to a substance released by crushed skin of conspecifics. (Waldman
Identification of the substrates mediating the alarm response in fishes is an active field of study.

Anatomical organization of the teleost olfactory system

The primary sensory neurons involved in olfaction are located in the epithelium of the olfactory rosette, the peripheral olfactory organ of the fish. These neurons send a single dendrite to the surface of the epithelium and an unbranched axon to the adjacent olfactory bulb. Here the axon terminals form discrete glomeruli in a stereotyped fashion. The olfactory epithelium (OE) contains a mix of three types of neurons, as characterized morphologically and by expression of differentially expressed cell-type markers. These are the ciliated, microvillous, and crypt neurons. In adult fish OE there is a roughly laminar organization of the cross-sectional epithelium, with the ciliated cells positioned more basally within the OE and the microvillous neurons situated more apically. Based on this organization and cell morphology, inferences have been drawn about what receptor type and signaling pathways are utilized by these respective cell types in teleosts. (A. Hansen et al. 2003) (A. Hansen et al. 2004)

The ciliated neurons express Olfactory Marker Protein (OMP) as well as Galpha(olf.) in situ hybridization detection of olfactory receptor mRNA suggests that a single ciliated OSN expresses one or a small number of ORs (Barth et al., 96 and 97) that are homologous to the canonical odorant receptors of terrestrial mammals. (L. Buck & R. Axel 1991) Like their counterparts in the mammalian main olfactory epithelium, they are thought to signal through a transduction pathway involving receptor mediated activation of adenylyl cyclase, production of cyclic AMP, and the subsequent opening of cyclic-nucleotide gated channels. In zebrafish, time of receptor expression onset differs between different receptor subfamilies. (Barth et al. 1996)

Microvillous neurons of fish express TrpC2 (transient receptor potential), a non-selective cation channel, as well as Galpha(o). (Anne Hansen & Zielinski 2005) This is likewise true of a subset of neurons found in the mouse vomeronasal epithelium, suggesting that the microvillous neurons may similarly signal through a phospholipase C dependent pathway. References In addition the receptors expressed in the microvillous cells are family C GPCRs homologous to the V2R family found in this subset of vomeronasal neurons. One notable parallel between the mammalian vomeronasal system and the zebrafish microvillous cell population is the apparent co-expression of a single broadly expressed receptor in a large percentage of neurons. (Martini et al. 2001) Studies undertaken in this thesis aim to determine the role of OlfCc1 in the zebrafish olfactory system, and to investigate the hypothesis that receptor heterodimerization is critical to olfactory responses in microvillous neurons. (Fig 1.5)

The least is known about the crypt cells, which are less numerous than the other classes of OSN. They have a short, rounded morphology and can be identified immunohistochemically by expression of S100B protein.
Similarly to the organization seen in mammals, axons of ORNs converge to form the olfactory nerve. They project to the immediately adjacent olfactory bulb where the axons defasciculate and form discrete clusters of neuropil known as glomeruli, targeting a single glomerulus with an unbranched axon. (Dynes & J. Ngai 1998) In zebrafish there is an approximate ratio of 100 neurons to each glomerulus. (H Baier & S Korsching 1994) ORNs expressing a given receptor appear to converge to a small number of glomeruli, roughly mirroring the organization of the olfactory bulb in mammals. (H Baier et al. 1994) The second order cells of the olfactory system are the mitral cells. The dendrites of these cells intercalate with the glomeruli while the axons project to the cortex by way of the medial and lateral olfactory tracts.

While there is a heterogenous mix of primary neuron types in the olfactory epithelium there appears to be a separation of these cell types as well as a topographic organization of different ligand responsive regions within the olfactory bulb. (Fig 1.6) The distinct ciliated and microvillous subpopulations target to complimentary non-overlapping regions of the bulb (Yuki Sato et al. 2005). The ciliated cells target to the dorsomedial glomeruli of the bulb while the microvillous population targets to more ventrolateral regions.

Examination of the responses of the second order cells to different ligands within bulb suggests it possesses a rough chemotopy which likely reflects this anatomical separation. Electrophysiology performed on mitral cells throughout the bulb of catfish as well as calcium dye imaging in the bulbs of developing and mature zebrafish reveal that the responses to amino acids fall largely within the ventrolateral bulb regions while the responses to bile salts fall within the more medial regions, suggesting that the ciliated and microvillous cell types respectively may mediate these responses. (Tine Valentincic et al. 2005) (R. W. Friedrich & S. I. Korsching 1998) (Sigrun Korsching 2002) The projections out of the bulb preserve this separation of odor response pathways, as the lateral olfactory tract, medial olfactory tract, and lateral medial olfactory tract projecting to different higher order regions of the brain. (Nobuhiko Miyasaka et al. 2009)

Many of the details of this map, such as whether a given glomerulus receives input strictly from a given receptor expressing population of ORNs for each of the neuron types, are not yet known for all of the neuron types. In the mouse there is a difference in the wiring of the second order cells between the main and accessory olfactory systems (Del Punta et al. 2002), raising the possibility that the organizational logic in the zebrafish olfactory bulb likewise differs between cell types and receptor families.

Receptor families, and OlfCc1

There are four major families of receptors currently believed to be involved in vertebrate olfaction. In fish, these families are the classical odorant receptors (ORs) (T. S. Alioto & J. Ngai 2005), receptors similar to mammalian type-1 vomeronasal receptors (V1R-like) (Pfister & I. Rodriguez 2005) (L. R. Saraiva & S. I. Korsching 2007), family C GPCRs similar to mammalian type-2 vomeronasal receptors (OlfCs) (T. S. Alioto & J. Ngai
2006), and the zebrafish trace-amine-associated receptors (zTAARs) (Liberles & L. B. Buck 2006) (Hashiguchi et al. 2008) (Hussain et al. 2009)

The members of these gene families are clustered within the zebrafish genome, as are their mammalian homologues. The ligand specificities of individual receptors are largely unknown. However, the chemotopy in the olfactory bulb described above as well as analysis based on sequence homology with well-characterized receptors has provided some insight into what chemical classes the different families may generally respond to.

The work performed in this thesis will focus on one of these families, the OlfC receptors, which have been predicted to bind amino acids based on a conserved amino acid binding residue motif in their ligand binding domain. In addition to the electrophysiological and imaging studies referenced above, it has been shown that the microvillous cells that express these receptors are critical for behavioral amino acid detection in adult fish (Koide et al. 2009). Some members of the homologous mammalian gene family, the V2Rs, have been shown to respond to peptides that secreted by mice and involved in assorted social behaviors, (Kimoto et al. 2005) (Chamero et al. 2007) suggesting an evolution of function and ligand selectivity may have occurred in at least some of these receptors. Another striking parallel between the microvillous cells expressing OlfCRs and the vomeronasal neurons expressing V2Rs is that both populations actually express two receptors per neuron, in contrast with the other neurons of the olfactory system. Specifically, these neurons express a single member of the gene family (OlfCc1 and V2R2) across the entire population, along with another seemingly random receptor. OlfCc1 and V2R2 are highly homologous to each other, and both are outliers from the larger family C gene family that they are part of. (T. S. Alioto & J. Ngai 2006) (Yang et al. 2005) The function of these widely expressed genes, and the significance of the clearly unique receptor selection mechanism employed in these cell types has not been investigated to date. The central experiments discussed in later chapters will in fact be aimed precisely at investigating this function, using zebrafish as a model organism.
Conclusions:

While less is known about the precise molecular mechanisms that underlie the formation of the zebrafish olfactory system than in mammals, recent research has provided a wealth of information about teleost olfactory function and odor processing. Further understanding of the zebrafish olfactory system will provide interesting details on the conservation of olfactory mechanisms across vertebrates; for instance the studies performed on the ubiquitous OlfC receptor OlfCc1 in this thesis may shed new light onto the unknown role of the homologous mouse vomeronasal receptor V2R2. In terms of understanding odor coding, zebrafish are particularly useful in that they have an order of magnitude fewer receptors in the canonical OR family but share a similar anatomical framework. They also share homologous receptor families and exhibit complex olfactory-mediated behaviors. Understanding the parallels and differences between mammalian and piscine olfaction promises to provide novel insight into both, and into the broad principles of neural coding and development in general.
1.4) Anatomy of the adult zebrafish olfactory system. The olfactory epithelium resides in a structure known as the olfactory rosette, which is densely folded to increase surface area. The olfactory rosettes are immediately adjacent to the olfactory bulb, which lies at the rostral tip of the telencephalon. ORNs project their axons to the OB and form glomeruli, an organization similar to the mammalian system.
1.5) The major neuron types in the zebrafish olfactory epithelium. The zebrafish OE is principally composed of two types of neurons, the ciliated and microvillous neurons, which are interspersed but segregated with respect to the location of their cell bodies along an apical-basal axis. The microvillous cells express the OlfCRs, the zebrafish homologues to the mammalian V2R family, while the ciliated cells express ORAs that are homologous to the canonical mammalian odorant receptors. A third cell type (unpictured, but see Fig 2.1), the crypt cell, is comparatively rare and expresses V1R-like receptors.
1.6) Organization of the olfactory bulb of a 3dpf zebrafish larva. The microvillous (red) and ciliated neurons (green) project to anatomically distinct regions of the olfactory bulb, a segregation that is maintained through adulthood. In general, microvillous cells target lateral glomeruli while ciliated ones project to more medial targets.
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Chapter two: Role of the broadly expressed olfactory receptor OlfCc1 in mediating amino acid detection in Zebrafish

Introduction

The olfactory systems of zebrafish and mammals have many conserved features, including homologous receptor families and similar principles of receptor expression. (Y Yoshihara 2009) While there are distinct anatomical differences in how the different receptor-family expressing classes of neurons are organized in mouse and fish, there are also many striking similarities within these populations of neurons.

In mammals, there are two closely associated olfactory organs, the main and accessory (vomeronasal) olfactory systems (see Fig 1.2). The vomeronasal epithelium contains neurons expressing the V1R or V2R receptor families, respectively parsed into apical and basal layers with respect to the position of their cell bodies. In contrast, zebrafish contain only a single peripheral olfactory organ, the olfactory rosette, in which different populations of neurons expressing the three receptor classes are intermixed but similarly stratified. (Fig 1.4, 2.1) The apically localized cells of the zebrafish olfactory epithelium (OE) express a class of family C GPCRs, the OlfCRs, that are homologous to the V2R family expressed in the basal cells of the mouse vomeronasal epithelium. Intriguingly, both the mammalian and zebrafish neurons expressing this receptor class actually co-express two receptors; one that seems to be chosen at random from the V2R or OlfCR family and one ‘ubiquitous’ receptor that is expressed across the entire neuronal population. The expression pattern of these ubiquitous receptors, V2R2 and OlfCc1 respectively, is unique among the various classes of olfactory receptors in vertebrates, pointing to a potentially novel role in vertebrate chemosensation. The goal of the experiments laid out in this work is to shed light on the role of the zebrafish OlfCc1 receptor in the teleost olfactory system.

