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Engineering of Olfactory Receptor OlfCc1 for Directed Ligand Sensitivity

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Engineering of Olfactory Receptor OlfCc1 for Directed Ligand Sensitivity

By

Allison Paige Berke

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Engineering of Olfactory Receptor OlfCc1 for Directed Ligand Sensitivity

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By Allison Paige Berke
Abstract

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Due to structural similarity, OlfCc1 and its mammalian analogue V2R2 are hypothesized to respond to amino acid ligands in a calcium-mediated fashion. By analyzing receptor structure and making targeted mutations, the specificity and sensitivity of the receptor should be tunable, within the range of OR response thresholds. OlfCc1 responds to the amino acids isoleucine and leucine, in a calcium-dependent manner. V2R2 shows a similar response profile, with additional bimodal responses to valine. Additionally, both function as amine receptors, responding to isoamylamine and 2-methylbutylamine. This represents a TAAR-independent amine-sending pathway. Targeted mutations to OlfCc1 have successfully altered the sensitivity of the receptor, through mutating residues in the proximal pocket that are predicted to be three Angstroms or less from the docked ligand. Additional docking with calcium in the binding pocket has clarified distal pocket residues that coordinate the side chain of the amino acid ligand. Mutations to these residues have successfully altered the specificity of the receptor, including mutations to align its binding profile with that of the calcium-sensing receptor. Residues affecting amine-group binding as well as side-chain stabilization and calcium binding have been identified through modeling and confirmed through expression and functional testing in HEK cells.

The utility of and interest in these findings have both engineering and biological significance. Being able to target receptor functionality through directed modeling and mutagenesis opens up the olfactory receptors, naturally occurring and highly sensitive native sensing elements, as a family of candidate in vitro sensing elements. Their ability to be tuned along a gradient of concentration-dependent responses (showing EC50
tunability) indicates that they may be useful as reporter elements. Additionally, the ability to make verifiable and accurate predictions about receptor functionality from an in silico model is useful to the study of many receptors. The utility of using OlfCε1 and V2R2 in particular stems from their unique expression profiles (expressed near-ubiquitously in certain populations of olfactory and vomeronasal neurons) and their close homology with LIVBP, MGlur, and PBP proteins. Showing through modeling and mutagenesis how OR regions of similarity correspond to binding profiles gives us insight into how receptors function combinatorially.
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Chapter 1: Overview of ligand binding in the olfactory system

Types and roles of receptors in the olfactory and vomeronasal systems

Introduction:

The olfactory system represents a complex and comprehensive evolutionary solution to the problem of sensing the chemical world around us. Functionalities as diverse as food sensing, predator awareness, reproductive communication, and kinship information are mediated by a single system of proteins responsive to volatile (or soluble) chemicals. The proteins in this system are G-protein Coupled Receptors (GPCRs), of either Family A (canonical odorant receptors) or Family C (mGluR-like or pheromone receptors). The two families can be distinguished by the presence, in Family C receptors, of a large N-terminal domain (Felder, Graul, Lee, Merkle, & Sadee, 1999). (See Figure 1-1). While the chemicals in odor space have in common molecular weights of less than 300 Daltons (Demole & Wuest, 1967), they span a wide range of structures, receptive concentrations, and functional groups. To respond to such a variety of chemicals, olfactory receptors are naturally highly sensitive, combinatorial, and differentially tuned to respond to multiple odorants (Araneda, Peterlin, Zhang, Chesler, & Firestein, 2004), (Malnic, Hirono, Sato, & Buck, 1999). These properties make them ideal candidates for use as in vitro sensing elements, provided their receptive sensitivities and specificities are suitably tunable.

Olfactory receptors were discovered relatively recently, in 1991 (Buck & Axel, 1991), a discovery that merited the Nobel Prize. The family of olfactory receptor genes in an organism is relatively large, and can represent up to four percent of an organism’s entire genetic library (Feldmesser et al., 2006). Not all of these olfactory receptor genes are functional, but the amount of genetic material devoted to olfactory recognition and processing underscores the importance and breadth of this sensory system. As a model for engineered sensing systems, the olfactory system has been studied again and again. E-noses, or electronic noses, encompass any electronic device built to detect odors or flavors on the basis of animal olfactory capabilities. The field of e-noses was established in 1982 (Persaud & Dodd, 1982), but the recognition nine years hence of the structure of olfactory receptors had the effect of introducing bioelectronic e-noses using olfactory receptors directly as sensing elements. Other electronic noses use quartz-crystal microbalances, metal oxide semiconductors, conducting polymers, carbon nanotubes, and surface acoustic waves to detect the presence of volatile odorant molecules (Saefvels, Lammertyn, & Berna, 2004), (Saefvels et al., 2004). While these techniques can be very sensitive, they are generally not as efficient or combinatorial as biological odorant recognition.

The mechanism underlying the recognition and binding of a ligand by an olfactory receptor is widely thought to be a combination of the ligand’s size, functional groups, and chemical properties such as hydrophobicity or charge. The sequence diversity present in the transmembrane domains of olfactory receptors is thought to provide the range of binding specificity and sensitivity required to respond to the broad range of olfactorily active chemicals. Even one or two amino acids of difference can mean that one recognizes an odorant or fails to, or can make the difference between recognizing an odorant as pleasant or as aversive (Conigrave & Hampson, 2010). In humans, two
nucleotides of difference distinguish those who smell androstenone as aversive and those who smell it as slightly sweet or who do not smell it at all (Menashe et al., 2007), (Keller, Zhuang, Chi, Vosshall, & Matsunami, 2007). Alternative theories propose that a chemical’s vibrational frequencies are important to the identification of characteristic functional groups, or that these portions of an odorant are recognized in place of the odorant as a whole (known as “odotope theory”) (Matsumoto et al., 2010), (Mori, Matsumoto, Tsuno, & Igarashi, 2009). Receptors may also be constrained by physical properties of ligands including chirality; the enantiomers S-carvone and R-carvone smell distinct (caraway and spearmint, respectively), indicating that they are recognized by different receptors, or by different combinations of receptors (Leitereg, Guadagni, Harris, Mon, & Teranishi, 1971), (Laska & Shepherd, 2007). In fact, most of the receptors that respond to either of the enantiomers respond to both at high enough concentrations, and most receptors that respond to S-carvone are broadly activated by similar odorants (Hamana, Hirono, Kizumi, & Sato, 2003). Certainly, some odorant receptors are more broadly receptive than others, and some odorants activate large populations of receptors while others are only known to activate a single receptor. With each of several million olfactory neurons expressing only one functional odorant receptor (out of approximately 1100 in mice, 300 in humans, and 150 in zebrafish (Alioto & Ngai, 2005), (Selbie, Townsend-Nicholson, Iismaa, & Shine, 1992), (Niimura & Nei, 2005), (Xiaohong Zhang & Firestein, 2009), (Young et al., 2003)), activation of multiple odorant receptors by the same odorant may be necessary both to span chemical space and to amplify the signal from responding receptors. These factors have made the in silico prediction of receptor-ligand interactions difficult, and necessitate the combined use of computer modeling and in vitro imaging of receptor activation.

Vertebrate olfactory and vomeronasal receptors and binding specificity and sensitivity

Development and specification of the olfactory and vomeronasal systems:

The olfactory and vomeronasal systems both comprise GPCRs (of primarily Family A and primarily Family C, respectively) (Alioto & Ngai, 2006), (Alioto & Ngai, 2005), (Pfister & Rodriguez, 2005), (Saraiva & Korsching, 2007), (Liberles & Buck, 2006), (Hashiguchi, Furuta, & Nishida, 2008), (Hussain, Saraiva, & Korsching, 2009). Olfactory receptors, found in the olfactory epithelium directly behind the nasal mucosa, are expressed in cilia on olfactory neurons. (See Figure 1-2). The olfactory epithelium is pseudostratified, with a basal layer of horizontal basal cells, the stem cells of the olfactory system, globose basal cells, an intermediate progenitor population, and sustentacular support cells that span the entire height of the epithelium (Carter, 2004), (Jang, Youngentob, & Schwob, 2003). Additionally, olfactory receptors are only expressed in certain regions of the olfactory epithelium, in zones along the dorsoventral access that correspond to regions of the olfactory bulb along the same axis (Vassar, Ngai, & Axel, 1993), (Ressler, Sullivan, & Buck, 1993), (Miyamichi, Serizawa, Kimura, & Sakano, 2005). The most dorsal of these regions is termed Zone 1, and numbering proceeds ventrally. Zone 1 is the most strictly delineated, and is thought to contain receptors that process innate olfactory behavior, such as responses to predator odors. Learned olfactory behaviors are thought to be mediated by more ventral regions of the epithelium (Kobayakawa et al., 2007).
Olfactory sensory neurons themselves are present in immature and mature forms, with the cell bodies of the immature forms residing basal to the mature neurons. In Family A receptors, each of these neurons expresses only one isoform of a receptor (though one neuron may express at its surface hundreds of copies of that one receptor) (Chess, Simon, Cedar, & Axel, 1994), (Serizawa et al., 2003), (Lewcock & Reed, 2004). The olfactory sensory neurons project their axons through the cribriform plate to the olfactory bulb, which is located anterior to the telencephalon and the frontal lobe. The organization of these axonal projections is delicate, and head trauma frequently jostles the cribriform plate, resulting in anosmia when axons cannot regrow to their previous positions (Jafek, Eller, Esses, & Moran, 1989). After reaching the olfactory bulb, the axons of olfactory sensory neurons synapse with mitral or tufted neurons, in regions of neuropil known as glomeruli. Glomerular organization roughly corresponds to features that odorants share in common. The glomeruli of the olfactory bulb represent a drastic pruning of the neuronal architecture devoted to olfaction; the number of mitral or tufted cells is less than the number of olfactory sensory neurons by nearly ten-fold (Zou, Chesler, & Firestein, 2009), (Mombaerts et al., 1996).

The organization of the olfactory bulb represents a chemotopic map, similar to other neurological maps of the somatosensory and visual systems. However, as this map is not fully known and involves the combinatorial responses of hundreds to thousands of receptors, functional screening involving in vivo recordings or electrophysiology is not practical to deorphan receptor responses.

In most terrestrial vertebrates (but not humans), a second system has developed for the detection of “pheromonal” or behaviorally active odorants, known as the vomeronasal system. Vomeronasal receptors are expressed in a population of microvillous neurons whose axons project from the spatially separate vomeronasal organ, which is located ventral from the nasal septum, and dorsal to the roof of the mouth, to the anterior olfactory bulb, located dorsal-posterior to the main olfactory bulb (See Figure 1-2). (Menco, Carr, & Ezeh, 2001), (Døving & Trotier, 1998). While the mechanisms of conveying odorants to either the olfactory receptors (by sniffing) or to the vomeronasal receptors (through the flehmen response) vary, their subsequent processing in discrete glomerular activation regions in either the main or accessory olfactory bulbs remains the same (Altieri & Müller-Schwarze, 1980). Furthermore, although individual olfactory sensory neurons (OSNs) responding to the same odorant (and expressing the same olfactory receptor) are distributed randomly across the olfactory epithelium, these neurons converge upon the same glomerulus in a spatially invariant manner that is replicated across individuals (F. Wang, Nemes, Mendelsohn, & Axel, 1998), (Oka et al., 2006). Distinct from the main olfactory bulb, however, vomeronasal neurons can project to multiple glomeruli, and the glomeruli of the anterior olfactory bulb do not seem to be organized in the same manner as glomeruli in the main olfactory bulb. Vomeronasal receptors are also expressed with a different class of G protein, Gaq, which nonetheless functions similarly to G_{olf} and decouples from the tripartite G protein to propagate a signaling cascade.

The mechanism of activation of the olfactory receptor begins with the binding of an odorant ligand to the binding pocket of the receptor. This binding causes a
conformational shift in the protein that releases the G\textsubscript{a} portion of the tripartite G protein, which in olfactory sensory neurons is a specific isoform known as G\textsubscript{olf}. G\textsubscript{olf} activates adenylyl cyclase 3 and catalyzes cyclic AMP (cAMP) production. Then, cAMP opens the cyclic nucleotide-gated channel A2, which creates an influx of calcium and sodium ions, depolarizing the cell (Belluscio, Gold, Nemes, & Axel, 1998), (Brunet, Gold, & Ngai, 1996), (Wong et al., 2000). This calcium influx can be detected by calcium-sensitive dyes. The opening of a calcium-activated chloride channel, through which chloride ions leave the cell, exacerbates the result of this influx of calcium. (See Figure 1-1). The efflux of chloride ions further depolarizes the cell and leads to an action potential. Desensitization and adaptation follow from the inhibition of cAMP binding by calmodulin, terminating signaling (Stephan et al., 2009), (Kurahashi & Menini, 1997), (Song et al., 2008).

In the vomeronasal neurons and in Family C olfactory neurons, the signaling process uses a slightly different pathway. Neurons in the vomeronasal organ expressing V1R receptors signal through the G protein variant Gi, while neurons expressing V2Rs signal through the G protein variant Go. In both cases, the alpha subunit of the G protein decoupling from the tripartite G protein results in the activation of phospholipase C, and the production of IP3 and diacylglycerol (DAG). DAG then colocalizes with TRPC2 ion channels and stimulates their depolarization of the neuron, resulting in an action potential (See Figure 1-1). When expressed in heterologous non-neuronal systems, like HEK cells, this signaling cascade results in the release of intracellular calcium stores from the endoplasmic reticulum, resulting in a localized increase in calcium concentration inside the cell that is analogous to the calcium current induced in a neuron.

Just as the microvillous neurons that express OlfCc1 respond to amino acids and influence feeding behaviors in zebrafish, the homologous family of vomeronasal receptor genes in mammals, the V2Rs, also mediate responses to behavioral cues (Koide et al., 2009), (Kimoto, Haga, Sato, & Touhara, 2005), (Chamero et al., 2007), (DeMaria & Ngai, 2010). This indicates that these two populations may have evolutionarily conserved functions and specificities. Therefore, deorphaning OlfCc1 and V2R2 may shed light on the function of these broadly expressed receptors, and may validate the relation between these two receptor families.

**Olfactory receptor tuning and expression:**

Olfactory receptors respond to odorants at concentrations as low as .0001 parts per billion (ppb) and reach saturation at concentrations as high as 500,000 ppb (T. Pearce, Schiffman, Nagle, & Gardner, 2006). This dramatic range in receptor sensitivity represents an extremely useful platform for discriminatory sensing, with proper tuning of responses. Odorant properties can also change drastically with concentration; some odorants that smell floral at low concentrations, like lilial, can smell harsh at higher concentrations. Another odorant, (E,E)-2,4-dienal, smells of chicken at 10 ppm, but smells of citrus at lower concentrations (Chang, Lo, & Lin, 2005). However, this is not true of all odorants, and although most responses to odorants are combinatorial, they are not strictly additive (Johnson & Leon, 2007). Typically, the half maximal effective concentration (EC50) for an odorant ranges between a few to a few hundred micromolar (M Michael Gromiha, 2011), (Katada, Hirokawa, Oka, & Suwa, 2005), (Adipietro,
Mainland, & Matsunami, 2012). Even a single receptor that responds to multiple odorants can respond to each of these odorants with a distinct EC50. As a result of this property, it is possible to identify to which odorant a receptor is responding if the receptor’s range of specificity and sensitivity is known. However, identifying the specificity range of an olfactory receptor can be difficult, partly due to the difficulty of expressing functional olfactory receptors in heterologous cells. Some olfactory receptors can be functionally expressed in heterologous cells, and other olfactory receptors can be expressed with the addition of sequence mutations, co-expression partners or “chaperone” proteins, and the use of long-term low-sensitivity assays like luciferase reporter assays. Still other receptors can be assayed within dissociated olfactory neurons or intact olfactory epithelium, and responses can be detected through the use of electrical patch-clamp recordings or calcium-responsive dyes (Zhuang & Matsunami, 2007), (T. Sato, Hirono, Tonoike, & Takebayashi, 1994), (Malnic et al., 1999), (Touhara et al., 1999), (Krautwurst, Yau, & Reed, 1998), (H. Zhao et al., 1998), (Kajiya et al., 2001), (Katada, Nakagawa, Kataoka, & Touhara, 2003), (Araneda et al., 2004), (Zhuang & Matsunami, 2008). Each of these methods has its advantages and its drawbacks; gains achieved in ease of imaging can be lost in the time spent cloning or modifying olfactory receptor sequences.

As mentioned, most olfactory neurons express only one olfactory receptor isoform. This is maintained by feedback from the receptor chosen for expression. This feedback ensures that a functional receptor is chosen, and may prevent the choice of an additional receptor through mechanisms that are still being elucidated, and are thought to involve histone acetylation. The importance of choosing a functional receptor was revealed by experiments using genes engineered to express a nonfunctional receptor under the control of an unaltered promoter, in essence tricking the cell into expressing a receptor that will not signal. These receptors were chosen, and then replaced by functional receptors, indicating that the olfactory neuron requires a functional, signaling receptor (Lewcock & Reed, 2004), (Imai & Sakano, 2009), (Serizawa et al., 2003), (Shykind et al., 2004). However, while monoallelic expression is the “rule” (and is frequently referred to as the one-receptor-one-neuron rule), some neurons express more than one receptor. In particular, OlfCc1 is near-ubiquitously expressed in its population of microvillous neurons, and V2R2 in mammalian vomeronasal neurons is expressed along with other V2Rs (Martini, Silvotti, Shirazi, & Ryba, 2001), (Silvotti, Moiani, Gatti, & Tirindelli, 2007). This pattern of receptor co-expression is also seen in the taste receptors; bitter taste receptors of the T2R family are co-expressed in the same taste buds, allowing these taste buds to be activated by any bitter tastant (Pydi, Upadhyaya, Singh, Pal Bhullar, & Chelikani, 2012), (Singh, Pydi, Upadhyaya, & Chelikani, 2011), (Dong, Jones, & Zhang, 2009). Similarly, the T1R1 and T1R2 receptors are both co-expressed with T1R3. T1R1 is a receptor for amino acid tastes, and T1R2 is a receptor for sweet tastes, while T1R3 is a low-sensitivity receptor for sweet tastes. The responses of cells that express a pair of these receptors are only governed by one member of the pair (either T1R1 or T1R2); T1R3 has no effect on the responses of these cells to tastants (Xiaodong Li, 2009), (Maitrepierre, Sigoillot, Le Pessot, & Briand, 2012).

Binding mechanisms and identification of behavior-motivating odorants
Olfactory receptors bind to odorants through hydrophobic and van der Waals interactions, with the occasional formation of stronger hydrogen bonds between receptor and ligand. These predominately weak interactions serve to allow the olfactory receptor to quickly be recycled once bound, and reach desensitization and adaptation to a novel odorant quickly. The speed of the process of binding, activation, and desensitization is also useful for the detection of changing gradients of physiologically relevant odorants. These odorants include pheromones, sensed through the vomeronasal organ, and odorants representing food and environmental cues. Fish are known to respond to amino acids, nucleotides, and bile salts, which are food and social cues (Koide et al., 2009), (Rolen & Caprio, 2007), (Kang & Caprio, 1995). Amino acids, including methionine, leucine, and alanine, are feeding cues for fish when present in water at micromolar concentrations (Valentincic & Caprio, 1994). Zebrafish respond to amino acids as feeding cues and are able to distinguish between nine amino acids (Valentincic, Kralj, Stenovec, Koce, & Caprio, 2000), (Friedrich & Korsching, 1998). Mammals respond to a wide variety of odors, gustatory to excretory and medicinal to aesthetic. Humans tend to classify amines and amino acids as fishy-smelling, thiol-containing compounds as noxious, and aldehyde-containing compounds as plant-like (Hellman & Small, 1974). While perhaps more obvious in some animals than in others, olfactory perceptions motivate behaviors ranging from food preference to predator avoidance to disease detection.

Family C olfactory receptors, glutamate receptors, and the calcium sensing receptor

Previous research has shown that Family C olfactory receptors have evolved to detect amino acid or amino acid-like ligands (Alioto & Ngai, 2006). When these receptors are added to Family C GPCRs, which include mGluR, the GABAb receptor, and the calcium sensing receptor (CaSR), a general receptive profile emerges that includes a range of amino acids, calcium, and GABA (Brauner-Osborne & Wellendorph, 2007). These receptors differ from the Family A or rhodopsin class of olfactory receptors in their long N terminal domains, where ligand binding occurs, and their responsiveness to non-volatile or internalized odorant cues. Many odorants identified by these receptors mediate behavioral responses, as do the vomeronasal receptors, and the proposed amino-acid-binding functionality of these receptors in fish is in agreement with the perception of amino acids as feeding cues by many species of fish (Alioto & Ngai, 2005), (Triballeau et al., 2008). Behavioral responses and chemosensitive attraction to 100 micromolar concentrations of amino acids are initiated on their fourth day post-fertilization, and continue throughout adulthood (Lindsay & Vogt, 2004). As zebrafish display attraction responses to 100 micromolar, but not 10 micromolar, amino acid, amino acid response may be correlated with a local increase in amino acid concentration around a source of food, as the background concentration of amino acid in the natural environments where zebrafish live are approximately 10 micromolar (Lindsay & Vogt, 2004), (Braubach, Wood, Gadbois, Fine, & Croll, 2009). Sensitivities of Family C receptors vary; mGluR has an EC50 of 10 micromolar glutamate, CaSR has an EC50 for calcium of four millimolar, and the GABAb receptor has an EC50 for GABA of 17 micromolar (Ito, Kohda, Tanabe, Hirose, & Hayashi, 1992), (S. H. Pearce et al., 1996), (Wise et al., 1999). Because of its sequence homology with OlfCc1, the calcium sensing receptor (CaSR) bears particular focus out of all Family C GPCRs. CaSR is activated by calcium and
magnesium ions at levels that occur naturally in the body (Hebert, 1996). It is also activated by L amino acids, which are considered modulators of its calcium signaling (Conigrave, Mun, & Lok, 2007). Additionally, allosteric modulators of its activity have been identified, and these modulators bind at regions physically distinct from the binding of amino acids; because of this and dose-response studies of its activation by calcium, these ligands are thought not to activate the receptor directly, and instead increase or decrease its affinity for calcium, potentially by changing the receptor’s conformation to make calcium sites more or less accessible (Chia, Brennan, Slawin, Riccardi, & O'Hagan, 2012). Physiologically, the CaSR functions to maintain extracellular levels of calcium. This is achieved through the secretion of parathyroid hormone, and in cases of hyperparathyroidism this functionality is lost. Unlike olfactory receptors, CaSR responds to ligands already present within the body (S. H. Pearce et al., 1996).

CaSR exists in vivo as a homodimer, in contrast with certain other Family C receptors that form heterodimers (or heterooligomers). Five residues in the N-terminal domain of the CaSR are thought to coordinate calcium binding therein, with additional stabilizing interactions provided by three more residues (Jensen, Hansen, Sheikh, & Bräuner-Osborne, 2002), (Silve et al., 2005). However, calcium ions do bind at multiple sites within this N-terminal domain, as suggested by mutation experiments and measurements of the Hill coefficient, which is between three and four for calcium and between two and three for magnesium, indicating cooperative binding (Y. Huang et al., 2009). Of the amino acids that activate CaSR, which include leucine, tryptophan, phenylalanine, alanine, histidine, glutamate, and arginine, all are thought to bind within the N-terminal domain of the receptor, but at only one binding site, and only one at a time. These amino acids are thought to bind concurrently with calcium, and in the same binding site, which helps to explain why the size of the binding site of CaSR is similar to the sizes of the binding sites in mGluR and the GABAb receptor, both of which bind amino acids (Acher, Selvam, Pin, Goudet, & Bertrand, 2011), (Xin Li et al., 2008). Amino acids and calcium have been proposed to stabilize different active conformations of the CaSR, and potentially to activate different signaling cascades, and the question of whether amino acids are true agonists or only allosteric activators of CaSR remains.

Structural similarities of Family C receptors: the VFTD

Family C receptors are known to possess a large, extracellular, N-terminal domain consisting of two lobes in the shape of a clamshell. This structure is commonly known as the Venus Flytrap Domain (VFTD) and is a recognizable feature of OlfCc1, V2R2, the CaSR, mGluR, and other proteins in Family C (S. Huang et al., 2011), (Cao et al., 2009). The identification of this structure was made from crystallographic studies of bacterial periplasmic binding proteins and activated and inactivated glutamate receptors (Felder et al., 1999). In ionotropic and metabotropic glutamate receptors, activation of the receptor depends upon the closure of the clamshell, which results in a change in the structural conformation of the receptor. Many Family C members are known to dimerize, and dimerization is suspected of OlfCc1. The activation of Family C receptor dimers is known to cause the two dimerized receptors to twist with respect to one another on a plane perpendicular to the hinge of the VFTD, allowing for easier activation of their respective G proteins (V. V. Gurevich & Gurevich, 2008). Furthermore, studies with
mGluR and the GABAb receptors, an obligate dimer, indicate that binding and activation of ligand in the binding pocket of one receptor can result in the activation of the G protein associated with its dimer pair, leading to asymmetric activation (Maurice, Kamal, & Jockers, 2011), (Damian, Mary, Martin, Pin, & Banères, 2008). Such an activation paradigm may provide a further role for the ubiquitous co-expression of OlfCc1, allowing it to activate its co-expressed receptor if heterodimers are formed. A similar modality has been discovered in taste receptors; T1R1 and T1R2 require the co-expression of receptor T1R3 to bind and signal functionally (Maîtreppierre et al., 2012). However, a caveat to this idea is the fact that receptor 5.24 and mGluR1 bind to their respective ligands with Hill coefficients of 1, indicating a single binding site with no cooperativity (Alioto & Ngai, 2006). Either these receptors form homodimers that both activate simultaneously, or binding data may not fully reflect receptor kinetics.
Figure 1-1: Schematic of the signaling cascade of an olfactory and vomeronasal receptor. Signaling begins when the odorant or pheromone is bound by the receptor, and involves
the phosphorylation of one subunit of the G protein, the translocation of that subunit to activate either adenylate cyclase or phospholipase C, and the activation of membrane ion channels, leading to the depolarization of the neuron. This process leads to the release of intracellular stores of calcium, which can be detected through calcium imaging with a calcium-sensitive dye.
Figure 1-2: The olfactory systems of the mouse and zebrafish. While the mouse has two separate organs for sensing traditional and pheromonal odorants, the olfactory epithelium and the vomeronasal organ, the fish integrates both types of sensing into one organ, the olfactory rosette. In both systems, axons from the neurons that innervate the epithelium, the vomeronasal organ, or the olfactory rosette, project to the olfactory bulb. In the mouse, these projections form spatially distinct regions known as the main olfactory bulb and the accessory olfactory bulb, whereas the fish olfactory bulb is not as clearly delineated.
Chapter 2: Deorphaning OlfCc1

Introduction

OlfCc1 is a Family C olfactory receptor found in the zebrafish. It is homologous to receptor 5.3 in the goldfish (Luu, 2004). It shares sequence homology with the mammalian calcium sensing receptor, the metabotropic glutamate receptor (mGluR), and the V2R family of receptors, including the mouse vomeronasal receptor V2R2. The vomeronasal and olfactory Family C receptors can be classified into sub-families based on expression; Family A and B receptors are expressed in a punctate fashion, while Family C receptors are expressed broadly in the vomeronasal or olfactory epithelium. Before examining the ligand binding of OlfCc1, certain aspects of fish olfaction should be detailed. Olfaction in the zebrafish proceeds with certain important differences from mammalian olfaction. The olfactory epithelium in the zebrafish is situated in the olfactory rosette, and hosts three types of olfactory neurons: ciliated neurons, most similar to Family A olfactory receptors, microvillous neurons, most similar to Family C olfactory and vomeronasal receptors, and crypt cells, similar to the trace amine-associated receptors (TAARs) (Jun Li et al., 2005). Although the ciliated cells are more basally positioned in the epithelium (and the microvillous more apical), the olfactory rosette is a heterogeneous mix of both types of neurons. Unlike mammalian olfactory systems, where each olfactory sensory neuron expresses only one olfactory receptor allele, ciliated neurons in fish express one or a few types of olfactory receptors. Microvillous neurons, which express receptors homologous to the mammalian V2R vomeronasal receptors, are thought to signal through a phospholipase C-dependent pathway different from that used by the ciliated cells (A. Hansen & Zielinski, 2005). Microvillous neurons also express more than one olfactory receptor per neuron; one receptor is broadly expressed in a large percentage of neurons that each additionally express a different olfactory receptor. This broadly-expressed olfactory receptor is OlfCc1.