The OlfCRs expressed in the apical microvillous neurons of zebrafish are predicted to be amino acid receptors on the basis of a conserved amino acid binding motif in their N-terminal domains. (Alioto & Ngai 2006) While the ubiquitously expressed receptor OlfCc1 is similarly predicted to bind amino acids, its broad expression makes it unlikely to be involved in the discrimination of a particular amino acid. Experiments from our lab have directly demonstrated by double in situ hybridization that OlfCc1 is coexpressed with other OlfC family members (E. Van Name, unpublished).

Based on the prediction that the OlfCRs are likely to be amino acid receptors, and the fact that OlfCc1 is ubiquitously co-expressed with the other OlfCRs, I hypothesized that OlfCc1 is playing a general role in facilitating amino acid detection across the microvillous cell population while the punctuate OlfCRs may be dictating the specificity of each neuron. To test this hypothesis, I employed a loss of function approach, using antisense morpholinos to knock down the expression of the OlfCc1 protein. I assessed the effect of this manipulation on the development and function of the larval olfactory system by a variety of methods.
My hypothesis predicts that loss of OlfCc1 should have a broad and deleterious affect on amino acid chemosensation. However, an alternative possibility is that the receptor could also or instead be playing a direct role in instructing the development of the olfactory system, or have an indirect role in which evoked activity in the microvillous cells could be important for their pathfinding, survival, or synapse formation. Finally, it could be acting instead as a solitary chemosensor or a mediator of adaptation. To distinguish between these possibilities, I examined expression and localization of the receptor itself, the anatomical development of the larval olfactory system, and odor-evoked activity within living larvae, with the knowledge that the results of the earlier experiments would guide interpretation of the later ones.

The specific aims of this study were to:

I) **Determine whether the subcellular and temporal localization of OlfCc1 are consistent with a chemosensory role.**

II) **Examine the effects of OlfCc1 knockdown on the histological development of the olfactory system.**

III) **Establish and test an assay for investigating odor-evoked activity in living zebrafish larvae.**

IV) **Use this assay to determine the effect of OlfCc1 knockdown on amino acid evoked activity in larval zebrafish**

When interpreting the results of the experiments that follow, the specific anatomical organization of the olfactory bulb with respect to the OlfC expressing microvillous cells will be important to keep in mind. The microvillous neurons are randomly interspersed with the ORA expressing ciliated neurons in the olfactory rosette but project to anatomically discrete regions of glomeruli in the olfactory bulb. (Fig 2.2) The microvillous innervated glomeruli are generally more lateral than the ciliated cell innervated ones, and are also present in a deeper plane than the more medial glomeruli. (Fig 2.3) This anatomical separation allows for differential effects on the microvillous cells to be compared with global effects on the olfactory system.

The following chapter will be divided into subsections based on the aims addressed in each. The first subsection addresses aims I & II; the second addresses aim III; and the third addresses aim IV. Results and implications of the sum of the experiments will be discussed in the conclusion.
2.1) Olfactory neuron types and their relative apical-basal localization in the zebrafish olfactory epithelium. Microvillous cells (red) express OlfC receptors and are localized apically in the cross-sectional OE. Ciliated cells (green) express the canonical family A olfactory receptors (ORAs) and have soma that are localized more basally. Crypt cells (blue) are relatively uncommon in the OE, express V1R-like receptors, and have squat, rounded soma located near the apical surface.
2.2) Medial-lateral segregation visualized in an OMP-UASxGAL4-GFP 4dpf zebrafish. Calretinin immunolabeling (red) marks the microvillous cells while GFP immunolabeling (green) marks the OMP-expressing ciliated population. The microvillous neurons particularly target lateral glomeruli adjacent to the OE, whereas the ciliated population projects to more medial regions. However, there are also sparse microvillous projections to other regions of the bulb.
2.3) Depth coded SV2 staining showing topographical layout of the olfactory bulb. SV2 marks synaptic vesicles, which are heavily concentrated at the glomeruli. Blue marks more rostral regions while red is more caudal. The entire depth of the bulb is ~30um. The medial glomeruli are more superficial while the lateral ones are deeper in the bulb. The most lateral glomeruli constitute an area known as the ‘lateral chain’ and are primarily targeted by microvillous cells.
Chapter 2.1: Histological investigation of the expression of OlfCc1 and the effects of its knockdown on the development of the zebrafish olfactory system.

Background

The first step in investigating the role of OlfCc1 was to confirm its widespread expression in microvillous cells, characterize the subcellular distribution of the receptor protein, and compare the temporal profile of the onset of receptor expression with the known onset of amino acid evoked activity in zebrafish larvae.

To these ends, I had a polyclonal antibody to a region of the OlfCc1 N-terminal domain commercially generated. The N-terminal domain of the gene represented an optimal region to target as it is more variable than the highly conserved 7-transmembrane region. (Alioto & Ngai 2006) To further minimize the possibility of cross-reactivity between different OlfCb receptors, the epitopic region for antigenic peptide generation was chosen after performing an alignment of all 62 of the OlfCb gene amino acid sequences and selecting a region of low conservation. I then affinity purified the antibody from the raw sera using the peptide with which immunization and boosts had been performed.

The features of the OlfCc1 genomic and coding sequences, a stylized schematic of receptor structure, and the sequence and target of the antigenic peptide used for antibody generation are shown in Fig 2.4.

Results

Applying the antibody to transverse sections of adult olfactory epithelium confirmed the broad distribution of expression previously observed by in situ hybridization, and showed that the expression of OlfCc1 was restricted to the apical cells of the sensory epithelium (Fig 2.5A), consistent with widespread expression in microvillous neurons.

In the developing larvae, the receptor protein begins to be expressed in the developing olfactory rosette by 48 hpf, with the number of expressing cells increasing over time. OlfCc1 expression broadly overlaps with that of calretinin, often used as a marker of microvillous cells in developing fish. (Fig 2.5B) (A. Germanà et al. 2007) Thus, expression of OlfCc1 begins slightly before odor-evoked activity is first detected in the olfactory bulb at 3 dpf. (J. Li et al. 2005) The timing of OlfCc1 expression is therefore consistent with the notion that it is involved in chemosensation.

Subjecting the adult sectioned tissue to an antigen-retrieval protocol revealed additional staining in the region of the apical microvilli, suggesting that there may be some degree of epitope masking in the mature receptor. (Fig 2.6) Interestingly, when the sequence of OlfCc1 is aligned with a homodimerizing Family C GPCR for which a crystal structure has been obtained, mGluR1 (Kunishima et al. 2000), the region containing the antigenic peptide overlaps with the predicted mGluR1 intermolecular dimerization interface. In mGluR1, homomeric dimerization at this site occurs via an intermolecular disulfide bond;
while the exact location of the cysteine involved in this bond is not conserved between the two sequences, there are two cysteines in the immediate location of the corresponding sequence in OlfCc1, including one within antigenic peptide sequence itself. (Fig 2.4) It is possible that the epitopic masking observed here is a direct result of receptor dimerization obscuring the antigenic epitope, in which case it is possible that the more difficult to detect microvillous protein represents a mature, dimeric species. Beyond this, the subcellular localization of OlfCc1 at the microvilli also places the receptor in a prime location for involvement in chemotransduction.

Background

Having confirmed that OlfCc1 is expressed in a broad subset of microvillous neurons and displays a subcellular distribution consistent with a possible role as an olfactory receptor or co-receptor, I next investigated the effects of decreasing or eliminating its expression in developing zebrafish larvae.

To this end, I employed an antisense morpholino oligonucleotide mediated knockdown approach. Morpholinos are synthetic nucleotide analogues that contain a 6-member morpholine ring in the place of the 5-member ribose ring that forms part of the backbone in RNA. Because of this modified backbone, endogenous nucleases involved in degrading RNA transcripts for rapid turnover do not recognize morpholino oligonucleotides (henceforth referred to simply as morpholinos). They can be employed in a similar fashion as siRNAs, targeting various regions of an mRNA transcript with an antisense sequence to complex with it and block translation. Instead of needing to be expressed off of a vector, they are directly injected into the yolk of a one cell embryo and remain stable over the course of days. In zebrafish, morpholinos have been shown to effectively inhibit the translation of their target genes for approximately five days. (A Nasevicius & S C Ekker 2000) After this time, protein expression gradually returns, as the morpholino is diluted in the rapidly expanding tissue of the developing larva until it is no longer present in sufficient concentrations to knock down expression.

I designed a perfect match (PM) morpholino that targeted the first exon/intron splice junction of the OlfCc1 transcript. Notably, this splice site is downstream of the epitope targeted by the OlfCc1 peptide antibody used in this study, ensuring that the antibody should be able to distinguish between complete loss of expression and expression of a truncated, mutant or mis-folded protein. As a control, I used a five-base mismatch (MM) morpholino in which five residues throughout the sequence had been scrambled. An important caveat of morpholinos is their tendency to show toxic, non-target specific effects. About 30% of morpholinos are known to exhibit such “off-target” effects, which also tend to disproportionally affect the nervous system and can be paradoxically sequence specific enough to exert different effects than their own five base mismatch control morpholinos. This neurotoxicity is believed to be p53 mediated, although it is unclear what triggers it or why it is so highly sequence specific. (Robu et al. 2007)

Knockdown efficacy was tested using the OlfCc1 antibody across the developmental time frame of 48hpf through 5 dpf, and was found to be robust and highly consistent. (Figure
2.7) The lowest amount of morpholino needed to induce complete knockdown of OlfCc1 expression was empirically determined to be ~10ng/embryo; this amount was therefore used throughout these experiments to minimize the possibility of toxicity. All of the subsequent experiments were performed multiple times for each marker, and the results were determined to be consistent across experimental repetitions.

Results

The widespread expression of OlfCc1 in most if not all microvillous olfactory neurons suggests that it could be playing a generalized role in some other process other than chemotransduction. To address this possibility, I first examined the effects of its knockdown on various morphological features of the developing olfactory system. The results of these experiments are summarized below and in Table 2.1.

The number of calretinin-positive cells, which should represent a large subset of the OlfCc1 expressing neurons, was examined in 48hpf and 3dpf fish. There were no gross anatomical differences in most fish and the axons of the calretinin positive cells projected to their correct lateral targets in both the morphant and mismatch fish. (Fig 2.8A) Quantitation of the number of calretinin cells in each population showed that there were between 20-30% fewer cells in the morphant fish at both times points, a statistically significantly difference compared to the mismatch injected control fish. (48hpf: p=0.001; 3dpf: p= 0.001)

As a control, I then looked at the non-OlfCc1 expressing population of ORNs, the OMP-positive neurons. As I lacked a direct immunohistological marker for this cell type, I injected OlfCc1 morpholinos into an OMP-Gal4 transgenic line generated by our lab (T. Ferreira, unpublished) crossed to a UAS-GFP reporter line. GFP positive fish were fixed at 3 dpf and stained with a polyclonal antibody against GFP to assess cell number. Surprisingly, there was an even greater effect of the morpholino on the number of OMP-positive cells (~45%, p = 3E-06) than on the calretinin expressing population (Fig 2.8B) Again, the axons of the remaining population appeared to largely target correctly across the population of injected fish.

To look more directly at effects on the formation of the glomerular map, morpholino injected fish were labeled with an antibody against the presynaptic marker protein SV2, which discretely marks the bounds of all gomeruli. SV2 staining did not seem to be grossly perturbed in the morphant fish, with discrete synaptically dense structures forming as expected in a roughly stereotyped fashion. This observation suggests that the functional synaptic architecture is still present in fish with repressed OlfCc1 expression, although the area of the bulb in general was smaller in some of the morphant fish. (Fig 2.8c) SV2 expression was used to mark the borders of glomeruli so that they could be counted discretely. Comparing the number of glomeruli between the mismatch and the morphant populations revealed a decrease similar in magnitude to that observed in the first order cells, with an average 1.5 fold decrease in the number of glomeruli averaged across ages (p= 1E-06, and 0.001 respectively) Reducing the amount of morpholino injected to 7ng/embryo did not eliminate this difference, although it did slightly reduce it.
(1.2 fold change; p=0.029). As the SV2 positive synapses reside in the two first order populations of cells already shown to be affected, it is not surprising that they likewise reflect this deficiency.

As the number of glomeruli is rapidly changing at this time point, with larger protoglomeruli refining into smaller ones without largely changing in size, I also compared the area of the outlined footprint of SV2 immunoreactivity relative to the area of the entire olfactory bulb, as morphologically identified using BOBO nuclear dye labeling. A similar reduction in size in the morphants was observed. (Table 2.1)

Discussion
OlfCc1 is, like the other OlfC family receptors, predicted to bind amino acids in its venus-flytrap extracellular domain. (Alioto & Ngai 2006) However, its strikingly different expression pattern suggests that it may have different or additional roles to the other OlfCs. The experiments described here confirm the widespread expression of OlfCc1 in the microvillous cell layer of the OE, and place the receptor protein at the odorant-binding microvilli exposed to the nasal cavity, localization consistent with a role in odor detection.

The OlfCc1 antibody also allowed me to confirm that I could achieve efficient protein knockdown in zebrafish injected with an antisense morpholino. I was thus able to investigate the question of whether the broad expression of OlfCc1 reflects some general developmental role.

The results of these experiments showed that the OlfCc1 morpholino had a significant and similar in magnitude negative effect on the number of calretinin positive cells, the number of OMP positive cells, and the number of glomeruli that these populations form in the olfactory bulb. While the loss of OlfCc1 appeared to cause deficiencies in various assorted olfactory markers, it is of important note that this effect did not appear to be restricted to the neurons expressing the receptor; the OMP expressing neurons were affected to an equal or greater degree than the calretinin expressing ones. There are two potential explanations for this observation; the first is that OlfCc1 has a non-cell autonomous affect on the proliferation or survival of the ciliated cells. The other is that the well-known potential neurotoxic effects of morpholinos are non-specifically acting on all of the neuronal populations observed. In light of this, it is interesting to note that some percentage of the morphant fish appeared to exhibit more widespread nervous defects, such as hypotrophic telencephalic regions. (S2.1) Such generalized defects could be due to p53 mediated apoptosis, or else reflect a developmental delay. A third possibility (not inconsistent with the other two) is that the morpholino is causing some sort of developmental delay, which may or may not be OE specific.

In spite of these widespread effects, OlfCc1 does not appear to be critical for guiding the development of any specific part the olfactory system. Future experiments, such as those using a different OlfCc1-specific anti-sense morpholino, perhaps one targeting the translational start site, will be needed to carefully dissect apart any OlfCc1 loss-mediated
effects from off-target but sequence specific ones. Also, future results obtained using this morpholino will need to be carefully interpreted in light of these results.

While the notion of a developmental role for OlfCc1 cannot be conclusively determined in these experiments, the receptor remains well placed spatially and temporally to be involved in odor-detection. This raises the question of whether OlfCc1 might have a chemosensory role, and if so, whether the receptor is acting solely to detect a particular ligand or is more broadly involved in mediating processes of signaling or desensitization. Based on its broad expression, my original hypothesis was that it would subserve one of the latter functions. To test this, I therefore needed a means by which to move beyond histology and investigate the functional properties of the olfactory system in a living fish. A commonly used approach in loss-of-function screens is behavioral assays; unfortunately, zebrafish do not begin to exhibit olfactory-related behaviors until well after the window of morpholino efficacy closes. However, experiments using calcium indicator dyes in the bulbs of zebrafish larvae have shown that they begin to show odor-evoked neural activity in the second order cells as early as 3 dpf, when the nares first begin to open. (J. Li et al. 2005) The observation that features of the basic anatomical framework are preserved in OlfCc1 morphant fish allows questions about its function to be asked in a relatively straightforward manner, as the other components that would need to be present for proper olfactory activity seem to be in place to some degree. The next section of this chapter discusses experiments performed to establish and validate a functional assay to further probe OlfCc1 function.
2.4) Features of OlfCc1 and Family C GPCRs. A) Exon/intron structure of OlfCc1. Unlike the canonical ORs, the OlfC receptors contain introns and are spliced pre-translation. The morpholino employed in these studies blocks the first splice junction. B) Coding sequence features of OlfCc1. Family C GPCRs are characterized by a large N-terminal domain, which comprises the majority of the coding sequence. The sequence used for antibody generation and putative epitope is shown in red and the predicted transmembrane domain helices in teal. C) Schematic of Family C GPCR structure. The large extracellular N terminal domain of Family C GPCRs folds into a 'venus flytrap' domain, which is thought to bind ligands within the hinge region. OlfCs are predicted to bind amino acids based on a conserved signature of residues within this region. D) Sequence of antigenic peptide used to generate OlfCc1 antibody.
2.5) Expression of OlfCc1 in adult and embryonic zebrafish olfactory tissue. A) Labeling in an 18um thick transverse section of the adult olfactory rosette. Acetylated tubulin (red) labels the long cilia of the non-sensory epithelium, illustrating the restriction of OlfCc1 expression to the sensory region. Inset is a close up showing neuronal morphology. B) Labeling in the developing olfactory epithelium of a 3 dpf larva. OlfCc1 shows broad but not universal overlap with the expression of calretinin, a marker of microvillous cells at this developmental stage. Scale bar = 20um.

Figure 2.6)
2.6) Antigen retrieval reveals additional OlfCc1 staining at the apical surface of the OE. A) Cross section of adult tissue stained with OlfCc1 antibody. The protein is evident throughout the cell bodies but not particularly concentrated at the apical surface. B) Robust apical staining is revealed in tissue briefly boiled in sodium citrate to partially unfold crosslinked proteins and potentially reveal hidden epitopic regions. Outlined arrow indicates the apical surface of the OE where microvilli are found; filled arrow indicates the more basal soma.
2.7) Effective knockdown of OlfCc1 protein by antisense morpholino injection. Injection of 5ng/embryo of perfect match morpholino abolished expression, within the detection limits of the OlfCc1 antibody. The morpholino maintained its efficacy from early in development (A) through 5 dpf (B), the developmental window that future experiments will span.
Figure 2.8) Effects of OlfCc1 morpholino on development of the zebrafish OE. A panel of representative images of morphant and mismatch injected fish stained with: A) calretinin (green) to mark the microvillous population and SV2 (red) to mark their target glomeruli at the bulb; B) GFP (green) in an OMP-Gal4xUAS-GFP stable transgenic line to mark ciliated cells and SV2 (red) to mark their targets; C) SV2 (green), using BOBO nuclear backlabel (red) to show an unabstructed view of the organization of glomeruli at the bulb.
Table 2.1) Summary results of OlfCc1 morpholino injections across all ages, markers tested, and amounts of morpholino injected. In all cases, a significant decline in the quantified parameter was observed in the morphant fish; these declines ranged from a 1.3 to 1.7 fold decrease. In the case of calretinin and OMP, 'value' represents the number of discrete neurons counted in a fish, averaged across both epithelia; in the case of glomeruli, it refers to the total number of anatomically discrete glomeruli defined by SV2 immunoreactivity; in the case of SV2 area, it represents the ratio of the footprint of SV2 immunoreactivity to the area of the olfactory bulb defined morphologically using BOBO nuclear stain to show features.

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Chapter 2.2) Functional imaging in live zebrafish larvae

Background

In order to determine whether OlfCc1 knockdown might have a functional affect on zebrafish olfaction, I established an imaging assay that would allow me to monitor neuronal activity in live, morphant zebrafish larvae.