This thesis presents the de-orphaning of OlfCc1 and mutagenesis experiments designed to engineer this receptor to respond in a directed fashion to a selection of amine and amino acid ligands with varying sensitivities. Experiments in this section are based on the methodology of a previous project in the Ngai lab that explored the responsivity of receptor 5.24, a goldfish receptor responsive to sub-micromolar concentrations of basic amino acids, particularly arginine (Luu, 2004) (Triballeau et al., 2008). While 5.24 is a Family C receptor, amino acids are not pheromonal ligands for fish, indicating that the segregation of Family A receptors as canonical olfactory receptors and Family C receptors as vomeronasal receptors that has been observed in mammals does not fully explain the functional distinctions between Family A and Family C receptors in fish. As OlfCc1 was also predicted to respond to amino acid ligands, the previous example of 5.24 was particularly useful. The 5.24 project used reproducible methods to functionally characterize a ubiquitously-expressed Family C receptor, and provided proof-of-principle for the use of in silico and in vitro techniques to characterize a receptor binding pocket.

Identification of homologous motifs between OlfCc1 and other amino acid-binding proteins
OlfCc1 is part of a group of OlfC receptors that are Family C olfactory receptors similar to the V2R family of vomeronasal receptors. They share sequence homology with certain well-characterized receptors, and because receptor sequence is generally thought to be related to ligand-binding specificity, ligand preferences may be conserved among receptors sharing sequence homology. In particular, the OlfC receptors have been predicted to bind amino acids, due to sequence homology and previous in vivo imaging of the zebrafish olfactory bulb and its responses to odorant cues (Nikaido et al., 2013). Further evidence supporting the response of OlfC receptors to amino acids includes studies showing that microvillous cells, which express OlfC receptors, mediate responses to amino acids in adult zebrafish (Koide et al., 2009), (Vielma, Ardiles, Delgado, & Schmachtenberg, 2008).

The V2R family in mammals, which are phylogenetically related to the OlfC family of receptors, are known to bind peptides and to express two receptors per neuron. This unique property of near-ubiquitous expression suggests an interesting function for the broadly-expressed receptor (OlfCc1 or V2R2), and indicates that the broadly-expressed receptor may be easily expressed in a heterologous system, as it is expressed in so many neurons in vivo. Additionally, from an engineering standpoint, the ability to tune the functionality of a receptor naturally expressed in so many neurons represents significant control over the response of a population of neurons. The experiments described in later chapters aim to provide this tunable functionality in the OlfCc1 receptor.

**Calcium imaging in HEK cells of predicted odorants**

Evidence that supports the hypothesis that OlfC receptors, including OlfCc1, respond to amino acids can be found in a conserved binding motif in the amino acid sequence of the N terminal domains of these receptors. This binding motif involves eight amino acid residues (Acher, 2005), is located in the binding pocket of the receptor (which for Family C receptors is within the large extracellular N-terminal domain), and is thought to coordinate ligand docking within the binding pocket. The binding motif is conserved in structurally homologous proteins including LIVBP in insects, metabolic glutamate receptor mGluR1, and the fish olfactory receptor 5.24, which is also known to bind amino acids (Acher & Bertrand, 2005). Conservation of this motif, as well as the functionality of the LIVBP protein to bind hydrophobic amino acids and the functionality of mGluR1 to bind the amino acid glutamate, suggest that OlfCc1 also binds and responds to amino acid ligands.

Based on previous research into OlfC receptors, and the sequence similarity discussed above, I hypothesized that OlfCc1 responds to amino acids. To test this hypothesis, I transfected a CMVI vector construct containing the full coding sequence of OlfCc1 into HEK 293 cells incubated with Fluo 4, a calcium-sensitive dye, and used calcium imaging with pools of odorants and individual odorants diluted in different concentrations of calcium imaging buffer to record the calcium responses of individual transfected cells. (See Figure 2-1). By comparing these cells to positive (5.24) and negative controls, I was able to assess the responses of OlfCc1 to the various odorants tested.

Experiments were conducted through calcium imaging, expressing OlfCc1 and V2R2 in HEK 293 cells to test their ability to release intracellular calcium stores in response to
receptor activation by a ligand solubilized in calcium imaging buffer. The receptor was expressed by means of a transfected vector construct with a CMV promoter and a C-terminal FLAG tag used to select for transfected cells through immunohistochemistry. The presence of the FLAG tag did not affect the ability of the receptor to traffic to the membrane or respond to ligand. (See Figure 2-1).

Testing the responses of V2R2 to amino acid odorants also assessed the predicted functional homology between OlfCc1 and V2R2. Calcium imaging has been used extensively to determine the activation of cell surface receptors whose activation cascades involve the release of intracellular stores of calcium. Calcium-sensitive dyes developed for this purpose include Fura-2 AM and Fluo-4. Both were tested, and Fluo-4 was used for experiments due to its nonratiometric properties and subsequent speed of imaging. Dose response curves and half-maximal activation (EC50) values are readily obtainable from calcium imaging traces of dose-dependent receptor activation. (See Figure 2-2 and Table 2-1).

**Calcium imaging in HEK cells of calcium responses**

Because the calcium sensing receptor (CaSR) shares extensive N-terminal domain sequence homology with OlfCc1, we hypothesized that OlfCc1 also shared functional similarities in its activation profile. Having discovered that calcium is required for OlfCc1 to be activated by amino acids, and knowing that CaSR is also potentiated by amino acids in its response to calcium, we investigated the half maximal response concentration of calcium necessary for activation in the presence of the amino acid agonist isoleucine. To perform these experiments, the responses of OlfCc1 to increasing concentrations of calcium imaging buffer with an invariant concentration of isoleucine were measured using the same protocol as used for our initial experiments with amino acids. (See Figure 2-1).

V2R2 has a pairwise sequence similarity of 59% with OlfCc1, and shares sequence homology with CaSR and other Family C receptors. Due to these similarities, it has also been predicted to respond to amino acids, and after testing OlfCc1, V2R2 was tested with the same pools of amino acids and with individual amino acids, in varying concentrations of calcium. Because vomeronasal receptors and Family C receptors use the same signaling cascade, responses were expected to be of similar magnitudes of activation. (See Figure 2-2 and Table 2-1).

**Results**

**Calcium imaging in HEK cells of predicted odorants**

OlfCc1 was probed against pools of amino acids and individual amino acids at increasing concentrations of calcium. The standard concentration for calcium imaging buffer is 1 mM; concentrations as low as 0.5 mM and as high as 20 mM were used in experiments. The pools of amino acids tested were Hydrophilic (Asn, Glu, Gln, His, Lys, Arg, Asp); Hydrophobic (Val, Leu, Ile, Met); Polar (Gly, Ser, Thr, Tyr, Cys); Non-Polar (Ala, Trp, Pro, Phe).
Representative results are shown in Figure 2-2 and Table 2-1. Figure 2-2 shows a representative averaged raw trace of one field of cells responding to pools of amino acids. Responses show typical response and desensitization kinetics for GPCR-mediated calcium response. Upon noticing initial responses to the hydrophobic pool, and no responses to any of the other pools, each amino acid was tested separately to confirm that a lack of response to a pool was not the result of any antagonistic interactions either between amino acids and the receptor binding pocket or between amino acids and any secondary regions of binding within the receptor. After testing each amino acid individually, it was determined that the only amino acids to activate OlfCc1 are isoleucine, leucine, and valine, and that activation only occurs in the presence of calcium. These results are shown in Figure 2-2 for OlfCc1 and V2R2. Figure 2-2 shows dose-response curves for isoleucine, leucine, and valine, tested in OlfCc1 and V2R2 in the presence of 10 mM calcium.

Further testing was completed in the presence of 10 mM calcium to ensure complete activation of the receptor. The EC50 of isoleucine in the presence of 10 mM calcium is 244.5; the EC50 of leucine in the presence of 10 mM calcium is 377.1, and the EC50 of valine in the presence of 10 mM calcium is 232.7. (See Table 2-1). Although valine would seem to be the most potent agonist, its binding curve indicates a less efficient binding process, and the amplitude of OlfCc1 response to valine was the lowest of the three amino acids. Taking into account this amplitude of response (the change in delta F as measured by the internal calcium-sensitive fluorescence of the dye used to measure internal calcium release), isoleucine is the most potent agonist for OlfCc1, followed by leucine. Responses to isoleucine are significantly different in comparison to responses to leucine and valine, and responses to leucine and valine are significantly different from responses to valine, meaning that the three odorants activate the receptor with distinguishable profiles. Control experiments were performed with ATP, to test cellular response to a known stimulant and confirm that cells were properly loaded with calcium-sensitive dye, and with arginine, a known ligand of receptor 5.24, to which OlfCc1 did not respond. Experiments performed with 0.5 mM calcium imaging buffer only demonstrated activity in response to ATP; no amino acid ligands activated OlfCc1 in this low level of calcium. These EC50 values are high, but the dose-response curves of receptors imaged in heterologous cells are frequently, as noted in the literature (Birnbaumer, Antaramian, & Themmen, 1992), (Severi et al., 1999). A possible explanation for this effect is that calcium release is several steps downstream of ligand binding and G-protein subunit decoupling, and any of these steps between ligand binding and intracellular calcium release may be non-linear. The magnitudes of calcium-imaging responses have been demonstrated to be in agreement with in vitro responses, but in vitro EC50 values may be up to ten-fold lower than those measured through calcium imaging in heterologous cells.

V2R2 (Vmn2R1, NM_019918.2 in GenBank), was also found to respond to hydrophobic amino acids, and its responses to isoleucine, leucine, and valine were compared to those of OlfCc1. The EC50s of V2R2 response to isoleucine, leucine, and valine are 290.4 uM, 260.5 uM, and 174.5 uM. (See Table 2-1). Additionally, V2R2 responses were also enhanced in the presence of increasing concentrations of calcium. These results indicate that OlfCc1 and V2R2 are homologous not only in sequence but also in functionality. The differences in each receptor’s response to isoleucine, leucine, and valine were
significant, indicating that the responses of each receptor may be used to distinguish the presence of these three amino acids both from one another and from other amino acids present in a mixture, when response magnitude is also measured. For OlfCc1, the p-value of an unpaired t-test between isoleucine and leucine was .0057, between isoleucine and valine .0009, and between leucine and valine .0237. For V2R2, the p-value of an unpaired t-test between isoleucine and leucine was .0086, between isoleucine and valine .0362, and between leucine and valine .0086. (See Table 2-1). Therefore, V2R2 is also activated by leucine, isoleucine, and valine with distinguishable profiles.

**Calcium imaging of HEK cells of calcium responses**

The responses of OlfCc1 to varying concentrations of calcium were measured in the presence of a static concentration of isoleucine (both 100 uM and 250 uM were tested). OlfCc1 showed standard dose-response kinetics, and its EC50 for calcium in the presence of 250 uM isoleucine is 4.7 mM. (See Table 2-1). This indicates that it is approximately as sensitive to calcium as the calcium sensing receptor. Interestingly, OlfCc1 did not demonstrate any activity to calcium in the presence of amino acids that do not activate the receptor, such as arginine, and did not show any response to calcium without the additional presence of amino acids. This pattern of activity raises the question of whether OlfCc1 should be considered an amino acid-activated calcium sensing receptor, or a calcium-activated amino acid sensing receptor. Until its physiological role is better determined, this question remains a more semantic than practical distinction.

In the previous section, V2R2 was shown to also respond to hydrophobic amino acids in the presence of calcium. The calcium dose-response profile of V2R2 was tested using the same protocol as that used to test OlfCc2, and the EC50 for calcium of V2R2 in the presence of 250 uM isoleucine was found to be 8.4 mM. (See Table 2-1). The physiological role of V2R2 in the mouse vomeronasal organ has yet to be determined. Preliminary functional imaging indicates that mouse vomeronasal neurons respond to amino acid cues, but the genetic tools to functionally knock out or repress V2R2 activity in vivo are still being developed.

As controls, the responses of OlfCc1 and V2R2 to increasing calcium concentrations in the presence of non-activating amino acids and in the presence of no amino acids were tested (See Figure 2-2). Additionally, cells incubated in 0.5 mM calcium imaging buffer were used in the same imaging protocol as cells incubated in 1 mM calcium imaging buffer, and no differences in their response patterns or amplitudes were evident. Lower concentrations of calcium imaging buffer were not tested due to concerns about cell viability throughout the experimental protocol.

CRAC channels, or Calcium Release Activated Channels, are plasma membrane calcium ion channels. These channels act to replenish intracellular stores of calcium when calcium is depleted from the endoplasmic reticulum. Because our calcium imaging protocol depends on the measurement of the release of intracellular calcium as part of the signaling cascade that follows ligand binding and receptor activation, if ligand binding were also in some way activating CRAC channels our measurements would be artificially high. Additionally, premature CRAC channel activation could alter the desensitization time course of receptor activation. To ensure that CRAC channels were not being
activated, we performed imaging studies in the presence of lanthanum ion (La3+), a CRAC channel blocker. Lanthanum is used at a very low concentration, 100nM, because it can be toxic to cells. These experiments indicated that in the presence of a CRAC channel blocker, OlfCc1 responded with the same intensity and half maximal activation concentration as in the absence of the CRAC channel blocker, implying that CRAC channels are not involved in the activation and cellular response to activation of OlfCc1 (See Figure 2-3).

Discussion

OlfCc1 was predicted, through homology modeling, sequence similarity, and previous in vivo functional experiments in our lab, to bind amino acids. The results shown here demonstrate that OlfCc1 does respond to three hydrophobic amino acids, isoleucine, leucine, and valine, but only in the presence of calcium. These results indicate that OlfCc1 does have some functionality as a low-sensitivity amino acid sensor, but the requirement of calcium for activation indicates that the receptor may be playing an additional role. Concentrations of calcium in the ambient liquid environment of the zebrafish (freshwater) range from 0.2 mM to 2mM, and may be further concentrated around food sources or in hard water (Lindsay, 2004). (Ambient saltwater has 10 mM calcium, but zebrafish are freshwater dwellers.) Additionally, OlfCc1 may be functioning similarly to the calcium sensing receptor (CaSR), which has an EC50 for calcium of 4 mM, which may account for the calcium sensitivity of OlfCc1.