The promoter-specific expression of genetically encoded sensors of neuronal activity promises to elucidate much about the functional circuitry of various neural systems. Calcium and voltage sensitive dyes have long been used as a means to monitor neural activity, in the olfactory system and elsewhere. However these approaches suffer from the drawback of allowing only for coarse anatomical targeting that does not specify which cell type is labeled. Expression of fluorescent calcium reporters in promoter-defined subsets of cells promises to circumvent this issue. GCaMP is a genetically encoded calcium indicator (GECI) constructed from a circularly permuted GFP that has been split in half and rejoined with the calcium-binding domain of myosin light-chain kinase. (J Nakai et al. 2001) Binding of calcium to this molecule causes a conformational change such that the fluorophore is reconstituted, causing an increase in fluorescence intensity. This property allows changes in GCaMP fluorescence to report neural activity in real-time, with sensitivity and a time-scale similar to that obtained with dyes. GCaMP 1.6, which is used in this study, can undergo changes in fluorescence over baseline in the tens of percents and has a KD of approximately 150nM. (Masamichi Ohkura et al. 2005)

Zebrafish constitute an optimal model organism with which to use these genetic tools due to their relative genetic tractability and transparency at a young age. A common approach utilizes the Gal4 – UAS trans-activation system adapted from yeast and Drosophila, in which the a promoter drives activation of the transcriptional activator Gal4 that binds an upstream activating sequence (UAS) to drive expression of whatever gene it is upstream of. Such a system allows stable promoter-Gal4 lines to be crossed with different UAS – reporter/effector lines interchangeably. (Scheer & Campos-Ortega 1999) (Davison et al. 2007) In this case, I used the UAS-GCaMP1.6 line generated by the Baier lab as a calcium-sensitive reporter. Additionally, Tol2 recombinase-mediated promoter trap screens have been used to generate a large number of transgenic lines driving Gal4 expression in random but interesting subsets of neurons and other cells, providing a library of potentially interesting drivers for future experiments. (Scott et al. 2007)

To establish and test the imaging setup, I crossed the UAS-GCaMP1.6 line with an OMP-Gal4 line produced in our lab (T. Ferreira, unpublished). My goal was to verify that I could reliably evoke differential responses to different classes of odorants, and to optically investigate for the first time odor-evoked activity in the first order ciliated cells.

Results

For these experiments, I focused on imaging during a window of 4-7 days post fertilization, as previous functional imaging experiments using calcium-sensitive dyes in
the olfactory bulb indicate that larval fish can exhibit evoked activity to odorants at this time (J. Li et al. 2005) and this period intersects with the approximately 5 day developmental window in which morpholinos maintain their efficacy. (A Nasevicius & S C Ekker 2000)

Zebrfish expressing GCaMP1.6 in their olfactory placode were immobilized with α-bungarotoxin (Shin-ichi Higashijima et al. 2003) and mounted in 1.2% low melting point agarose. After the agarose was carefully cleared from their heads, with particular care paid to the nostril region, they were mounted in custom imaging chamber in an anterior up orientation, such that confocal slices provided roughly transverse sections of the olfactory bulb. (Fig 2.9) Odor application was synchronized with the initiation of image acquisition for consistency across experiments with respect to photobleaching and dark-state conversion of GCaMP.

To test the maximum responsiveness of GCaMP1.6, I exposed the fish to IBMX/forskolin (1mM/50uM, respectively). Forskolin is an activator of adenylate cyclase that causes crosses the cell membrane and causes a large increase in cAMP concentration, while IBMX is a phosphodiesterase inhibitor that blocks its immediate breakdown. Together, they cause a large increase in cAMP, activating the cAMP dependent signaling cascade and triggering calcium influx. As can be seen in Fig 2.10, IBMX/forskolin application results in extremely strong GCaMP activation, as indicated by the change in fluorescence over baseline. Food extract (FEX), which has been shown to cause robust and widespread calcium influxes in the mitral cells of the olfactory bulb at this age (J. Li et al. 2005), also evoked a strong response, although only a fraction of the magnitude of IBMX/forskolin. Notably, the FEX-evoked response was constrained to the apical dendrites of the neurons and activated only a small subset of cells in the OE and synapses at the bulb. The localization of the latter response is generally representative of odorant-evoked activity, where the calcium influx seems to be concentrated in the region dense with olfactory receptors and the calcium channel rich synaptic terminals.

These responses were consistent across many fish and experiments. Additionally, I was able to show that the responses at the synapses in the olfactory bulb, while sparse, were differentially activated by different pools of odorants, including complex pools. An example of this finding is shown in Fig 2.11, where pools of FEX, an extract of crushed zebrafish skin, and a 10uM pool of the bile acids Glyccholic acid, Taurocholic acid, Taurodeoxycholic acid, and Taurochenodeoxycholic acid evoked spatially differential activity.

Discussion

Using the OMP-Gal4XUAS-GCaMP1.6 fish, I was able to establish an optical imaging assay which allowed me to reproducibly image odor-evoked responses in 4-6 dpf zebrafish larvae in vivo. The activity of the first order cells in response to even complex odors in this line of fish was surprisingly sparse, although intense IBMX/Forskolin activation demonstrated that the signal transduction machinery downstream of the OR was present and functional.
A degree of chemotopy was evident in the synapses of the bulb, where different odors activated synapses in different regions.
Figure 2.9)

2.9) Schematic of imaging setup. A paralyzed fish is mounted such that the objective is closest to the most anterior region, with the optimal angle for mounting shifting slightly as the fish ages.
Figure 2.10) Forskolin/IBMX and Food Extract both evoke robust calcium changes in an OMP-Gal4xUAS-GCaMP1.6 fish. A) Forskolin/IBMX (1mM/50uM) induces a huge and long lasting calcium influx throughout the cell bodies and synaptic terminals of all GCaMP1.6 expressing neurons. B) Food extract (FEX) evokes a response that is localized to the cilia of a subset of neurons in the same fish; this response has a much shorter time scale and activates only a few discrete terminals at the olfactory bulb.
2.11) Differential responses to assorted odorants (3s application) in the OB of a 5dpf zebrafish. Responses to complex stimuli (food odorant, skin extract) as well as a pooled class of odorants (bile salts) could be detected in small subsets of synaptic terminals. Bars mark odor application. (FO) Food odorant; (BA) bile acids; [10µM each of TCA, GCA, TDCA, TCDC2, TLCA]; (SE) skin extract; (B) bath. Fluorescence traces in arbitrary units.
Chapter 2.3: Investigation of functional consequences of OlfCc1 knockdown

Background

Having determined that OlfCc1 is well-placed to be involved in chemosensation and that the basic architecture of the olfactory system can form in its absence, I next sought to investigate the role of the receptor in odor-evoked signaling. To this end, I used the in vivo calcium imaging system established using the OMP-Gal4xUASGCaMP1.6 transgenic line to examine the effects of morpholino-mediated knockdown on evoked responses to a variety of odorants in a line of fish expressing GCaMP1.6 widely in the nervous system.

Because OMP promoter driven expression is specifically excluded from the calretinin-expressing microvillous cells, a different transgenic line was needed to detect evoked activity in the microvillous cells. HuC is known to be a nearly pan-neuronal reporter, and its widespread expression in the second order mitral cells of the olfactory bulb of zebrafish has been previously reported. (J. Li et al. 2005) Notably, HuC-positive regions of the bulb respond to amino acids in 4 dpf zebrafish, indicating that it would be potentially useful for this assay.

For the following experiments I used a Huc-Gal4 line to drive GCaMP1.6 expression. This approach is advantageous in that it allows different classes of odorants to be examined in parallel in the same fish. Based on previous reports showing that the microvillous cells mediate behavioral chemotraction towards amino acids in adult fish (Koide et al. 2009), it seemed likely that the prediction that the OlfC family should bind amino acids would hold true. If microvillous cells are primarily responding to amino acids and the bulk of the amino acid response is mediated by microvillous cells, it can be predicted that the loss of OlfCc1 will have a specific affect on amino acid signaling while leaving detection of other odorants unperturbed.

Experimental design

The experimental protocol for these experiments involved injecting perfect match or mismatch OlfCc1 morpholinos into the progeny of HuC-Gal4xUASGCaMP1.6 crosses, and imaging them as described in the Materials and Methods section between 4-4.5 dpf. The injections were repeated across three experiments that were further split into a total of five imaging days. Due to time constraints, the number of fish imaged in a given day varied from three to eight. In total, data from 12 mismatch and 14 morphant fish were included in these analyses.

Importantly, HuC driven GCaMP was not excluded from any glomerular regions, as can be visualized using BODIPY vital dye as a backlabel. (Fig 2.12) HuC-driven GCaMP is also expressed in some subset of the first order neurons, with an expression pattern very similar to that of OMP.
For all experiments, the following common set of four odor pools, plus a control channel that contained the Ringers solution used as the bath, was used:

1) Food extract (FEX)
2) Pool of 9 amino acids at 100uM each: Glu, Ala, Val, Phe, Ile, Lys, Arg, Trp, Met
3) Pool of 4 bile acids at 10uM each: TCDCA, GCA, TCA, TDCA
4) Pool of 5uM each of the following amines: cyclohexamine, phenylethylamine, trimethylamine, N-methylpiperipine, tyramine, octopamine.

The amino acids in the pool were selected such that each of the four amino acid classes was represented: acidic, basic, short-chain neutral, and long chain neutral. In the latter two experiments, subset pools of 100uM leucine plus isoleucine and 100uM arginine + lysine were also used in order to investigate differential effects.

In all experiments, morpholino efficiency was confirmed, either retrospectively in the fish that had been functionally imaged or in non-imaged clutchmates from the same injection day.

Results

All of the tested pools of odorants were able to evoke broad patterns of activity at the olfactory bulb in uninjected or mismatch injected HuC:Gal4xUAS:GCaMP1.6 fish, with FEX eliciting the most robust response. Both the morphants and mismatch injected fish appeared to display similar responses to food extract and the bile acid and amine pools. In contrast, the response of the OlfCc1 morphants to the amino acid pool was strikingly reduced in magnitude and than that of the control fish. This effect was consistent across multiple experiments and at all of the planes imaged. Visual representation of this result is shown in Fig 2.13, which shows activity maps of fluorescence changes in response to each odor pool for a representative mismatch and morphant fish.

To further quantify and compare odor-evoked responses in both populations, I measured the total area that changed in fluorescence over baseline in one hemisphere of the bulb in a similar plane in each fish. Activity was therefore measured at a consistent anatomical location from animal to animal. To control for differences in size, the arbitrary pixel area was normalized to either the area of the hemi-bulb, as traced in a fluorescent image, or to the total area that responded to FEX, as traced in an activity map. The values calculated in these analyses thus represent either the ratio of response area to bulb area or the ratio of response area to FEX response area.

The rationale for choosing these two normalization methods was as follows: normalizing all responses to the odorant which evokes the maximal response might control for generalized, non-specific effects caused by the morpholino. Conversely, if the amino acid response comprised a significant percentage of FEX-evoked activity, comparing the odor-evoked activity vs. bulb area should highlight this. Finally, if amino acid evoked activity is specifically affected and leaves other odor responses, including FEX, unperturbed, the results of each approach should corroborate the results of the other.
Given the earlier immunohistochemistry experiments, which demonstrated that the OlfCc1 morpholino reduced the number and sizes of OMP and calretinin positive neurons and glomeruli, respectively, a generalized deficit in odor-evoked activity was thought to be likely in the morphant fish. As expected, OlfCc1 injected fish displayed slightly depressed responses compared to their mismatch injected counterparts across the panel of tested odorants. However, for the non-amino acid odor pools, these differences were not statistically significant by either method of normalization. In contrast, amino acid evoked activity was decreased in morphants to a small fraction of the mismatch response in an extremely significant fashion. (Fig 2.14)

The non-significant changes in responses evoked by FEX (63% vs. 56%; p = 0.32), the bile acid pool (36% vs. 27%; p = 0.133) and the amine pool (27% vs. 23%; p = 0.131) in the bulb-normalized data, and likewise in the bile acid (56% vs. 49%; p = 0.5) and amine pool (44% vs. 40%; p = 0.655) in the FEX normalized condition are in stark contrast with the changes in amino acid evoked activity. In morphant fish, there is a nearly 8-fold reduction in amino acid responses (24% vs. 3%; p = 9.5E-10) when normalized against the area of the bulb, and a 6-fold reduction (39% vs. 6%; p = 2E-05) when normalized against the FEX response. Notably, many of the amino acid morphant fish exhibited no amino-acid evoked activity whatsoever, and the OlfCc1 knockdown fish with the most extensive response still fell below the control fish with the lowest responding area.

The difference between morphant and mismatch fish is also clear when looking at the magnitude of responses instead of the footprint of all responding regions. This is illustrated in Fig 2.15, which shows deltaF/F0 response traces specifically for the anterior lateral glomerulus of four control fish and four morphants. This glomerulus is part of the lateral chain targeted by the microvillous cells (as shown in Fig 2.2, 2.3) and is generally responsive to amino acids in mismatch injected fish. For instance, fluorescence change traces from this specific region of the bulb show robust responses to the amino acid pool in the control fish, with increases ranging from 10-30%. In contrast, there is little to no response in the morphants.

While previous studies in both larval and adult zebrafish have demonstrate that fish across a spectrum of developmental ages show differential but broadly overlapping patterns of activity in response to individual amino acids in the bulb, (J. Li et al. 2005) (Sigrun Korsching 2002) (R. W. Friedrich & S. I. Korsching 1998), one possible explanation for the results observed here is that OlfCc1 is acting as a broadly tuned OR and could be principally directly responsible for amino acid detection at this age. If this were the case, loss of amino acid evoked activity after knockdown could be due to the fact that OlfCc1 itself is binding and directly responding to the majority of amino acids in the control fish. To rule out this possibility, I compared two 100uM class-specific subset pools of amino acids to the 9 member 100uM pool. The first pool contained the neutral amino acids isoleucine (I) and leucine (L) while the second consisted of the basic amino acids arginine (R) and lysine (K). The rationale for performing this comparison is that if OlfCc1 is acting as the principal broadly tuned amino acid receptor and is expressed universally in microvillous neurons, then different amino acids should evoke almost completely spatially overlapping patterns of activity. There may be different degrees of
activity based on receptor affinity, but there should not be mutually exclusive activation of one odor class versus another unless individual receptors with varying selectivities are being differentially activated and projecting to unique bulb locations. Looking in different control fish, I found that the IL and RK pools elicited different but often overlapping response patterns. Fig. 2.16 shows an example of this, wherein 100um IL and RK strongly activate different subsets of the regions activated by the 100um 9 amino acid pool, which contains all four of them. This finding is important as it suggests that the widespread decrease in amino acid signaling after OlfCc1 knockdown is universally affecting activity that is normally differential; that is, neurons expressing different OlfC receptors are all affected.

It should be noted that there was a fair amount of variability in the degree of responses to all classes of odorants from fish to fish; some of these differences seemed to be dependent on depth of the plane imaged, with the amine pool always evoking a broader response in the rostral planes and amino acids always evoking more activity in the caudal ones. Much of the variability simply seemed to be intrinsic animal-to-animal differences; experimental variance cannot be ruled out as a source for this variance. However, both the morphant fish and the mismatch injected ones displayed similar differences, suggesting that this variability is not a consequence of OlfCc1 knockdown. Notably, similar variability had been previously observed using unmanipulated OMP-Gal4xUAS:GCaMP1.6, and it may be the case the degree of olfactory development is not absolutely fixed across an aged-matched population at this age range. The normalized data for each fish included in figure 2.14 can be seen in Table 2.2.

**Discussion**

The results of these experiments demonstrate that knockdown of OlfCc1 has a profound and specific effect on amino acid evoked activity in the olfactory bulb of larval zebrafish. Due to its restricted expression, HuC driven GCaMP1.6 is likely reporting calcium influx in the postsynaptic second order cells, at least in the microvillous cell targeted glomeruli. As OlfCc1 was not detected in the olfactory bulb by immunohistochemistry, this decrease in activity is very likely due to decreased input from the first order cells synapsing on them. While the second order cells have complex topographic and temporal response profiles that evolve over time and during shifts in odor presentation (Emre Yaksi et al. 2007) (Niessing & Rainer W Friedrich 2010), they can also be more coarsely used as a general readout of olfactory activity.

One competing explanation for the loss in signaling would be that the cells or synapses needed to mediate amino acid transduction are absent. While the morphant fish had a slightly decreased response to all odorant pools, the responses to all odorants except amino acids seemed relatively similar. Thus, there did not seem to be systemic deficiencies in the olfactory system. The lateral anterior glomeruli were still apparent in the morphant fish by both HuC and BODIPY visualization, indicating that some physical part of the detection apparatus was still present. Coupled with the observations that calretinin positive cells appeared to correctly find their targets in 2-3 dpf fish and lateral
SV2 staining was still present in the morphants, it would appear that the microvillous ORNs are still projecting to and synapsing on targets in the correct region of the bulb. While there may be fewer cells and therefore fewer synapses, there is not a fundamental absence of any of the necessary components.

These observations support the notion that the almost complete abrogation of amino acid evoked activity in the morphant fish is not due to a fundamental defect in the wiring of the circuit, but a decrease in the activity of the neurons involved in it. This strongly supports the hypothesis that OlfCε1 is involved directly in the chemosensation process, and is in fact critical for the function of different OlfC family members. The broader implications for this finding, a proposed molecular model for its mechanisms, and the experiments that will need to be done to answer remaining questions will be expanded upon in the conclusion to this chapter.
2.12) HuC-Gal4 drives UAS-GCaMP expression throughout the olfactory bulb. BODIPY vital dye labels membranes and allows for the dense neuropil glomerular regions to be visualized. GCaMP is widely but not uniformly expressed in the olfactory bulb; however, colocalization with BODIPY makes it clear that it is expressed in all glomerular regions. The dotted line indicates the bounds of the olfactory bulb. The outlined arrow indicates an anterior lateral glomerulus proximal to the olfactory epithelium that is in a microvillous cell innervated region.
2.13) Representative activity maps in 4.5 dpf mismatch and morphant HuC-Gal4xUAS-GCaMP1.6 fish. Each map consists of a false color $\Delta F/F_0$ maximum activity map superimposed over the F0 composite image used to generate the activity map. Food extract (FEX) elicits the most widespread response in both fish. The response to the 9 amino acid pool at 100uM is localized to the anterior lateral region of the bulb while the corresponding region in the morphant fish shows no activity (open triangles.) Scale bar = 15um; maximum $\Delta F/F_0 \approx 30\%$. 
Figure 2.14) Relative responses to odor pool application in mismatch and morphant fish. A) FEX: $p = 0.32$; Amino acids $p = 2E-05$; Bile acids: $p = 0.133$; Amines: $p = 0.655$. B) Amino acids: $p = 0.655$; Bile acids, $p = 0.5$; Amines, $p = 0.655$. Mismatch $n = 12$ for all conditions but bile acids, 11 for bile acids. Morphant $n = 14$ for all conditions. Error bars represent SEM.
2.15) Responses to pooled amino acids in a lateral glomerulus in control and morphant fish. A) ΔF/F0 traces after 100um amino acid pool exposure (marked by arrow) in a roughly anatomically equivalent region in 4 mismatch injected fish (blue toned lines) and four morphants (red toned lines). B) The actual ROIs from which the traces were generated. The top four panels are mismatch, the bottom four are morphants; the panel outline color matches the trace color it represents. The general anatomical stereotypy can be seen from the ROI location.
2.16) Differential responses to IL vs RK pools of amino acids. Activity maps from one fish (fluorescent inset shown above) demonstrating the responses to an aa pool, R+K, and I+L respectively. R+K and I+L activate overlapping but differential subregions of the bulb and subsets of the 9 aa pool response.
Table 2.2)

Response normalized to area of bulb for each fish in every condition:

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Conclusions and future directions

The results of the experiments presented here suggest that, as hypothesized, OlfCc1 is playing a broad and critical role in amino acid detection in larval zebrafish. Among the immediate future directions will be to confirm this finding in a TrpC2-Gal4xUAS;GCaMP1.6 transgenic line, which should allow a clear view of the direct response of receptor knockdown in the first order cells, as opposed to an inference based on the activity in the second order cells. This will also allow us to determine whether the microvillous cells are solely amino acid responsive and, if not, whether OlfCc1 knockdown affects all microvillous cell odor-evoked activity in a similar fashion.

There are a number of potential explanations for why OlfCc1 might be critical for amino acid evoked activity. One possibility is that OlfCc1 is directly binding to the punctate receptors it is coexpressed with. Dimerization is a common means of GPCR regulation, particularly among the family C GPCRs. The canonical example and one of the earliest studied cases of GPCR dimerization is that of the GABA\(_B\) receptors. (White et al. 1998) In this case, the ligand binding GABA\(_B\)R1 subunit requires an interaction with a coiled-coil domain in the C-terminus of the non-ligand binding GABA\(_B\)R2 receptor to mask an ER retention signal and allow the heteromeric protein to exit the secretory pathway and reach the cell surface. (Kammerer et al. 1999) While OlfCc1 and the other OlfC receptors lack equivalent ER retention or coiled-coil domains, there are other means by which such regulation of trafficking could take place.