The previously identified Family C receptor 5.24 is activated by every naturally occurring amino acid, but demonstrates its most robust responses to arginine (Luu, 2004). OlfCc1 did not show the same broad range as 5.24, indicating that Family C receptor activation specificity is not conserved. However, this lack of broad activation is useful for our purpose of testing the ability of an olfactory receptor to be engineered for designated specificity or sensitivity, as changes to its specificity result not in an increased sensitivity to a previously activating ligand, but in an entirely new modality of activation. When designing a receptor to work as a sensor, this type of engineered activation is useful to control against native activation of un-engineered receptor. The ability of the responses of OlfCc1 to be used to discriminate between isoleucine, leucine, and valine is promising, as it indicates that one receptor could be used to determine the presence of three amino acids and to distinguish each from the others, rather than requiring the activation of three separate receptors.

These results raise the question of whether OlfCc1 is acting solely to detect its preferred ligands, or whether its activation and subsequent signaling are secondary to a potential role in receptor expression or desensitization. OlfCc1 is expressed nearly ubiquitously, but it is unclear whether its activation results in near-ubiquitous signaling to the olfactory bulb. Another open question is whether OlfCc1 is expressed in greater total quantities in the zebrafish olfactory epithelium; this question could be answered through quantitation of OlfCc1 mRNA transcripts compared with the mRNA transcripts of a traditionally expressed punctate receptor. Further experiments are necessary to determine whether the broad expression of OlfCc1 decreases the intensity of its subsequent inputs to the olfactory bulb upon activation, and whether OlfCc1 is normally activated with or without the cotemporaneous activation of the additional receptor present in the neuron in which
OlfCc1 is expressed. Additionally, experiments discussed later will probe the limits of the OlfCc1 binding pocket and identify an additional class of ligands to which OlfCc1 responds. Odorant space is broad, and OlfCc1 may respond with greater sensitivity to different ligands that were not identified in our initial screen.

The extensive sequence homology between OlfCc1, V2R2, and the calcium sensing receptor (CaSR) translate to functional homology between the three receptors as well. The activation of broadly-expressed olfactory and vomeronasal receptors by calcium in the presence of amino acids, and by amino acids in the presence of calcium, indicates that amino acid sensing plays a role in the determination of behavior, and may represent a low-sensitivity response to the identification of food cues in the environment. Fish perceive amino acids as food cues, and it is reasonable to assume that they would be behaviorally motivated to follow an increasing gradient of amino acids. In mice, the behavioral motivation underlying a low-sensitivity amino acid response is less clear. However, vomeronasal receptors usually respond to non-volatile chemicals, and amino acid sensing may be mediated by licking behavior or oral inhalation.

Conclusions and Future Directions

The results of the experiments presented here demonstrate that OlfCc1 is activated by calcium in the presence of certain hydrophobic amino acids, and by these same hydrophobic amino acids when in the presence of a sufficient concentration of calcium. Neither calcium alone nor amino acids alone are sufficient to activate OlfCc1. Furthermore, OlfCc1 is a low-affinity receptor, requiring concentrations in the hundreds of micromolar amino acids to activate fully. In comparison to other olfactory receptors that are commonly activated by concentrations in the tens of micromolar amino acids, activation at concentrations one order of magnitude higher indicates that OlfCc1 does not function solely to inform the fish of the presence of hydrophobic amino acids at concentrations normally found in the environment.

The responsivity of OlfCc1 to three hydrophobic amino acids places it in the middle of the dynamic range exhibited by other Family C receptors. While some, like mGluR, are gated to respond to only one amino acid, others such as 5.24 respond with low sensitivity to all 20 amino acids, and with higher sensitivity to preferred ligands. The responsivity of OlfCc1 may be related to its function as a broadly-expressed olfactory receptor; unlike mGluR, which responds to glutamate as a neurotransmitter, OlfCc1 responds to ligands it must filter through the liquid medium in which a zebrafish lives, swims, and eats. This requires the receptor to filter out only a small range of amino acid side chain diversity from the regions of chemical space with which it is presented. Though OlfCc1 responds with low sensitivity, it responds to chemically similar substances (hydrophobic amino acids) and its signals may be interpreted at the level of the olfactory bulb in concert with signals from other receptors that are responding to more stringently to functional moieties. If OlfCc1 signals together with the receptor with which it is co-expressed, its signals may be amplified by, or gated by, the responses of this other olfactory receptor.

Other explanations for the function of OlfCc1 as a calcium-sensitive amino acid receptor may be related to its function as a dimerization partner, trafficking aid, or calcium indicator. The dimerization of OlfCc1 has been proposed as a reason for its co-expression
with a variety of receptors in the microvillous cells that do not express at the cell surface when transfected alone into heterologous cells. The hypothesis behind this reasoning is that OlfCc1 brings these receptors to the surface because they are bound together. Co-expression in HEK cells indicates that receptors are capable of trafficking to the membrane when they are transfected in concert with OlfCc1, indicating that OlfCc1 may be able to interact with these “punctate” or normally expressed receptors in vivo, shedding light on a possible explanation for its function and co-expression (DeMaria et al., personal communication).

The fact that OlfCc1 is activated by, and requires for its activation, calcium, is not entirely surprising. OlfCc1 shares sequence similarity and has been phylogenetically clustered with the human calcium sensing receptor (CaSR). Furthermore, CaSR is also activated by amino acid ligands, which indicates that the two are functionally similar receptors. Functional imaging of OlfCc1 in zebrafish indicates that its knockdown affects responses to all classes of amino acids, not only hydrophobic amino acids. This discrepancy in activation may be a result of the interaction of OlfCc1 with other receptors, such as the punctate receptors with which it is coexpressed in vivo, or the result of imaging in heterologous cells, which could interfere with some of the in vivo functionality of the receptor in its natural cellular environment.

Future directions reported in the following chapter include the characterization and directed engineering of the OlfCc1 binding pocket to direct its specificity and sensitivity. The aim of these experiments was to provide proof of principle for the ability to engineer a receptor in a directed manner to respond to selected ligands. Other interesting avenues for future research involve providing definitive proof of the dimerization of OlfCc1 and ascertaining its role as a heterodimerizing chaperone for the surface expression of other receptors. Furthermore, the behavioral utility to the zebrafish of a calcium-requiring low-affinity amino acid sensor has yet to be determined.

Materials and Methods

Cell culture and transfections

HEK293 cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum. Cells were transfected in ply-d-lysine coated 8-chamber slides using Lipofectamine 2000 regent (Invitrogen, CA) according to the manufacturers instructions. The cells were fixed 24-48 hours after transfection in 4% paraformaldehyde for 30 min at room temperature.

Immunohistochemistry

Twenty-four to forty-eight hours after transfection, cells were treated according to a detergent-free protocol to detect surface localization of the genetically encoded FLAG tag within the CMVI vector. Rabbit anti-Flag antibody (Invitrogen) was added to cells at a 1:1000 dilution and cells were incubated at 4 °C for 1 hour. Subsequently, cells were washed with PBS, fixed with 4% paraformaldehyde (PFA), and washed with TTBS. Secondary antibody (anti-rabbit Alexa 488, Invitrogen) and nuclear counterstain (Hoechst) were added at 1:200 and 1:1000, respectively, in TTBS buffer with 10% HINGS for 30 minutes. Cells were then washed again with TTBS and PBS, rinsed with
PBS, and a coverslip was applied with VectaShield mounting medium. Cells were imaged on a Nikon E600 epifluorescence microscope at 20x magnification.

**HEK cell calcium imaging**

All experiments were carried out on HEK 293 cells plated on poly-D-lysine coated glass cover slips in 35mm tissue culture dishes, and transfected with 3µg of a FLAG-tagged CMV1 vector containing the OlfCc1 coding sequence (UniGene ID 66392213), or the V2R2 coding sequence (UniGene ID 273000). After 24 hours of growth, cells were incubated in 1mM Calcium Imaging Buffer (121 mM NaCl, 6 mM NaHCO3, 5.4 mM KCl, 5.5 mM D-glucose, 0.8 mM MgCl2, 25 mM HEPES, 1 mM CaCl2, pH 7.4) and 2µM Fluo-4, a calcium-sensitive dye. Cells were imaged on a Nikon inverted microscope using Imaging Workbench v.6 (INDEC BioSystems), which measures total fluorescent response and baseline-subtracted fluorescent response (DeltaF).

DNA constructs used were Qiagen miniprepped in accordance with the manufacturer’s instructions. All constructs were sequence verified. For transfections, 2ug of each construct were used with 6 uL Lipofectamine, in accordance with the Lipofectamine protocol.

HEK 293 cells were maintain in 1xDMEM with high glucose and were supplemented with 10% FBS (Gibco) and 1% Pen-Strep when not being transfected. Passage number was maintained below 20. Cells were carefully maintained to avoid overgrowth; cells were transfected at 70-80% confluency.

Fluo-4 and Fura-2 AM were used as calcium-sensing dyes. Each 50ug aliquot of dye was resuspended in 25uL of 20% Pluronic in DMSO to a final concentration of 2mM. Calcium Imaging Buffer was made with fresh Na Bicarbonate and 0.22uM filter sterilized. Cells were plated on poly-D-lysine-coated coverslips. For dose response curves measuring increasing concentrations of amino acids, 99.9% pure amino acids, leucine and isoleucine (SIGMA chemicals), were added to 10mM Calcium Imaging Buffer to obtain the appropriate dilution, and vortexed to mix. Solutions were added to live cells in imaging dishes via a customized profusion/vacuum system, and fluorescent responses were recorded with a CCD camera. Error bars represent Standard Error, and were calculated between separate cell populations, with each cell population encompassing between 15 and 25 cells. Each figure represents data from between 10 and 15 cell populations. Cells are marked as responding when a peak of fluorescence change is three or more times average baseline variation, as calculated from a control experiment using incubating 1mM Calcium Imaging Buffer in each profusion channel for the entire length of the experiment. The experimental protocol used is reprinted below:

**Calcium imaging protocol:**

HEK 293 cells are plated for transfection on 35mm dishes or MP6 wells. They are seeded at 3-400,000 cells per well. The cells should be 70-80% confluent the next day. The total seeding volume is 2mL. Cells are transfected with Lipofectamine 2000 (Invitrogen) in OPTI-MEM (Gibco) and the appropriate DNA. Cells are incubated overnight and replated on PDL-coated coverslips in sterile 2 cm petri dishes. Cells are incubated for a minimum of four hours before dye loading. Then, 1 mL of 5 uM Fluo-4 is added to each
coverslip, and incubated for 30 minutes at 37 °C. After this incubation period, cells are washed twice with calcium imaging buffer at room temperature and protected from light.

Calcium imaging buffer (CIB) with 1 mM calcium contains 121 mM NaCl, 6 mM NaHCO3, 5.4 mM KCl, 5.5 mM D-glucose, 0.8 mM MgCl2, 25 mM HEPES, and 1 mM CaCl2, at pH 7.4. The concentration of calcium was adjusted to produce 0.5 mM CIB, 1 mM CIB, 2.5 mM CIB, 5 mM CIB, 7.5 mM CIB, 10 mM CIB, and 20 mM CIB.

Images are acquired with a Sutter Instrutech Data Translation 3155 camera on continuous acquisition with 488 nM emission and 520 nM acquisition.

Stock solutions of L-amino acids were made to 100mM in 20mM HEPES pH 7.5 and stored at –20°C with the exception of L-Asp and L-Glu which were made in 0.1M HCl. Perfusion solutions were made fresh before each run in CIB.

Dose response curves measuring increasing concentrations of calcium were obtained using fixed-volume solutions of increasingly concentrated calcium, with the same molar concentration of amino acid. In this case, all cells responding in 20mM calcium were isolated and taken to represent 100% response, and the percentage of these cells responding at lower calcium concentrations were recorded as a normalized percentage of responding cells. Error bars represent Standard Error, and were calculated between separate cell populations, with each cell population encompassing between 15 and 25 cells. Each figure represents data from between 10 and 15 cell populations. Cells are marked as responding when a peak of fluorescence change is three or more times average baseline variation, as calculated from a control experiment using incubating 1mM Calcium Imaging Buffer in each profusion channel for the entire length of the experiment.

Dose response curves to amino acid pools represent four separate trials on the same cells. Amino acid pools were composed of 100uM, 250uM, and 500uM concentrations of each amino acid, in 10mM Calcium Imaging Buffer. Amino acid pools were added to incubating 1mM Calcium Imaging Buffer at 130s (100uM), 160s (250uM), and 190s (500uM), corresponding to the black lines on the image, and left on for 20s, then vacuumed off and replaced with incubating 1mM Calcium Imaging Buffer. Pools were composed as follows: Hydrophilic (Asn, Glu, Gln, His, Lys, Arg, Asp); Hydrophobic (Val, Leu, Ile, Met); Polar (Gly, Ser, Thr, Tyr, Cys); Non-Polar (Ala, Trp, Pro, Phe).

Data Analysis

Data were exported from Advanced Imaging Workbench (AIW) 5.1 software as Excel spreadsheets (Microsoft) with background-subtracted and raw data. Data were then thresholded to control responses and processed to generate Delta F/F values for fluorescence intensity. These values were recorded and calculated for each individual cell, represented as a single user-selected region of interest during recording. Delta F/F values were averaged across a single field of view and a single experiment, from which standard error values were also calculated, and response curves were calculated for each experiment separately. Average EC50s were obtained from these response curves, as well as the standard error of the EC50. Software used to plot response curves and calculate statistical analyses was GraphPad Prism v.5.0c. (GraphPad Software).
**Figure 2-1**: Schematic of calcium imaging experiments in HEK cells. Immunocytochemistry of FLAG-tagged protein construct is shown in A and B. C shows a representation of the FLAG tag on a Family C receptor and on a Family A receptor, and a schematic of the signaling cascade and imaging process is shown in D.
Figure 2-2: Response of OlfCc2 and V2R2 to excitatory ligands. Sample traces of OlfCc1 response to pools of amino acids in 10 mM calcium is shown in A. Responses of OlfCc1 to increasing concentrations of isoleucine and leucine in 10 mM calcium is shown in B. Responses of OlfCc1 to increasing concentrations of calcium in 250 uM isoleucine is shown in C. Responses of V2R2 to increasing concentrations of isoleucine and leucine in 10 mM calcium and to increasing concentrations of calcium in 250 uM isoleucine are shown in D and E. EC50 values for OlfCc1 are 245 uM for isoleucine, 377 uM for leucine, 233 uM for valine, and 4.7 mM for calcium. EC50 values for V2R2 are 263 uM for isoleucine, 279 uM for leucine, 183 uM for valine, and 8.4 mM for calcium.
Figure 2-3: Responses of isoleucine and leucine in the presence and absence of lanthanum. Lanthanum is a known CRAC channel blocker; the lack of statistically significant differences in the dose responses of OlfCc1 to isoleucine and leucine in the presence and absence of lanthanum indicates that CRAC channel activation does not account for the calcium sensitivity or amino acid sensitivity of OlfCc1.
Table 2-1: Statistics of OlfCc1 and V2R2 responses. This table shows the EC50s, peak responses (maximum amplitude), n values, and statistical significances of OlfCc1 and V2R2 responses discussed in this chapter. Amino acid responses are presented in a background of 10 mM calcium, and calcium responses are presented in a background of 250 uM isoleucine.