In light of this, it is of interest that the majority of OlfCRs fail to traffic to the plasma membrane when expressed in heterologous cells. In general, the receptors form inclusion bodies or hang up somewhere in the secretory pathway. This inability to surface express has greatly hampered attempts to determine the ligand selectivities of the OlfCRs via classic heterologous cell assays. OlfCc1 again contrasts the other members of its family; surface immunochemistry against an N-terminal HA epitope in non-permeabilized HEK cells transfected with an HA-tagged OlfCc1 shows that the receptor efficiently expresses to the plasma membrane. (Fig S2.2) Similarly, there is evidence that the mammalian homologous receptor V2R2 can be successfully surface expressed in heterologous cells (Silvotti et al. 2005) (and personal correspondence, A. Berke.) As the remaining V2Rs are also difficult to surface express, there appears to be some conserved feature of OlfCc1 and V2R2 permitting them to be trafficked. Exploring the possibility that this feature may reflect the ability of OlfCc1 to act as a shuttling vector for the punctate receptors, previous work performed in our lab (E. Van Name, unpublished) found that OlfCc1 coexpression with punctate OlfCRs could facilitate the expression of many of the otherwise retained punctate receptors in HEK cells. This suggests that these receptors can interact in some fashion and could provide the basis of an explanation for the necessity of the ubiquitous receptors in vivo.

Even if OlfCc1 is not functioning as a shuttling vector, the suggestion that it may interact directly with the punctate receptors yields other potential molecular mechanisms for its role. For instance, again in the case of the GABA\(_B\) receptor, signaling through the heteromeric receptor involves trans interactions, in which the GABA\(_B\)1 subunit binds
ligand while it is GABA\(_\alpha 2\) that is critical for activating the downstream signal transduction cascade. (Kuner et al. 1999) More subtly, dimerization in GPCRs can underlie both positive and negative cooperativity. (Franco et al. 2007) OlfCc1 could thus contain some critical determinant for OlfCR activation.

This raises the question as to whether OlfCc1 itself is capable of triggering signal transduction. A student in our lab has been working to determine whether OlfCc1 expressed in heterologous cells is functional and what its ligand selectivity might be. (A. Berke, personal correspondence.) These experiments have revealed that the receptor is capable of triggering calcium influx, indicating that receptor detected at the surface is not simply misfolded protein, and is a low-affinity receptor for the hydrophobic amino acids isoleucine and leucine in high calcium conditions. My finding that the response to all classes of amino acids, not just I and L, is affected by loss of OlfCc1 in zebrafish suggests is not simply functioning as the “I/L” receptor, per se. However, the fact that OlfCc1 contains the determinants necessary to couple to the GPCR signal transduction machinery and is capable of binding particular ligands supports the idea that it could be strictly necessary for trans signaling and that it could have cooperative effects with punctuate OlfCR signaling.

A summary of my proposed models for how OlfCc1 might be acting as a coreceptor with the other OlfC receptors is summarized in Fig 2.15

Further experimentation will be needed to tease apart these potential mechanisms, particularly as they are by no means necessarily mutually exclusive. The first step will be to determine whether the other OlfCRs target to the microvilli in the absence of OlfCc1. This could be achieved by the generation of a custom antibody for one of the punctuate receptors, as was done for OlfCc1. Failure of the punctuate OlfCRs to traffic in the OlfCc1 morphants would provide convincing evidence for this defect being the source of the amino acid signaling deficiency in these experiments, but would not necessarily rule out the involvement of OlfCc1 in other aspects of chemotransduction. If, on the other hand, the receptors were to target correctly, the evidence for OlfC1’s direct involvement in the transduction of odorant binding becomes much stronger. A more comprehensive set of experiments would be to engineer an OlfCc1 receptor that is insensitive to the morpholino and express it under the control of the TrpC2 promoter to first see whether responses to amino acids could be rescued. If so, then this approach would also allow the different domains of OlfCc1 to be mutated in potential rescue constructs, to further investigate which regions are important in conferring mediating chemoreception.

The structural differences between OlfCc1 and the other OlfCRs may hold the key to unlocking what it is about this receptor that allows it to fulfill its unique function. Compared to the other OlfC family genes, OlfCc1 has an expanded 3\(^{rd}\) intracellular loop, an area that, along with the C-terminus, has been associated with interactions with \(\beta\)-arrestin in other GPCRs. (Ferguson 2001) Of additional interest is the fact that OlfCc1 is a phylogenetic outlier among the OlfC family. OlfCc1 clusters more closely with the mammalian calcium sensing receptor CaSR than with the other OlfCRs (Alioto & Ngai 2006), and may similarly bind calcium or other divalent cations. CaSR, meanwhile, is
potentiated by amino acids (Leech & Habener 2003), underscoring the parallels between the receptors. A potential dimerization OlfCc1 interface can be predicted in the N-terminus, based on similarity to CaSR and mGluR1. All of these regions represent potentially interesting targets once an in vivo assay can be refined to look at exogenously expressed receptors.

While future experiments will shed further light on how OlfCc1 exerts its role in zebrafish olfaction, the experiments presented here put forth a conclusive case that it is critical for amino acid detection and build towards a model explaining why this might be the case. Given the conservation between the OlfC family and the peptide-detecting mouse V2R family, it is likely that these results also will also offer some insight into the role of V2R2 in the mammalian accessory olfactory system. Taken together, these experiments advance our understanding of the field of vertebrate olfaction and may allow for better understanding of general mechanisms of GPCR regulation.
2.17) Models for possible roles of OlfCc1 heterodimerization. A) OlfCc1 could be critical for the targeting and localization of the other OlfCRs, such that knocking it down leaves them unable to reach the microvillous membrane. B) OlfCc1 could be a critical part of the signal transduction machinery between odorant binding and G-protein activation.
Materials and Methods:

Zebrafish husbandry and genetic backgrounds:

Zebrafish were kept in a dedicated facility on a 12h light/dark cycle at 29° C and fed on a regular schedule. Wildtype Tubueng Longfin and AB* fish were obtained from the Zebrafish International Resource Center (ZIRC; Eugene, OR). The OMP-Gal4 transgenic line was developed by Todd Ferreira in the Ngai lab (UCB). The UAS-GCaMP1.6 and HuC-Gal4 transgenic lines were provided by the Baier lab (UCSF). Morpholino and DNA injected embryos were raised in E3 embryo media (150 mM NaCl, 4.5 mM KCl, 1 mM CaCl₂, X mM Na₂HPO₄ *2H₂O, X mM KH₂PO₄ *2H₂O). Transgenic fish were screened for reporter expression by fluorescence or by fin clip PCR-based genotyping, as described in ZIRC resources. Fish used in imaging experiments were raised in embryo media containing 0.2mM 1-phenyl 2-thiourea (PTU) in order to prevent pigmentation.

Cell culture and transfections

HEK293 cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum. Before transfection, cells were seeded into eight chamber culture slides coated with poly-d-lysine or into 12-well plates for immunochemistry or biochemistry, respectively. Cells were transfected at an approximate density of (75,000 cells per cm²) using Lipofectamine 2000 regent (Invitrogen, CA) according to the manufacturers instructions. For immunoprecipitation experiments using the secreted N-terminal domains of receptors, the supernatant was collected 24 hours after transfection. For immunocytochemistry, cells were fixed 24-48 hours after transfection in 4% paraformaldehyde for 30 min at room temperature.

OlfCc1 polyclonal antibody production and validation:

A custom polyclonal antibody directed against the N-terminal domain of OlfCc1 was generated in guinea pig (COVANCE, Denver PA). The epitopic region of the protein was chosen based on its antigenic properties and low sequence conservation with all other members of the OlfC family in order to minimize the chances of cross reactivity. The synthesized peptide used for inoculation and boosting was: CSLVSTDSNTTDPPEVSD-amide.

Affinity purification of the antibody from guinea pig sera was performed by running the collected sera over a column packed with thiol-sepharose beads coupled to the antigenic peptide by its terminal cysteine. Bound antibodies were eluted off by low pH glycine and antibody-rich fractions were identified by Bradford assay. The specificity of the OlfCc1 antibody was verified in both receptor-transfected HEK cells and in situ by cross-adsorbing the antibody with the peptide used to generate it.

Morpholino injections:
A morpholino oligonucleotide blocking the first splice junction of the OlfCc1 transcript and a five base mismatch were obtained from Gene Tools LLC (Philomath, OR). The sequences of these morpholinos were as follows:

**OlfCc1 Perfect Match (PM):** ATATTTTATCATACCCCTGGCAATC

**OlfCc1 Mismatch (MM):** ATtTTaTATCTAgCCCTcGCAtTC

Lyophilized morpholinos were resuspended in distilled water to a stock concentration of 20ng/nl (25mM). For injection, morpholinos were diluted to a final concentration of 2ng/nl in a 10ul solution containing water, 0.2mM KCl and 0.5ul of Phenol Red. The morpholino mix was injected into the yolk of 1-2 cell embryos through a pulled glass capillary tube with a 2-3um tip width. A micromanipulator mounted nanoPinjection system (WPI) was used to deliver precise volumes. The minimal volume/concentration of morpholino required to achieve efficient knockdown was experimentally determined to be 2.3nl/embryo. Determination of knockdown efficacy was determined by IHC using the OlfCc1 antibody, as described below. Quantification of immunohistochemical features in morphants and controls was performed on coded images and later decoded for statistical analysis. Images were analyzed in NIH ImageJ and cell counts, glomerular counts, and area analysis were performed by hand. Quantified parameters were recorded on each image and saved as experimental records.

**Zebrafish immunohistochemistry:**

**Embryonic whole-mount IHC:** Zebrafish embryos (24 hpf -7 dpf) were briefly cooled to 4º C to anesthetize them and then immersed in a fixative solution containing 4% paraformaldehyde PBS for 2 hours at 25 ºC. For SV2 immunoc hemistry, embryos were fixed in 2% Trichloroacetic acid in PBS for the same amount of time. Embryos were then washed 3 times for 15m in PBS containing 0.1% Triton X-100, 0.1% Tween-20 (PBSTT) and then blocked for 1h at room temp in a blocking solution composed of PBSTT + 10% HINGS, 1% DMSO. Embryos were incubated overnight at 4 C blocking solution with an appropriate dilution of primary antibody. Subsequently, embryos were washed three times in PBSTT, and incubated in secondary antibody diluted by 1:200 in blocking solution overnight at 4 C. Secondary antibodies appropriate to the origin species of the primary conjugated to either Alexa Fluor 488 or Alexa Fluor 548 were used for visualization. In some experiments, a nuclear backlabel, BOBO (Molecular Probes, ect), was used to define tissue boundaries. BOBO was applied at a 1:2000 dilution in PBSTT for 15m after secondary labeling during one of the final wash steps. After 5 further 15m PBSTT washes, embryos were mounted in a drop of 1.2% Low Melting Point Agarose (Sigma) in custom cast 2% agarose trays attached to glass coverslips. Z-series through the entire depth of the olfactory epithelium and bulb were acquired using an upright confocal microscope (Nikon)

**Adult section IHC:** Adult zebrafish were anesthetized by MS-222 and chilling and quickly decapitated. Heads were placed in 4% PFA for 2h at room temp, and then cryoprotected by sinking in 30% sucrose overnight at 4 C. Heads were then mounted in blocks of tissue freezing medium and cut into 18um coronal sections adhered to glass slides. Slides were blocked in a solution containing PBS, 0.1% Triton X-100, and 10%
HINGS and incubated in primary antibody overnight at 4 C. The slides were then washed 3 times for 15m, and secondary antibody was applied for 1h at room temp.

The following primary antibodies were used in these experiments: α-SV2 (DSHB) 1:20; α-calretinin (SWANT, Switzerland) 1:500; α-OlfCc1 (Ngai Lab) 1:100; α-GFP (Abcam, MA) 1:200; α-acetylated tubulin (Sigma, MO) 1:1000. Alexa-fluor 488 and alexa fluor 568 coupled secondary antibodies (Molecular Probes, CA) were used at a dilution of 1:200.

In vivo functional imaging:

4-7 day old zebrafish expressing GCaMP1.6 in different neuronal populations were used for functional imaging experiments. Larvae were briefly cooled, mounted in 2% LMP agarose dissolved in Zebrafish Ringers Solution (116 mM NaCl, 2.9 mM KCl, 1.6 mM CaCl₂, 5 mM HEPES, 11 mM glucose), and then paralyzed by injection of 2.3nl of 1mg/ml alpha-bungarotoxin (Sigma) into the spinal column at the base of the hindbrain. Injections were carried out on a dissecting microscope (Nikon) using a micromanipulator mounted nanoliter injector and an oil filled glass capillary tube in order to ensure precise volume and anatomical targeting. After paralysis, the heads of the larvae were cut free from the agarose and they were remounted in a custom imaging chamber designed to allow laminar flow of Ringers solution and aqueous odorants over the nostrils and head. An 8 channel pneumatically controlled perfusion system (AutoMate Scientific, CA) was used to switch between a constant 2ml/min flow of Ringers solution and odorants dissolved in Ringers during the experiments. Images were acquired at 4Hz on a Zeiss Live 5 line scanning confocal microscope using a 40X or 60X water immersion objective. For most experiments, each odorant was applied in independent trials 7.5s after image acquisition was triggered. Odorant application occurred for 4s before switching back to the bath channel, and image acquisition continued for another 40s. Two minutes of washout and recovery without laser exposure was allowed between each odor application. This interstimulus interval was experimentally determined to generously allow complete recovery from desensitization, in order to prevent cross-desensitization between odorants. At the beginning of each acquisition period, the focus was manually adjusted to a reference plane to ensure the same features were in the field of view for each trial. Each fish was imaged at multiple planes through the OE or OB. For some experiments, the vital membrane dye BODIPY (Molecular Probes, CA) was used as a backlabel to provide better identification of glomerular position.

Amino acids, amines, IBMX, forskolin, and bile acids were obtained from Sigma. (St. Louis, MO) Working concentrations of each odorant were prepared from aliquoted 10-100X stocks prior to each experiment. Food extract was freshly prepared as described in (J. Li et al. 2005).

Each fish was imaged at 2-3 different confocal planes to give a comprehensive picture of evoked responses throughout the bulb. All experiments contained a bath channel as a common control; odorant pools were otherwise chosen as appropriate by experiment.
Functional data analysis:

Data were acquired using the Zeiss LSM 510 software (Zeiss, Germany). Individual trials were imported as .lsm files into NIH ImageJ (NIH, Maryland) running the MacBiophotonics plugin collection. The first 20 frames of each trial were removed due to the initial rapid decay of GCaMP signal. Every image series was processed with the Image Stabilizer plugin (K. Li 2008) using translation settings to minimize any potential artifacts due to lateral X-Y shifts. Image series’ demonstrating shifts along the Z-axis were not analyzed, as anatomically stable ROIs could not be chosen.

In the HuC morpholino quantifications, a single plane out of the 2-3 that were imaged was chosen for quantification in each fish, such that n=1 represents one bulb hemisphere of a unique animal. For consistency, the most caudal plane of the bulb, corresponding to the region containing the lateral chain of glomeruli, was the plane chosen for every animal.

For the generation of deltaF/F0 intensity vs. time plots for a particular ROI, raw average intensity values for each frame was exported into Excel 2003 (Microsoft, WA). F0 was calculated as the numerical average of the first 6 frames, and subsequent frames were normalized against this value. For the generation of activity maps, the first six frames of the image series were averaged to produce an F0 image and subsequent frames were divided by this image. Five frames spanning the maximal response in the resultant deltaF/F0 image series were Z-projected. For quantification of the percent area of the bulb responding, an average threshold mask was applied to the activity map to clearly mark non-responding areas, and the area of responding regions was calculated from ROIs drawn around unmasked regions.

For visualization of the activity maps, a smooth RBG spectrum LUT was applied to the activity map. In most cases, the false color map was set to 50% transparency and superimposed over the average F0 image in order to provide anatomical reference points for the responses and to clearly delineate the out of focus regions subject to stochastic noise without cropping the image. If an image was cropped, a reference F0 image is provided to indicate out of plane (and thus non-fluorescent) regions.

For all morpholino experiments, pairwise comparisons between values for mismatch and morphant populations were calculated using Students T test to determine the two-tailed p. The threshold for statistical significance was set at p < 0.005.
Supplementary Figure 2.1)

<table>
<thead>
<tr>
<th>OlfCc1 Mismatch</th>
<th>OlfCc1 Perfect Match</th>
</tr>
</thead>
</table>

S 2.1) Morphological evidence of a general off-target effect of OlfCc1 antibody. There is some evidence that the OlfCc1 morpholino is having broad off-target neurotoxic effects in addition to knocking down receptor protein. For instance, some of the morphant fish possess smaller heads and show what appears to be a defect in the ability of the dorsal telencephalon to fuse around its central vesicle.
S2.2) OlfCc1 is expressed on the surface of transfected heterologous cells. HEK 293 cells transfected with HA-OlfCc1 and stained with α-HA in detergent-free, non permeabilizing conditions demonstrate that the receptor is efficiently transported to the plasma membrane.
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Chapter 3: The role of Forkhead transcription factor FoxG1 in development of the zebrafish olfactory placode.

Background

The identity of a particular cell is established by precise programs of gene expression carried out by progenitor cells during their specification. Transcription factors are the master regulators of this process, acting in concert to turn on and off multitudes of genes involved in proliferation and differentiation. During the developmental process, pluripotent stem cells migrate to the correct anatomical location, proliferate and differentiate into the different cell types that comprise the mature tissue. The peripheral sensory systems derive from discrete thickenings of endothelium known as placodes. These thickened ectodermal structures eventually give rise to neurons, cranial ganglia, and other sensory cell types. (Schlosser 2005) (Kathleen E Whitlock 2004)

Understanding how the olfactory system develops begins with identifying the factors involved in this first step of specification.

In the olfactory system, there is an additional facet of interest to understanding these processes, as olfactory tissue is one of the few regions of robust adult neuroregeneration (Whitman & Greer 2009) (Frankland & Miller 2008), and there has been considerable question as to whether the programs guiding the adult neural stem cell to its correct neuronal lineage overlap with those acting to specify the olfactory epithelium in the first place.

Development of the mouse olfactory epithelium:

The development of the vertebrate olfactory epithelium has been studied extensively in mouse, with particular focus on the maturation of the neuronal lineage. In the pro-neural pathway thus elucidated, Mash1 expressing transit amplifying cells give rise to Neurogenin1 and then NeuroD expressing neuronal precursors, which in turn give rise to Gap-43 positive immature neurons and finally mature olfactory neurons that express Olfactory Marker Protein (OMP). (Cau et al. 1997) (Frankland & Miller 2008) (Murray et al. 2003) (Manglapus et al. 2004)

Identities of and interactions between the factors that set up the earlier stages of olfactory epithelial development are less well known, particularly with respect to which factors are specifically expressed in the embryonic olfactory stem cells. These include the cues which guide specification of the progenitor population and the formation of the olfactory placode from which the olfactory epithelium is derived. There is evidence for the involvement of the morphogenic growth factor Fgf8 signaling in the early specification of placode formation, (Kawauchi et al. 2005) possibly through regulation of the transcription factors Six1 and Six4 (Chen et al. 2009). Fgf8 acts to specify pro-neural identity in an antagonistic fashion with BMP signaling, which instead promotes respiratory epithelial fates in chick. (Maier et al. 2010)
In rodents, the forkhead transcription factor FoxG1 is first expressed in the anterior neural tube (Tao & Elai 1992), and widely in rostral telencephalic structures. (Hatini et al. 1994) It is known to have widespread roles in the developing CNS, where it is critical for such processes as specification of the ventral telencephalon (Maier et al. 2010), midline crossing of retinal ganglion cell axons (Pratt et al. 2004), and the specification, differentiation, and proliferation of cortical progenitors. (Hanashima et al. 2002) (Hanashima et al. 2004)

FoxG1 was identified as highly expressed in the developing OE in a microarray screen performed in our lab (C. Duggan), leading us to investigate its role in the development of the olfactory system. (Duggan et al. 2008) FoxG1 was found to be expressed in the earliest stages of olfactory system development in the cells migrating into the nascent OE from the neural crest and putative progenitor population. To elucidate the roles of FoxG1 in olfactory development, a mouse in which the coding sequence of FoxG1 had been replaced with Cre was investigated. Strikingly, all of the olfactory structures in the FoxG1 -/- mutant mouse were severely compromised, with an effect apparent as early as the first olfactory tissue at embryonic day 10 and increasing in severity through development. The mutant mice died at birth, presumably due to gross airway defects and failure to respire. However, by the latest observable time point, the olfactory system and associated structures were almost completely absent. The severity of this phenotype demonstrated that FoxG1 is strictly necessary for olfactory development from the earliest stages of the process. In accordance with this, analysis of epistasis placed the transcription factor upstream of the earliest known markers of the olfactory neuronal lineage. To assess the origin of this phenotype, an immunohistochemical analysis of assorted markers associated with cell division, proliferation, and apoptosis was performed on the mutant mice. The results suggested that a severe deficiency in proliferation was primarily responsible for the olfactory deficiencies in FoxG1 -/- mice.