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<th>EC50</th>
<th>Max Amplt</th>
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<td>10</td>
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Chapter 3: Structure/Function Analysis of OlfCc1

Introduction

Homology modeling of receptors

To generate a homology model of OlfCc1, important residues were first identified through a sequence alignment of OlfCc1 with other Family C receptors and previously-identified amino acid-binding receptors like 5.24. (See Figure 3-1). Included in the alignment were mGluR1, the human CaSR, 5.24, LIVBP, goldfish 5.3, Z06, Lamprey OlfCaa, and V2R2. Residues were aligned based on an eight-residue sequence motif known to bind amino acids that had been previously identified (Acher & Bertrand, 2005), known regions corresponding to alpha helices and beta sheets, and knowledge of the structure of the N-terminal binding domain of Family C receptors. This alignment was later used to identify residues evolutionarily conserved among Family C receptors, and residues changed in OlfCc1 from the residues at those positions in 5.24 and the CaSR, whose binding profiles are most similar to that of OlfCc1.

Knowledge of the sequential and phylogenetic similarities of Family C receptors lends itself to homology modeling on the backbone of a crystallized receptor within the family. Crystallization of GPCRs is accompanied by the formidable challenges of disassociating the receptor from the membrane while maintaining intact its structure, and therefore few candidates are available as models for a crystal structure of an as-yet uncrystallized receptor like OlfCc1. Nearly all models of olfactory receptors are based on the crystal structure of bovine rhodopsin, but rhodopsin-like receptors, including canonical ORs and V1Rs, are Family A receptors, and Family C receptor N-terminal domains cannot be modeled on structures that lack these domains. For the following experiments, a homology model was built on the backbone of the crystal structure of mGluR1, which in the PDB database is referenced as 1ewk. This receptor provides crystal structures in unliganded forms and when bound to glutamate, providing some information on the closing of the VFTD during ligand binding, and indicating the potential location of the binding pocket, which is thought to be conserved among Family C receptors. The receptor mGluR1 is known to dimerize, and ligand binding stabilizes the bound dimer and the unbound or resting monomer in dynamic equilibrium. Homology modeling was accomplished using Accelrys Discovery Studio software, and was refined with a sequence alignment of Family C receptors, to determine conserved binding, loop, and helical regions. (See Figure 3-2 and Figure 3-3). Proposed ligands are positioned in the binding site using CHARMM-based molecular dynamics. Random ligand conformations are then generated, and these conformations are tested for fit in the binding site, after which energy minimization determines the optimal positioning of the ligand in the proposed binding site. (See Figure 3-4). Ligand flexibility is taken into account during this process, and in subsequent refinements the backbone of the receptor is held unchanged while side chains are allowed to rotate to permit the ligand to attain its optimal conformation. In this manner, candidate ligands were identified for OlfCc1, including isoleucine and leucine, and residues predicted to be in the binding pocket were identified.
A 3D homology model of the N terminal domain (VFTD) of OlfCc1 was constructed as described, and its binding pocket was modeled off of the crystal structure of mGluR1 (PDB ID 1ewk). Residues predicted to coordinate the glycine moiety of amino acids were identified in the proximal portion of the binding pocket, and are numbered with reference to the numbering of mGluR residues in the sequence alignment between Family C members. These residues are S127, S146, E148, S150, and Y198. (See Figure 3-5). Residues identified in the distal portion of the binding pocket are predicted to coordinate the specificity of the amino acid ligand and the properties of its side chain. These residues are R51, F87, S252, D253, A278, L369, R370, and I371. (See Figure 3-5). Anticipated ligands were docked in the binding pocket, and upon ascertaining the receptor’s affinity for isoleucine and leucine, the dockings of these amino acids were refined in the model. As seen in other Family C receptors, the binding pocket of OlfCc1 is located within the bilobate “clamshell” VFTD, which consists of an upper and lower lobe (lobes 1 and 2) and a flexible hinge that allows the two lobes to close around the ligand upon binding, thereby initiating the activation cascade that results in the release of cellular calcium stores. (See Figure 3-4). Many residues within the binding pocket have the potential to contact the ligand or to stabilize it through van der Waals or pi-orbital interactions. Additionally, water molecules present in the receptor’s natural and artificial surroundings are thought to stabilize the ligand within the binding pocket by filling excess space and providing polar interactions.

Identification of proximal pocket residues and their mutational effects on the OlfCc1 binding profile

Residues thought to be involved in the binding and stabilization of OlfCc1 ligands were sorted into proximal residues (those thought to interact with the glycine moiety or amine portion of the amino acid) and distal residues (those thought to interact with the side chain of the ligand). A proximal binding site motif of amino acids was previously identified among 5.24, LIVBP, mGluR, CaSR, and ZO6 (all Family C receptors), and these eight residues were paid particular attention. The motif, and the alignment of the sequences of the Family C receptors in which it is conserved, can be seen in Figure 3-1.

In Chapter 2, we identified the response of OlfCc1 to isoleucine, leucine, and valine in the presence of calcium. As our intention in mutating the binding site of OlfCc1 is to alter its specificity and sensitivity, we focused on both proximal and distal residues. The motif residues identified are R370, F87, E148, S150, S127, E277, Y198, and A278. (See Figure 3-1). The binding pocket motif residues were initially identified as part of a five-residue motif binding the glycine moiety of amino acids in the periplasmic binding protein-like family. Upon the creation of the homology model and the computation of intra-binding pocket distances, additional residues were identified in the sequence alignment and in the homology model, and tested through mutation to assess their effect on ligand stabilization within the binding pocket.

All residues predicted to be within three Angstroms of the ligand were also selected as defining the scope of the modeled binding pocket, and these residues included R370, A278, E277, D253, S252, S251, K225, Y198, D196, A194, I167, S150, S149, E148, S127, G126, G125, F87, and R51. (See Figure 3-5).
Identification of amine ligand binding

The residue corresponding to S127 in OlfCc1 was previously identified, in 5.24 and other Family C receptors, as being critical to the stabilization of the carboxyl group of amino acid ligands in the binding pocket. Because the mutant S127A did not demonstrate any change in sensitivity to isoleucine or leucine binding (See Figure 3-6), we hypothesized that the carboxyl group was not necessary for ligand recognition, and further reasoned that amine ligands, particularly those ligands that are the result of decarboxylation of an amino acid that has already been shown to activate OlfCc1, would also function to activate the receptor. Amine ligands dock in the binding pocket in the same orientation, and contact many of the same residues, as their related amino acid ligands. Testing receptor responsiveness to these ligands followed the same calcium imaging protocol as used for amino acid ligands.

Amines generally have fishy or ammoniac odors, and are predominately sensed by the trace amine-associated receptors (TAARs) expressed in crypt cells in the zebrafish, and are associated with food, as well as with odors of decay (Cichero, Espinoza, Gainetdinov, Brasili, & Fossa, 2013). Zebrafish are known to be sensitive to amines, but previous functional studies have only shown amine identification through activation of the TAARs.

Identification of distal pocket residues and their mutational effects on the OlfCc1 binding profile

In keeping with the goal of directionally engineering the specificity and sensitivity of the binding pocket of OlfCc1, attention turned to distal pocket residues as mediators of specificity. (See Figure 3-5). While proximal pocket residues are conserved among Family C receptors, distal pocket residues are not conserved, which leads to the diversity of ligand responses seen among such Family C members as CaSR, mGluR1, and 5.24. OlfCc1 has a smaller range of receptivity than CaSR and 5.24, which led us to attempt to lower its specificity and introduce receptor recognition of, and activation by, new amino acid ligands. Because of the extensive sequence homology between CaSR and OlfCc1, mutations were made specifically to binding pocket residues that differed between CaSR and OlfCc1, in an attempt to engineer the responsivity of OlfCc1 to more closely resemble that of CaSR.

Identification of calcium-sensitive sites and their mutational effects on the OlfCc1 binding profile

Between three and five distinct calcium binding sites have been identified in the calcium sensing receptor. (See Figure 3-7). At least one of these binding sites resides in the binding pocket, consistent with data indicating that calcium is required for amino acid or amine activation of the receptor. (See Figure 3-8). The other binding regions are within the VFTD N-terminal region of the receptor, but lie outside the binding pocket. One such region, Site 3 in the calcium sensing receptor, is close to the proposed site of receptor dimerization. In the calcium sensing receptor, calcium is coordinated at this site by four glutamate residues. Three of these glutamates are conserved in OlfCc1, and mutations
were made to these residues to determine whether they play a role in coordinating a binding site for calcium in this receptor. (See Figure 3-9).

Because the Hill coefficients for OlfCc1 binding vary, and are generally greater than one, it is very likely that OlfCc1 has multiple calcium binding sites, inside and outside of the binding pocket. Docking of a calcium ion in the binding pocket of the OlfCc1 homology model indicates there is room in the binding pocket and a suitable group of residues to coordinate the calcium ion, but mutagenesis of these residues did not conclusively demonstrate calcium effects. To probe a calcium binding site not directly involved with the binding pocket, and therefore presumably unaffected by ligand binding or any steric effects on the binding pocket from the presence of ligand, I focused instead on a calcium binding site on the outer face of Lobe 2. (See Figure 3-7). In the calcium sensing receptor, mutations to the residues at this site resulted in up to three-fold increases in the EC50 for calcium of the CaSR, indicating a decrease in sensitivity related to the disruption of a calcium binding site. Because the total number of calcium binding sites in OlfCc1 is unknown, I expected disruption of the three glutamates coordinating the binding site that I targeted to decrease the receptor’s sensitivity for calcium but not to remove it completely. To further probe the sensitivity of this putative binding site for calcium, I mutated the glutamate residues in question to alanines and to asparagines. Alanine has a much shorter side chain than glutamate and lacks its negative charge; in the CaSR, mutations of glutamate to alanine had severe effects. Asparagine is a less severe mutation; both asparagine and glutamate have polar side chains and are similar in length.

Results

Identification of proximal pocket residues and their mutational effects on the OlfCc1 binding profile

The proximal pocket residue mutations tested were S127A, S150A, S146T, Y198A, E148T, E148A, E148C, S150T, and F87NE148A, a double mutant containing a proximal mutation and a distal mutation. (See Figure 3-5). Of these, mutations to E148 and S150 had noticeable effects on the sensitivity of OlfCc1 to isoleucine and leucine, while mutations to other residues failed to affect isoleucine or leucine response significantly. The EC50 for isoleucine of S150A was 185 uM; that of E148T was 513 uM; that of E148A was 358 uM; that of S150T was 306 uM; and that of E148C was 192 uM. (See Table 3-2). The increase in EC50 for E148T may be explained by the difference in the side chain lengths of glutamate and threonine, and the potential steric interactions between the ligand and the side chain of threonine. (See Figure 3-10). In the unmutated receptor, the side chain of glutamate lies parallel to the side chain of the ligand and may stabilize it through van der Waals interactions; the side chain of alanine is too short to interact with the side chain of the ligand, and may prevent it from stabilizing within the binding pocket. The side chain of cysteine provides a charged moiety and extends alongside the side chain of the ligand, which may help with its stabilization and contribute to the lower EC50 observed for E148C. (See Figure 3-11). The mutant S150T also appears to have steric hindrances with the glycine moiety of the ligand (See Table 3-2); S150A may in fact permit more room for the ligand to bind, while not disrupting the
putative hydrogen bonds forming between adjacent residues and the amine group of the ligand. (See Figure 3-12).

The mutants Y198A, S127A, and S146T have EC50s for isoleucine of 277 uM, 255 uM, and 243 uM, respectively, while the EC50 of the unmutated receptor for isoleucine is 244.5 uM. (See Table 3-2). The failure of these mutations to affect the sensitivity of OlfCc1 to isoleucine indicates that the mutations were not drastic enough to affect ligand stabilization in the binding pocket. In the case of Y198A (See Figure 3-13), the alanine in this position is still able to form a putative hydrogen bond with the carboxyl group of the ligand; S127A loses one putative hydrogen bond with the carboxyl group of the ligand but maintains one. (See Figure 3-6). S146 is not predicted to directly contact the ligand, and so S146T does not have a significant effect on isoleucine binding. (See Figure 3-14).

Proximal residue mutations did have effects on the calcium sensitivity of OlfCc1, but these effects were varied. S127A, S146T, E148T, S150T, and Y198A had decreased sensitivities to calcium; S150A and E148C had increased calcium sensitivities, and the calcium sensitivity of E148A did not differ significantly from that of the unmutated receptor. (See Table 3-2). The trends of increased sensitivity to both calcium and amino acid ligand observed in S150A and E148C are consistent with enhanced activation of the receptor, while the stable calcium sensitivity of E148A indicates that this mutation only affected ligand binding, and not calcium docking within the binding site. Residues predicted to coordinate the binding of calcium in the proximal pocket are S150, E177, D170, and Q173. (See Figure 3-8). Unfortunately, mutations to D170 and E177 did not express at the cell surface, but the mutants S150A and S150T may shed some light on the binding of calcium in this position; S150A did not have a significant effect on calcium sensitivity, while S150T significantly increased (p < .005, one-way ANOVA with Dunnett post-test) the EC50 for calcium. (See Table 3-2).

Some of the most interesting mutations were E148A, E148C, and F87NE148A. These mutations decreased the specificity of OlfCc1, allowing it to bind additional ligands (histidine and phenylalanine in the case of E148A and E148C; because of the effects of F87N discussed in a later section, the double mutant also bound glutamate.) (See Table 3-2 and Table 3-1). Unmutated receptor shows no sensitivity to either histidine or phenylalanine; presumably mutations to E148 allow amino acids with longer side chains to bind, as the side chain of E148 in its position appears to be aligned parallel to the side chain of the ligand and may limit its branching or charge. Phenylalanine, being nonpolar and having a hydrophobic side chain, benefits from many of the same hydrophobic interactions in the binding pocket as isoleucine and leucine. Histidine also contains an aromatic ring in its side chain, and can form pi stacking interactions, which may help to stabilize it in the binding pocket. These interactions are disrupted when the pH of its solution drops below 6, but calcium imaging buffer is slightly basic (pH 7.4) and should not inhibit pi interactions.

As controls, all mutants were tested initially with the pools of amino acids described in Chapter 2. If additional pools activated the receptor, then the individual components of these pools were tested, which resulted in the identification of new ligands for E148A, E148C, and F87NE148A. (See Figure 3-15, Figure 3-11, and Figure 3-16). All ligands were tested in increasing concentrations of calcium, and calcium dose-response curves
were generated in increasing concentrations of isoleucine. Although isoleucine is no longer the most potent agonist for some of these mutants, isoleucine was used for calcium dose-response experiments to provide continuity with previous experiments and with results from the unmutated receptor. Additionally, all mutants were tested with previously identified amine ligands isoamylamine and 2-methylbutylamine, as well as phenylethylamine, the decarboxylated form of phenylalanine, which activated mutants that were also activated by phenylalanine, further validating the amine responsivity of this receptor.

**Identification of amine ligand binding**

Calcium imaging experiments were performed using the same protocol as that used for amino acid ligands, but decarboxylated amino acids (i.e., amines) were used instead. The amine versions of isoleucine and leucine are 2-methylbutylamine (2MBA) and isoamylamine (IAA), respectively. Phenylethylamine (PEA) was also tested with those mutants responsive to phenylalanine. Histamine (the decarboxylated version of histidine) was tested with mutants that responded to histidine, but no activation was observed.

Generally, responses of mutants to the amine versions of activating amino acid ligands demonstrated the same trends in activation and relative sensitivity as compared to the responses of the unmutated receptor. Unmutated OlfCc1 responded to 2MBA and IAA, with EC50s in 10 mM calcium of 179 uM and 169 uM, respectively. (See Table 3-2). These values indicate that the receptor is slightly more sensitive to amines than to amino acids, raising the question of whether OlfCc1 should be characterized as an amine receptor as well as an amino acid receptor.

**Identification of distal pocket residues and their mutational effects on the OlfCc1 binding profile**

The distal pocket residue mutants tested were I371A, I371G, L369G, R370G, F87A, F87Y, A278L, A278T, A278K, A278G, F87N, R51E, D253G, S252T, and F87NS252T. (See Table 3-1). I371G, L369G, and R370G were all nonresponsive to any ligands, indicating that their side chains may be important to ligand binding. (See Figure 3-17, Figure 3-18, and Figure 3-19). I371A, however, did not have a significant effect on the EC50 of isoleucine; for this mutant it was 244 uM. (See Figure 3-20). It is posited that alanine maintains the hydrophobic contact between this residue and the ligand, while mutations to glycine remove this contact. F87A was also nonresponsive (See Figure 3-21); the EC50 of F87Y for isoleucine in the presence of 10 mM calcium is 720 uM, which is nearly three-fold higher than that of the unmutated receptor, indicating that F87 is critical for binding and stabilization of amino acids in the binding pocket. (See Table 3-1 and Figure 3-22).

The residue A278 was mutated to leucine, threonine, lysine, and glycine. (See Figure 3-23, Figure 3-24, Figure 3-25, and Figure 3-26). Mutations to lysine and glycine resulted in EC50s for isoleucine in the presence of 10 mM calcium of 507 uM and 555 uM, respectively, whereas mutations to leucine and threonine resulted in EC50 values for isoleucine in the presence of 10 mM calcium of 185 uM and 243 uM, respectively. (See Table 3-1). It appears that leucine and threonine in this position help maintain the
hydrophobic pocket, whereas a charged or very short amino acid does not stabilize the ligand sufficiently.

As shown with two proximal pocket mutants above, five distal pocket mutants served to expand the range of ligands that activate OlfCc1. These mutants are F87N, which responds to histidine, R51E, which responds to phenylalanine, D253G, which responds to phenylalanine, S252T, which responds to histidine and glutamate, and F87NS252T, which responds to histidine, glutamate, and phenylalanine. (See Figure 3-27, Figure 3-28, Figure 3-29, Figure 3-30, and Figure 3-31). F87N expands the size of the distal binding pocket, and the position aligned with F87 in CaSR is also an asparagine. R51E and D253G may serve to move S252 away from the side chain of the ligand, permitting larger ligands to bind. The mutant S252T is also accompanied by a shift in the aromatic ring of the sidechain of F87; this may serve to both widen the binding pocket for larger amino acids to bind while also contributing to pi interactions between F87 and phenylalanine as a ligand. (See Figure 3-32). Additionally, at a pH of 7.4, glutamate and threonine are both polar, and weak interactions between the two may help stabilize glutamate as a ligand in S252T. (See Figure 3-33 and Figure 3-37).

As controls, all mutants were tested initially with the pools of amino acids described in Chapter 2. If additional pools activated the receptor, then the individual components of these pools were tested, which resulted in the identification of new ligands for F87N, R51E, D253G, and S252T. All ligands were tested in increasing concentrations of calcium, and calcium dose-response curves were generated in increasing concentrations of isoleucine. (See Table 3-1). Although isoleucine is no longer the most potent agonist for some of these mutants, isoleucine was used for calcium dose-response experiments to provide continuity with previous experiments and with results from the unmutated receptor. Additionally, all mutants were tested with previously identified amine ligands isoamylamine and 2-methylbutylamine, as well as phenylethylamine, the decarboxylated form of phenylalanine, which activated mutants that were also activated by phenylalanine, further validating the amine responsivity of this receptor.

Identification of calcium-sensitive sites and their mutational effects on the OlfCc1 binding profile

Mutations to the three glutamates identified as a potential calcium-binding site, E208, E211, and E212, decreased the sensitivity of the receptor for calcium, indicating that these three residues do coordinate the binding of one or more calcium ions. Mutations to alanine had greater effects than did mutations to asparagine, although both sets of mutations resulted in decreases in calcium sensitivity, as measured by the EC50 of the dose-response binding curve. (See Table 3-3). Mutations to alanine also indicated decreased sensitivity to amino acid and amine ligands, perhaps as a result of the cooperativity of binding and signaling in this receptor. Asparagine mutants E208N, E211N, E212N, E208NE211N, and E208NE212N showed sensitivities for amine and amino acid ligands similar to those of the unmutated receptor. (See Figure 3-9 and Table 3-3).

Mutations of glutamates to asparagines generally had less prominent effects than alanines, likely because asparagine and glutamate are both polar, while alanine has a
shorter side chain than glutamate. The EC50 values for calcium in the presence of 250 uM isoleucine of E208A, E211A, E212A, E208AE211A, and E208AE212A are 6.7 mM, 6.8 mM, 8mM, 8.14 mM, and 8.79 mM, respectively. The EC50 values under the same conditions for E208N, E211N, E212N, E208NE212N, and E208NE212N are 6.44 mM, 7.45 mM, 5.3 mM, 7.43 mM, and 7.6 mM. (See Table 3-3). The double mutants in each case have greater effects than do the single mutants; E208AE212A did not respond to any stimulant and was therefore considered nonresponsive, so the mutant E208NE212N was not tested. These values also indicate that E212 has the most central role in coordinating the calcium ion at this site, as it has the largest effect of the single mutants when mutated to alanine, but has a much smaller effect when mutated to glutamine. This indicates that arginine at this position is still capable of coordinating calcium, but to a lesser extent than glutamate. (See Figure 3-9).

Mutations to this calcium site should not affect the binding pocket, as they are physically well separated. Mutations to asparagine generally did not greatly affect the receptor’s sensitivity to isoleucine or leucine, whereas mutations to alanine did; this suggests that calcium binding at multiple sites on the receptor affects the receptor’s sensitivity toward amino acid ligands, or at least its ability to signal upon binding.

Discussion

Identification of proximal pocket residues and their mutational effects on the OlfCcl binding profile

Proximal residue binding is conserved among many Family C receptors, including 5.24 and Z06. The failure of some proximal residues to affect ligand binding led us to speculate that the carboxyl group of the ligand is not providing significant stabilization within the binding pocket. In particular, S127 was previously identified as critical to the stabilization of amino acid ligands in the binding pocket, but mutation to alanine and the disruption of one putative hydrogen bond had no significant effect on ligand sensitivity. This discovery and analysis led to a series of experiments with amine ligands (decarboxylated amino acids), described in the following section. Generally speaking, proximal residues closer to Lobe 1 had more of an impact on ligand binding and receptor sensitivity than those closer to Lobe 2, which may give an indication of how the ligand docks in the open conformation of the receptor. Data from these mutants allow us to predictively define which residues are involved in binding the glycine moiety of the amino acid ligand, and suggest an explanation for the low sensitivity of this receptor to its amino acid ligands, if the carboxyl group of the ligand is not being effectively bound by the receptor.

These experiments demonstrate the ability of homology modeling to correctly predict mutations that can expand the dynamic responsive range of a Family C receptor to amino acid ligands. The identification of E148 as a residue that can mediate greater or lesser specificity within the binding pocket may indicate that, because in OlfCcl the residue I371 is shorter than the residue with which it aligns in mGluR and CaSR, an intermediate hydrophobic residue (E148) is needed to contact the ligand. (See Figure 3-34, Figure 3-
and Figure 3-36). CaSR also responds to histidine and phenylalanine as intermediately sensitive ligands; the addition to OlfCc1 of responses that are shared in CaSR validates the homology of the binding pockets of both receptors, as E148 in CaSR is a cysteine, and the responses of E148C in OlfCc1 partially reconstitute the responsive range of CaSR.

Testing of these residues, and particularly those previously identified as coordinating the glycine moiety in periplasmic binding protein-like proteins, verified their importance in amino acid binding, and the importance of the conservation of these residues evolutionarily. Three additional residues that constitute the eight-residue motif previously identified (Acher, 2005) serve to distinguish plant-based, teleost, and vertebrate groups from within the periplasmic binding protein-like proteins.

Identification of amine ligand binding

The identification of amine activation in a Family C receptor expressed in the microvillous neurons represents a novel pathway for amine activation in the zebrafish, and the first non-TAAR to mediate amine sensation. Interestingly, the amine forms of amino acids activate this receptor at concentrations on the same order of magnitude, meaning that OlfCc1 is a low-sensitivity amine receptor and a low-sensitivity amino acid receptor, and both of these functionalities require the presence of calcium. CaSR is not known to bind amine ligands; 1-arylmethyl pyrrolidin-2-yl ethanol amines have been identified as antagonists of the CaSR (Saidak, Brazier, Kamel, & Mentaverri, 2009; M. Wang & Hampson, 2006), though it is not clear whether these molecules bind in the binding pocket of the VFTD, or to the seven transmembrane domains, where other allosteric modulators of the CaSR are known to bind. Based on their dose-response curves and activation profiles, the amines tested here are believed to bind in the VFTD binding pocket.

Physiologically, the need for two distinct amine-responsive pathways, the TAARs and OlfCc1, in the zebrafish olfactory system, is unclear. Because of OlfCc1’s expression pattern, it is possible that its responses to both amines and amino acids are of secondary importance to another function that it serves in microvillous neurons. Perhaps it helps co-expressed receptors to signal, or to traffic to the membrane and attain functionality. Future experiments are necessary to determine the extent of in vivo zebrafish response to amines, and to what extent microvillous cells mediate these responses. Reporter-specific knockout lines of zebrafish that can selectively block signaling from microvillous cells would shed light on the functional basis for amine signaling through OlfCc1.

Identification of distal pocket residues and their mutational effects on the OlfCc1 binding profile

Distal pocket residues are considered distal because amino acid ligands are predicted to bind in VFTD pockets with the glycine moiety situated closest to the hinge. These residues are therefore predicted to contact and stabilize the side chain, and to delimit the size and shape of the available binding pocket. As predicted, mutations to these residues decreased the specificity of the receptor, and conferred the ability to bind additional ligands also known to activate CaSR. Additionally, none of these mutants had
substantially lower peak activation values than the unmutated receptor, indicating that receptor expression level was not affecting receptor activation, the receptor’s structure was being globally maintained, and the receptor was able to close properly and enter an active conformation. Because an active conformation is determined by the degree of closure of lobes 1 and 2 of the clamshell, we can infer that the N-terminal domain was able to properly close, and that the functional translation of closed NTD to signal transduction was preserved.

The sequence alignment presented in this chapter predicts a number of distal pocket residues that are important in binding to amino acid side chains, and determine the specificity of the binding pocket for large or charged amino acids. Alignment of OlfCc1 to other Family C receptors known or predicted to bind amino acids yields specific predictions about individual residues and areas of the binding pocket that stabilize amino acid side chains. Mutation of these residues (for example, in D253G or E148A) increases the size of the binding pocket, decreasing steric effects that would normally hinder the binding of amino acids with longer side chains like phenylalanine. Additionally, residues aligning with position E148 in OlfCc1 are predicted to stabilize and lie parallel to the side chain of the amino acid ligand. This position is very important in the sequence alignment, and mutation of E148 to alanine or cysteine affects the size of the amino acid ligand activating the receptor.

Binding of OlfCc1 to a variety of amino acid ligands implies that the binding pocket is permissive despite requiring a precise closing angle for maximal receptor activation. In fact, the binding pocket is likely filled with a number of water molecules as well as the ligand and the calcium ion, and the number of water molecules filling the binding pocket changes with the size and hydrophobicity of the ligand. (See Figure 3-34 and Figure 3-35). Preferred ligands form the maximal number of contacts between ligand and each lobe, and non-preferred ligands form fewer contacts or less favorable contacts, resulting in a less stable closed state for the receptor. This explains the permissivity of the receptor for large ligands like phenylalanine that still may not activate the receptor as fully as smaller ligands like isoleucine; the larger ligand is displacing water molecules that normally fill the binding pocket, but the larger ligand is not forming the same interlobular contacts and is not stabilizing the bound configuration of the receptor as effectively.