Extending the investigation of the role of FoxG1 in early olfactory specification into zebrafish offered two advantages: 1) it allowed us to examine whether the role of FoxG1 is conserved across multiple vertebrate species, and 2) the hypomorphic nature of the morpholino-mediated knockdown phenotype in zebrafish eventually allowed us to examine the mechanisms that lead to the observed olfactory defects in a way which was precluded in mouse by the severity of the phenotype.

In zebrafish, an anterior preplacodal field is thought to give rise to the lens, trigeminal, otic, adenohypophyseal, and olfactory placodes. The prevailing model holds that the preplacodal field is formed by a convergence of ectodermal cells migrating from the anterior neural crest. (K.E Whitlock & Westerfield 2000) There is evidence from lineage tracing experiments that the eventual fates of these migrating precursors are specified early in development (Kozlowski et al. 1997) The nascent olfactory placode (as well as the immediately adjacent adenohypophyseal placode) express dlx3b, and the refinement of the expression domain of dlx3b over time marks the coalescence of these precursors into a defined placodal region. (Bhattacharyya & Bronner-Fraser 2008)

**Experimental setup**
In order to first ensure that a parallel examination to the mouse phenotype in zebrafish would constitute a valid comparison, the expression of FoxG1 in the developing zebrafish olfactory placode was examined by in situ hybridization and found to be widely present in both the telecephalon and olfactory epithelium of 36 hpf to 48 hpf fish. (Duggan et al. 2008)

In order to study the role of FoxG1 in the zebrafish olfactory system, I used an antisense morpholino to knock down expression of the protein and examined whether histological changes in the morphant paralleled those observed in the mutant mouse. Because there are two FoxG1 homologues in zebrafish, BF1a and BF1b, we first designed and tested morpholinos against both of them, singularly and in combination. Unfortunately, there is not an effective zebrafish antibody to FoxG1 to test protein knockdown and the gene and its variants are intronless, precluding splice analysis as a readout of morpholino efficacy. Therefore, I used a known phenotype of FoxG1 deficiency in mouse, a defect in the ability to form an organized optic chiasm in the absence of FoxG1 (Pratt et al. 2004), as a phenotypical readout of morpholino function. I found that, of the two variants, the morpholino against BF1a was effective at recapitulating the phenotype of the FoxG1 deficient mouse. (Fig S3.1) Injection of both morpholinos did not compound this or any detected effect. Thus, the BF1a morpholino (subsequently referred to as FoxG1) was used for the remainder of the study, injected at 10ng per embryo.

Results

The number of calretinin expressing cells, representing one of the two major cell types of the zebrafish olfactory epithelium, was determined in FoxG1 morphants vs. mismatch injected fish. (Fig 3.1A) The morphant fish were found to have ~58% of the number of calretinin positive cells compared to the control fish (Table 3.1) This decrease parallels the loss of olfactory receptor neurons in mice but is nowhere as severe, suggesting that the morphant may be acting as a hypomorph, exhibiting incomplete knockdown of FoxG1 at the concentration ranges tested. (Fig 3.1A)

Phosphohistone 3, which marks dividing cells in the M cycle of mitosis, is likewise decreased in the morphant fish across three separate experiments, (Fig 3.1B) suggesting that neurogenesis may also be compromised in this population, as is seen in the mutant mice. Again, the differences in severity between the FoxG1 knock-down fish and the mutant mouse is likely due to a hypomorphic effect in the fish, revealing some degree of dose-dependence for FoxG1 loss. The results of these experiments are summarized in Figure 3.2 and Tables 3.1 and 3.2.

Finally, I examined the expression pattern of acetylated tubulin to assess the degree of organization in the olfactory bulb and found a striking affect; the axons in the morphants were disorganized and failed to coalesce into the contralaterally crossing olfactory nerve, similar to the effect observed in the optic chiasm. (Fig 3.1C)

Discussion
The results of these experiments support the hypothesis that the role of FoxG1 is fundamental to olfactory development and conserved in zebrafish, indicating that the principles underlying the development of the olfactory placodes are generally in place across vertebrates. The hypomorphic nature of the morphant phenotype also offered the unique opportunity to further examine the basis of FoxG1’s role in olfactory neurogenesis. Further experiments conducted in the lab sought to determine the cellular autonomy of the knockdown effect using cells from the hypomorphic zebrafish in order to determine whether the defect in olfactory organ development was directly due cells lacking FoxG1 or instead to a general defect in the formation of the environment. To study this, a series of transplantation experiments were undertaken by (Duggan et al. 2008). They found that when a lineage-traced morphant cell was implanted into a wildtype embryo, the cell still had difficulty entering the olfactory lineage. Conversely, a non-morphant lineage traced cell implanted into the morphant tissue was competent to form a neuron. Taken together, these results suggests that despite the widespread effects of FoxG1 in the rostral central nervous system, the transcription factor is playing a cell-autonomous role in the generation of the olfactory epithelium.

The results of this study demonstrate that FoxG1 is required for the formation of the vertebrate olfactory system and is acting upstream of known markers of the pro-neural lineage. Defects in cell division are largely responsible for the massive hypotrophy of olfactory tissue seen when FoxG1 is deleted in mice and mirrored when it is knocked down in zebrafish, and its effects appear to be cell-autonomous. The use of zebrafish in parallel with mice strengthened these conclusions, demonstrated that the role and effect of this transcription factor is conserved in vertebrates, and allowed insights into the cell autonomy precluded by the severity of the phenotype in mouse. Further studies in both mice and fish may yield further information as to the nature of the role of FoxG1, and identify its upstream regulators and downstream effectors.
Immunohistological markers demonstrate the effect of FoxG1 knockdown on the developing zebrafish olfactory placode. A) Calretinin, a marker of one of the two major cell types in the zebrafish OE, reveals a decrease in the number of olfactory neurons in 48 hpf fish. B) Phosphohistone 3 staining is likewise decreased, mirroring the effect seen in the mutant ouse. C) Acetylated tubulin, an axonal marker, shows severe disruptions in the organization of the olfactory bulb and formation of the olfactory nerve. Scale bar 50um.
3.2) Quantification of calretinin and PH3 staining in FoxG1 morphant and mismatch fish. p<<0.001 in both cases.
Table 3.1. Summary of calretinin-positive cells in the olfactory epithelium of 48 hpf zebrafish embryos injected with *Foxg1* and mismatch morpholino oligonucleotides

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Morpholino</th>
<th>Average calretinin+ cells/epithelium</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mismatch</td>
<td>31</td>
<td>7</td>
<td>$3.6 \times 10^{-7}$</td>
</tr>
<tr>
<td></td>
<td><em>Foxg1</em></td>
<td>18</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mismatch</td>
<td>31</td>
<td>8</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td><em>Foxg1</em></td>
<td>23</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Mismatch</td>
<td>30</td>
<td>12</td>
<td>$3.5 \times 10^{-6}$</td>
</tr>
<tr>
<td></td>
<td><em>Foxg1</em></td>
<td>16</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

*n*, Number of fish assayed; *p*, *p* value from two-tailed Student's *t* test.
Table 3.2. Summary of PH3-positive cells in the olfactory epithelium and telencephalon of 48 hpf zebrafish embryos injected with Foxg1 and mismatch morpholino oligonucleotides

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Morpholino</th>
<th>Average PH3+ cells/epithelium</th>
<th>n</th>
<th>P</th>
<th>PH3+ cells/telencephalon</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mismatch</td>
<td>8.5</td>
<td>9</td>
<td>0.017</td>
<td>12</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>Foxg1</td>
<td>5.6</td>
<td>14</td>
<td></td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mismatch</td>
<td>11</td>
<td>24</td>
<td>2.0 x 10(^{-7})</td>
<td>17</td>
<td>2.6 x 10(^{-6})</td>
</tr>
<tr>
<td></td>
<td>Foxg1</td>
<td>7.3</td>
<td>28</td>
<td></td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Mismatch</td>
<td>12</td>
<td>15</td>
<td>0.007</td>
<td>17</td>
<td>1.3 x 10(^{-5})</td>
</tr>
<tr>
<td></td>
<td>Foxg1</td>
<td>7.7</td>
<td>11</td>
<td></td>
<td>8.6</td>
<td></td>
</tr>
</tbody>
</table>

n, Number of fish assayed; p, p value from two-tailed Student's t test.
S3.1) FoxG1 morpholino disrupts formation and organization of the optic chiasm. Immunostaining against the axonal marker acetylated tubulin shows widespread disorganization in the region of the optic chiasm, an expected finding based on the previously characterized phenotype of the mutant mouse.
Materials and Methods:
Zebrafish husbandry and genetic backgrounds:

Zebrafish were kept in a dedicated facility on a 12h light/dark cycle at 29º C and fed on a regular schedule. Wildtype Tubueng Longfin and AB* fish were obtained from the Zebrafish International Resource Center (ZIRC; Eugene, OR). Morpholino injected embryos were raised in E3 embryo media (150 mM NaCl, 4.5 mM KCl, 1 mM CaCl₂, X mM Na₂HPO₄ *2H₂O, X mM KH₂PO₄ *2H₂O). To prevent pigmentation, X mg/ml 1-phenyl 2-thiourea (PTU) was added to the media in which the embryos were raised.

Morpholino injections:

A morpholino oligonucleotide blocking the start site of the BF1a gene and a five base mismatch were obtained from Gene Tools LLC (Philomath, OR). The sequences of these morpholinos were as follows:
BF1a Perfect Match (PM): ATATTTTATCATACCCCTGGCAATC
BF1a Mismatch (MM): ATtTTaTATCTAgCCCTcGCAtTC
Morpholino injections were carried out as described in the previous chapter. 10ng of morpholino was injected per embryo.

Zebrafish immunohistochemistry:

Zebrafish embryos (36 hpf - 48 hpf) were fixed and immunolabeled following the protocol described in the previous chapter. Labeled embryos were mounted in 2% Low Melting Point agarose and imaged with a Nikon confocal microscope.

The following primary antibodies were used in these experiments: α-SV2 (DSHB) 1:20; α-calretinin (SWANT) 1:500; α-OlfCc1 (Ngai Lab) 1:100; α-PH3 (Santa Cruz Biotechnology) 1:500; α-acetylated tubulin (Sigma) 1:1000. The secondary antibody was an appropriately directed Alexa-Fluor 488 at a concentration of 1:200.

Data Analysis
For each embryo, a confocal stack through the entire OE and OB was obtained and Z-projected. Quantification of calretinin and PH3 positive cells was performed on the projected images, and the cell counts were independently confirmed by another researcher.
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The Journal of Comparative Neurology, 479(2), 216-233.