Identification of calcium-sensitive sites and their mutational effects on the OlfCc1 binding profile

Calcium binding sites are thought to be conserved among several Family C members, including OlfCc1. Mutations to three glutamates in a putative calcium binding site that is conserved in a sequence alignment between OlfCc1 and CaSR appears to affect the response of OlfCc1 to calcium in the presence of amino acid and amine ligands, and may also affect the ability of the receptor to dimerize. Although the calcium binding site within the binding pocket should have a greater effect on the calcium responsiveness of the receptor, results from mutations to this calcium binding site are promising in their ability to alter and control calcium responsiveness, while not eliminating amino acid or amine responses.
In CaSR and mGluR1, ligands that bind within the VFTD are activators, while ligands that bind to regions along the seven transmembrane domains are allosteric modulators. Binding of calcium ions to the VFTD confirms that they are ligands, rather than modulators, and that OlfCc1 requires both calcium and an amino acid or amine ligand for activation and signaling.

An interesting future experiment would be to measure the ligand binding capability of a single receptor, rather than measuring aggregate calcium release from a cell that is expressing many copies of the same receptor simultaneously. Single-receptor recording would give a much better understanding of the dynamics of ligand binding and the order in which calcium and amino acid or amine ligands bind. For example, the homology model indicates that calcium binds in the binding pocket, closer to the hinge than the amino acid or amine ligand, indicating that it must enter the binding pocket first. However, does calcium bind at more than one site on the receptor? And if so, does it bind at all site simultaneously, or does its binding at one site cooperatively affect other sites, making it easier to bind more calcium ions to the same receptor? Can the receptor signal if one of its amino acid or amine ligands is bound but not all calcium sites are occupied with calcium? If it does signal with partial calcium binding, is this signal weaker or does it take longer to signal than a receptor with calcium bound at every potential site? Currently, calcium imaging is limited to whole-cell recording, but other assays that detect decoupling of the G protein or the release of G protein subunits, coupled with superresolution microscopy for intracellular localization, would allow imaging of the signaling events taking place at a single receptor.

Conclusions and Future Directions

OlfCc1 is a unique candidate for functional mutation studies and directed engineering of ligand binding because it is expressed nearly ubiquitously in the microvillous cell population of the zebrafish olfactory epithelium. Its unique expression pattern gives it the potential to simultaneously affect an entire cell population, and means that it is somewhat robust in maintaining its surface expression after mutations to its N-terminal VFTD. The wide array of mutations described above have succeeded in reducing the specificity of the receptor and permitting it to respond to larger and more varied ligands, as well as increasing and decreasing the sensitivity of the receptor, in a directed fashion. The homology model of the binding pocket of OlfCc1 was verified through selected mutations to expand the binding pocket or remove key stabilizing interactions, and the picture of OlfCc1 that emerges is of a receptor that behaves similarly to the mammalian CaSR in its calcium and amino acid responsiveness.

The identification of amine responsivity in OlfCc1 is interesting as well, as it indicates a second TAAR-independent amine-sensing pathway in the zebrafish olfactory system. TAARs and TAAR-like receptors are expressed in crypt cells in the zebrafish, whereas OlfCc1 is expressed in microvillous cells, indicating that amine sensing does proceed through two distinct pathways. While in many receptors that share the eight residue amino acid binding motif previously identified (Acher & Bertrand, 2005), the residue corresponding to position S127 in OlfCc1 mediates binding of the glycine moiety of the
amino acid, OlfCc1 does not lose its ability to bind amino acids in the S127A mutant. This not only indicates that OlfCc1 has a different mode of binding from other, homologous amino acid binding proteins, it also indicates that this position is less important in OlfCc1 than in other receptors, and that amine ligands fit into the pocket just as well as, and in the same orientation as, amino acid ligands.

The role of calcium in OlfCc1 is also very interesting. Because of the sequence homology of OlfCc1 and the CaSR, the effects of calcium as a ligand were partially predicted. The fact that both calcium and an amino acid or amine ligand are required for activation of the receptor, however, indicate that these two ligands may stabilize one another in the binding pocket. The carboxyl (COO-) moiety of the amino acid ligand may be shielded by the positively-charged calcium as they both dock in the main binding pocket, and calcium may also stabilize amino acid docking within the pocket, providing an explanation for the lower magnitude of amine responses. If calcium and the carboxyl group of the amino acid ligand do serve to stabilize the bound configuration, the lack of this stability with an amine ligand that lacks the carboxyl group would lead to a less stable closed (or bound) receptor configuration, and therefore a lower magnitude of response.

While many responses reported herein diverged from the known binding behavior of homologous receptors in Family C, many distal pocket residues identified through sequence alignment as being important for coordinating the R group moieties of amino acid ligands were shown to have evolutionarily conserved functions. The importance of these conserved residues lies in their ability to predict amino acid binding in similar receptors, and demonstrate the tunability of amino acid binding receptors through alterations to the size and charge of the binding pocket. As seen with the modeling and imaging of receptor 5.24, these distal pocket residues show conserved abilities to broaden the binding specificity of the receptor and permit the binding of amino acids with longer side chains.

The analysis presented herein presents proof of principle for the directed engineering of receptor binding using in silico homology modeling and in vitro calcium imaging as verification. This thesis also suggests the utility of OlfCc1 as a partial mimic of the functionality of the CaSR, and further research aiming to express a mutated version of OlfCc1 to direct its signaling in vivo would be of great interest. However, it is still very important to determine the in vivo function of OlfCc1, and whether it signals independently or only in conjunction with a receptor with which it is co-expressed.

Materials and Methods

Cell culture and transfections

HEK293 cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum. Cells were transfected in ply-d-lysine coated 8-chamber slides using Lipofectamine 2000 regent (Invitrogen, CA) according to the manufacturer’s instructions. Cells to be used for immunohistochemistry were fixed 24-48 hours after transfection in 4% paraformaldehyde for 30 min at room temperature.

Immunohistochemistry
Twenty-four to forty-eight hours after transfection, cells were treated according to a detergent-free protocol to detect surface localization of the genetically encoded FLAG tag within the CMVI vector. Rabbit anti-Flag antibody (Invitrogen) was added to cells at a 1:1000 dilution and cells were incubated at 4 °C for 1 hour. Subsequently, cells were washed with PBS, fixed with 4% paraformaldehyde (PFA), and washed with TTBS. Secondary antibody (anti-rabbit Alexa 488, Invitrogen) and nuclear counterstain (Hoechst) were added at 1:200 and 1:1000, respectively, in TTBS buffer with 10% HINGS for 30 minutes. Cells were then washed again with TTBS and PBS, rinsed with PBS, and a coverslip was applied with VectaShield mounting medium. Cells were imaged on a Nikon E600 epifluorescence microscope at 20x magnification.

TTBS: 0.1M Tris-HCl, pH 7.5; 0.15M NaCl; 0.1% Triton X 100.

Mutagenesis

Site directed mutagenesis was conducted using the QuickChange system (Stratagene). Custom oligonucleotides for single codon mutations were constructed according to the manufacturer’s suggestions. Complementary oligonucleotides generally overlapped the mutation by 10-15 nt 5' and 20 nt 3'; were 40-50%GC and had approximate melting temperatures of 78 °C. Forward and reverse oligos were added to plasmid DNA in the following reaction: 100ng plasmid, 125ng forward and reverse mutagenic primers, 5uL 10x reaction buffer, 1uL dNTP mix, ddH2O to 50 uL, and 1uL Pfu Turbo. The primers used were as follows:

R51E  5'-ccaggttaaccttgagggcatccccgctgggctc-3'
      5'-gagccgcagcgaattgctcggaagtaagccctgg-3'

F87N  5'-atgtcatctagactctcttgtgataattcgatctccaaagctgtgg-3'
      5'-ccacagctttggaatttgcaggtcatagatgacat-3'

F87A  5'-atgtcatctagactctctgttcgtacactttccaagctgtgg-3'
      5'-ccacagctttggaatttgcaggtcatagatgacat-3'

F87Y  5'-gtcatctagactctctgttcgtacactttccaagctgtgg-3'
      5'-ccacagctttggaatttgcaggtcatagatgacat-3'

S127A 5'-gctagtaagcagtccgcatctcgtggtcctgg-3'
      5'-ccagaaagcagtcctcgtacatgac-3'

S146T 5'-ctattttactgacacaggtctcagctatgtct-3'
      5'-agcatgagcagactgtaagtcggagtaaagatag-3'

E148A 5'-ccacaagtcagttacgcctcagctctcgtg-3'
      5'-aagcagcagcagagactgtaacctcgtg-3'

E148T 5'-tttcacagagacagctctcagctctcgtg-3'
5'-cagaacggagcatgaggacgtgtaactgacttgtgggaaa-3'

E148C 5'-tatttccacaagtcagttacgtctcttgctctggtaactgactgttttggagaat-3'
5'-ttccagaacggagcatgaggacgtgtaactgactgttttggagaat-3'

S150A 5'-gtcagttacgagtccgcatgctctttgttggcatactgactggt-3'
5'-ccagaacggagcatgaggacgtgtaactgactgtttggaat-3'

S150T 5'-caagtctgagttacgtctttggtggcatactgactgtttggaat-3'
5'-ctttccagaacggagcatgaggacgtgtaactgactgtttggaat-3'

E208A 5'-ctaaaagagaaggggaggtttgtggtggaagaacgaggag-3'
5'-ctgtctttccaccacccgcttaaacctcttaatg-3'

E208L 5'-ggaaataccggcatattaagggaaaaggtgttgggagaagcaggag-3'
5'-ctcctgctttccaccactgttaaacctcttaatgcccctat-3'

E208N 5'-atacggcatattaagggaaaaggtgttgggagaagcaggag-3'
5'-ctgtctttccaccacccgcttaaacctcttaatgcccctat-3'

E208AE211A 5'-ttaagagctagaagggggaggtttgtggtggaagaagcaggag-3'
5'-cacactctctgttctcaccacccgcttaaacctcttaatg-3'

E208AE212A 5'-ctaaagagaaggggaggtttgtggtggaagaagcaggag-3'
5'-ctgtctttccaccacccgcttaaacctcttaatg-3'
5'-ggaggtgtttgggagaagcaggagcaggtgtgca-3'
5'-tgacactctctgttctcaccacccgcttaaacctcttaatg-3'

E208AE211N 5'-ggaaataccggcatattaagggaaaaggtgttgggagaagcaggag-3'
5'-atgacactctctgttctcaccacccgcttaaacctcttaatgcccctat-3'

E208NE211N 5'-ggaaataccggcatattaagggaaaaggtgttgggagaagcaggag-3'
5'-atgacactctctgttctcaccacccgcttaaacctcttaatgcccctat-3'

E211A 5'-ttaagagctagaagggggaggtttgtggtggaagaagcaggag-3'
5'-gcacactctctgttctcaccacccgcttaaacctcttaatg-3'

E211N 5'-gaggtttagaggtgtgttgggagaagcaggagcaggtgtgcatat-3'
5'-atgacactctctgttctcaccacccgcttaaacctcttaatg-3'

E211AE212A 5'-gttaagagctagaagggggaggtttgtggtggaagaagcaggag-3'
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<td>5'-atatgcaacatctctgcatttccaccacctctttaaaacct-3'</td>
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<td>5'-tgcaacactctctgtgctgctccacccctcc-3'</td>
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</tr>
<tr>
<td></td>
<td>5'-ccttatagacgtgtatgttatccctatcctgcgactac-3'</td>
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</tbody>
</table>

PCR reactions were conducted according to the manufacturer’s instructions, and 1uL DpnI (10u) was added to the reactions and incubated for 1 hour at 37 °C. Then, 1uL of the reaction was used to transform 50uL of supercompetent XL1 Blue cells (Stratagene). After 30’ preincubation with DNA on ice in Falcon 2059 tubes, reactions were heat shocked for 45s at 42 °C. Finally, 500 uL of SOC media was added and the
transformations were shaken at 37 °C for an additional hour prior to plating. Individual colonies were grown and miniprepped (Qiagen) prior to confirmation by sequencing.

**HEK cell calcium imaging**

All experiments were performed on HEK 293 cells plated on poly-D-lysine coated glass cover slips in 35mm tissue culture dishes, and transfected with 3µg of a FLAG-tagged CMV1 vector containing the OlfCc1 coding sequence or mutated versions of the OlfCc1 coding sequence. After 24 hours of growth, cells were incubated in 1mM Calcium Imaging Buffer (121 mM NaCl, 6 mM NaHCO3, 5.4 mM KCl, 5.5 mM D-glucose, 0.8 mM MgCl2, 25 mM HEPES, 1 mM CaCl2, pH 7.4) and 2µM Fluo-4, a calcium-sensitive dye. Cells were imaged on a Nikon inverted microscope using Imaging Workbench v.6 (INDEC BioSystems), which measures total fluorescent response and baseline-subtracted fluorescent response (DeltaF). The protocol used for calcium imaging is the same as that reported in Chapter 2.

For dose response curves measuring increasing concentrations of amino acids, 99.9% pure amino acids (SIGMA chemicals), were added to 10mM Calcium Imaging Buffer to obtain the appropriate dilution, and vortexed to mix. Additional chemicals tested include isoamylamine (Cole Palmer), 2-methylbutylamine (Sigma), histamine (Sigma), phenylethylamine (Sigma), lanthanum (Bautista Lab), and terbium (Sigma). Solutions were added to live cells in imaging dishes via a customized profusion/vacuum system, and fluorescent responses were recorded with a CCD camera. Error bars represent Standard Error, and were calculated between separate cell populations, with each cell population encompassing between 12 cells. Each figure represents data from 4 different cell populations, recorded from separate dishes of transfected cells. Cells are marked as responding when a peak of fluorescence change is three or more times average baseline variation, as calculated from a control experiment using incubating 1mM Calcium Imaging Buffer in each profusion channel for the entire length of the experiment. For experiments with amines, response to isoleucine was used to identify transfected cells.

Dose response curves measuring increasing concentrations of calcium were obtained using fixed-volume solutions of increasingly concentrated Calcium Imaging Buffer, with the same molar concentration of amino acid. In this case, all cells responding in 20mM calcium were isolated and taken to represent 100% response, and the percentage of these cells responding at lower calcium concentrations were recorded as a normalized percentage of responding cells. Error bars represent Standard Error, and were calculated between separate cell populations, with each cell population encompassing 12 cells. Each figure represents data from 4 different cell populations, recorded from separate dishes of transfected cells. Cells are marked as responding when a peak of fluorescence change is three or more times average baseline variation, as calculated from a control experiment using incubating 1mM Calcium Imaging Buffer in each profusion channel for the entire length of the experiment.

**Data Analysis**

Data were exported from Advanced Imaging Workbench (AIW) 5.1 software as Excel spreadsheets (Microsoft) with background-subtracted and raw data. Data were then thresholded to control responses and processed to generate Delta F/F values for
fluorescence intensity from responses within the recorded time period after the addition of a new stimulus. These values were recorded and calculated for each individual cell, represented as a single user-selected region of interest during recording. Delta F/F values were averaged across a single field of view and a single experiment, from which standard error values were also calculated, and response curves were calculated for each experiment separately. Average EC50s were obtained from these response curves, as well as the standard error of the EC50. Software used to plot response curves and calculate statistical analyses was GraphPad Prism v.5.0c. (GraphPad Software). Student T-tests and ANOVA tests were used to determine significance between mutants; statistical significance was set at p<0.05.
Figure 3-1: Alignment of Family C receptors, including OlfCc1, used to construct homology model. Alpha helices are colored in red, beta sheets are colored in green, and predicted homology regions are indicated above. X markings denote residues predicted to bind the glycine moiety of the ligand and the distal portion of the ligand, as predicted from the binding pocket of mGluR.
Figure 3-2: Isoleucine (top) and leucine (bottom) docked in the binding pocket of OlfCc1. Residues thought to directly contact isoleucine are shown in orange, with space-filling spheres. In the space-filling model below, leucine is shown in the binding pocket of OlfCc1, surrounded by residues proposed to interact with the ligand. Putative hydrogen bonds are shown as dashed green lines, carbon atoms are represented in green, hydrogen atoms are represented in white, nitrogen atoms are represented in blue, and oxygen atoms are represented in red.
Figure 3-3: Space-filling models of the OlfCc1 and V2R2 binding pockets docked with isoleucine. In purple is a space-filling representation of the size of the binding pocket after docking of the ligand. The V2R2 binding pocket is predicted to be larger than that of OlfCc1. Orientation here shows the proximal pocket to the left and the distal pocket to the right, with lobe 1 on the top and lobe 2 on the bottom.
Figure 3-4: Homology model of isoleucine docked in the binding pocket of OlfCc1. The top figure shows the proximal and distal residues of the pocket in relation to isoleucine and calcium. The bottom figure shows two rotated views of the binding pocket, with lobe 1 above lobe 2. Alpha helices are colored red, and beta sheets are colored blue. In the amino acids shown, hydrogen molecules are white, oxygen molecules are blue, and carbon molecules are red.
Figure 3-5: Proximal and distal residues in the OlfCc1 binding pocket. The residues shown here are grouped according to their placement in the binding pocket and their predicted contact with either the distal or the proximal portion of the ligand; isoleucine is shown here. Predicted hydrogen bonds are shown in dashed green lines. Oxygen molecules are in red, hydrogen molecules are in white, and nitrogen molecules are in blue for isoleucine; for other amino acids shown, sulfur molecules are in yellow, nitrogen molecules are in blue, and oxygen molecules are in red.
Figure 3-6: Homology model showing the S127A mutation, and graphs of the responses of this mutation to isoleucine, leucine, valine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-7: Predicted calcium-binding sites of OlfCc1. Calcium site 2, in purple, was mutated and demonstrated calcium sensitivity. Calcium site 1 includes residues previously mutated, but had ambiguous effects on calcium sensitivity.
Figure 3-8: Calcium coordination by residues in Calcium site 1. The carboxyl group of the ligand may help to stabilize the calcium ion in this position.
Figure 3-9: Homology model showing the locations of the three glutamates E208, E211, and E212, and graphs showing the responses of mutants involving these residues to calcium in the presence of 250 uM isoleucine.
Figure 3-10: Homology model showing the E148T mutation, and graphs of the responses of this mutation to isoleucine, leucine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-11: Homology model showing the E148C mutation, and graphs of the responses of this mutation to isoleucine, leucine, histidine, phenylalanine, 2-methylbutylamine, isoamylamine, and phenylethylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-12: Homology model showing the S150A mutation, and graphs of the responses of this mutation to isoleucine, leucine, valine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
**Figure 3-13:** Homology model showing the Y198A mutation, and graphs of the responses of this mutation to isoleucine, leucine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-14: Homology model showing the S146T mutation, and graphs of the responses of this mutation to isoleucine, leucine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
**Figure 3-15**: Homology model showing the E148A mutation, and graphs of the responses of this mutation to isoleucine, leucine, histidine, phenylalanine, 2-methylbutylamine, isoamylamine, and phenylethylamine in the presence of 10 mM calcium; and calcium in the presence of 250 µM isoleucine.
Figure 3-16: Homology model showing the F87NE148A mutation, and graphs of the responses of this mutant to isoleucine, leucine, valine, histidine, phenylalanine, glutamate, 2-methylbutylamine, isoamylamine, and phenylethylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
**Figure 3-17**: Homology model showing the I371G mutation; this mutant did not respond to known ligands of OlfCc1 including isoleucine, leucine, valine, or calcium, but did express at the surface of HEK 293 cells.
Figure 3-18: Homology model showing the R370G mutation; this mutant did not respond to known ligands of OlfCc1 including isoleucine, leucine, valine, or calcium, but did express at the surface of HEK 293 cells.
Figure 3-19: Homology model showing the L369G mutation; this mutant did not respond to known ligands of OlfCc1 including isoleucine, leucine, valine, or calcium, but did express at the surface of HEK 293 cells.
Figure 3-20: Homology model showing the I371A mutation, and graphs of the responses of this mutation to isoleucine, leucine, valine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-21: Homology model showing the F87A mutation; this mutant did not respond to known ligands of OlfCc1 including isoleucine, leucine, valine, or calcium, but did express at the surface of HEK 293 cells.
Figure 3-22

**Figure 3-22**: Homology model showing the F87Y mutation, and graphs of the responses of this mutation to isoleucine, leucine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-23: Homology model showing the A278L mutation, and graphs of the responses of this mutation to isoleucine, leucine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-24: Homology model showing the A278T mutation, and graphs of the responses of this mutation to isoleucine, leucine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-25: Homology model showing the A278K mutation, and graphs of the responses of this mutation to isoleucine, leucine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
**Figure 3-26:** Homology model showing the A278G mutation, and graphs of the responses of this mutation to isoleucine, leucine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
**Figure 3-27**: Homology model showing the F87N mutation, and graphs of the responses of this mutation to isoleucine, leucine, valine, histidine, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-28: Homology model showing the R51E mutation, and graphs of the responses of this mutation to isoleucine, leucine, valine, phenylalanine, 2-methylbutylamine, isoamylamine, and phenylethylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-29: Homology model showing the D253G mutation, and graphs of the responses of this mutation to isoleucine, leucine, valine, phenylalanine, 2-methylbutylamine, isoamylamine, and phenylethylamine in the presence of 10 mM calcium; and calcium in the presence of 250 μM isoleucine.
Figure 3-30: Homology model showing the S252T mutation, and graphs of the responses of this mutation to isoleucine, leucine, valine, histidine, glutamate, 2-methylbutylamine, and isoamylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-31: Homology model showing the F87NS252T mutation, and graphs of the responses of this mutant to isoleucine, leucine, valine, histidine, phenylalanine, glutamate, 2-methylbutylamine, isoamylamine, and phenylethylamine in the presence of 10 mM calcium; and calcium in the presence of 250 uM isoleucine.
Figure 3-32: Binding pocket residues of OlfCc1 shown with their proposed connections to isoleucine. Putative hydrogen bonds are shown in dashed green lines, and the residues in orange are proposed to limit the size of ligand accommodated within the binding pocket.
Figure 3-33: Docking of isoleucine in the OlfCc1 binding pocket. Residues in purple restrict the size of the binding pocket. The space-filling representation in grey indicates the contacts between E148 and I371, which create a hydrophobic pocket.
Figure 3-34: Isoleucine docked in the OlfCc1 binding pocket. Residues in orange make hydrophobic contact with the ligand.
Figure 3-35: Isoleucine docked in the OlfCc1 binding pocket after MD minimization. Residues in orange make hydrophobic contact with the ligand.
Figure 3-36: Isoleucine and residues of the OlfCc1 binding pocket, showing hydrophobic contacts and the predicted interaction between E148 and R370.
**Figure 3-37:** Isoleucine docked in the OlfCc1 binding pocket showing residues from Lobe 1 making hydrophobic contact. The amine group of the ligand is shown here making three putative hydrogen bonds with E148, underscoring the importance of this residue in stabilizing amino acid ligands.
Table 3-1: Statistics of distal pocket mutants. This table shows the EC50s, peak responses, n values, and statistical significances of distal pocket mutants discussed in this chapter.
### Table 3-2: Statistics of proximal pocket mutants

This table shows the EC50s, peak responses, n values, and statistical significances of proximal pocket mutants discussed in this chapter.

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<tr>
<th>Indicateur</th>
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<th>n</th>
<th>SEM</th>
<th>Sign from WT</th>
<th>EC50 (µM)</th>
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<td>10</td>
<td>10</td>
<td>5 &lt;p&gt;</td>
<td>2.6</td>
<td>18</td>
<td>18</td>
<td>5 &lt;p&gt;</td>
</tr>
<tr>
<td>S527A</td>
<td>4.5</td>
<td>42</td>
<td>42</td>
<td>5 &lt;p&gt;</td>
<td>2.6</td>
<td>18</td>
<td>18</td>
<td>5 &lt;p&gt;</td>
</tr>
<tr>
<td>S530A</td>
<td>3.7</td>
<td>66</td>
<td>66</td>
<td>5 &lt;p&gt;</td>
<td>3.1</td>
<td>35</td>
<td>35</td>
<td>5 &lt;p&gt;</td>
</tr>
<tr>
<td>S447T</td>
<td>4.4</td>
<td>16</td>
<td>16</td>
<td>5 &lt;p&gt;</td>
<td>3.2</td>
<td>20</td>
<td>20</td>
<td>5 &lt;p&gt;</td>
</tr>
<tr>
<td>Y358A</td>
<td>4.1</td>
<td>61</td>
<td>61</td>
<td>5 &lt;p&gt;</td>
<td>3.7</td>
<td>70</td>
<td>70</td>
<td>5 &lt;p&gt;</td>
</tr>
<tr>
<td>E148T</td>
<td>4.1</td>
<td>12</td>
<td>12</td>
<td>5 &lt;p&gt;</td>
<td>2.9</td>
<td>16</td>
<td>16</td>
<td>5 &lt;p&gt;</td>
</tr>
<tr>
<td>E148A</td>
<td>3.9</td>
<td>12</td>
<td>12</td>
<td>5 &lt;p&gt;</td>
<td>2.5</td>
<td>15</td>
<td>15</td>
<td>5 &lt;p&gt;</td>
</tr>
<tr>
<td>S530T</td>
<td>4.14</td>
<td>16</td>
<td>16</td>
<td>5 &lt;p&gt;</td>
<td>2.6</td>
<td>16</td>
<td>16</td>
<td>5 &lt;p&gt;</td>
</tr>
<tr>
<td>E148C</td>
<td>4.5</td>
<td>15</td>
<td>15</td>
<td>5 &lt;p&gt;</td>
<td>2.6</td>
<td>8</td>
<td>8</td>
<td>5 &lt;p&gt;</td>
</tr>
<tr>
<td>FBP6148A</td>
<td>4.6</td>
<td>7</td>
<td>7</td>
<td>5 &lt;p&gt;</td>
<td>2.3</td>
<td>4</td>
<td>4</td>
<td>5 &lt;p&gt;</td>
</tr>
</tbody>
</table>

**Table 3-2**: Statistics of proximal pocket mutants. This table shows the EC50s, peak responses, n values, and statistical significances of proximal pocket mutants discussed in this chapter.
Table 3-3: Statistics of calcium binding site mutants. This table shows the EC50s, peak responses, n values, and statistical significances of calcium binding site mutants discussed in this chapter.

<table>
<thead>
<tr>
<th>Position</th>
<th>Calcium with 50 MIBK</th>
<th>Max. Amp. (E50)</th>
<th>n</th>
<th>SEM</th>
<th>Sig. from WT</th>
<th>Calcium with 50 MIBK</th>
<th>Max. Amp. (E50)</th>
<th>n</th>
<th>SEM</th>
<th>Sig. from WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>E208A</td>
<td>5.3 ± 3.436 ns</td>
<td>30.6 ± 3.436 ns</td>
<td>4</td>
<td>3</td>
<td>0.39 ± 0.039</td>
<td>5.3 ± 3.436 ns</td>
<td>30.6 ± 3.436 ns</td>
<td>4</td>
<td>3</td>
<td>0.39 ± 0.039</td>
</tr>
<tr>
<td>E211N</td>
<td>4.0 ± 2.347 ns</td>
<td>48.5 ± 2.347 ns</td>
<td>4</td>
<td>5</td>
<td>0.55 ± 0.255</td>
<td>4.0 ± 2.347 ns</td>
<td>48.5 ± 2.347 ns</td>
<td>4</td>
<td>5</td>
<td>0.55 ± 0.255</td>
</tr>
<tr>
<td>E212N</td>
<td>4.0 ± 2.347 ns</td>
<td>48.5 ± 2.347 ns</td>
<td>4</td>
<td>5</td>
<td>0.55 ± 0.255</td>
<td>4.0 ± 2.347 ns</td>
<td>48.5 ± 2.347 ns</td>
<td>4</td>
<td>5</td>
<td>0.55 ± 0.255</td>
</tr>
<tr>
<td>E213N</td>
<td>4.0 ± 2.347 ns</td>
<td>48.5 ± 2.347 ns</td>
<td>4</td>
<td>5</td>
<td>0.55 ± 0.255</td>
<td>4.0 ± 2.347 ns</td>
<td>48.5 ± 2.347 ns</td>
<td>4</td>
<td>5</td>
<td>0.55 ± 0.255</td>
</tr>
</tbody>
</table>

Note: The table should be read as follows:
- The first column represents the position of the mutants.
- The second column represents the calcium with 50 MIBK.
- The third column represents the Max. Amp. (E50) for each position.
- The fourth column represents the SEM for each position.
- The fifth column represents the Sig. from WT for each position.
- The last two columns show similar data for calcium with 50 MIBK.
Chapter 4: A Microfluidic Platform for Olfactory Sensing Applications

Introduction

Microfluidic devices are used as sensing systems in the fields of health and medical testing, to detect volatile biomarkers in human bodily fluids; biochemical defense, to detect weapons signatures and analytes indicative of bioterrorism; in environmental science, to detect pesticides and determine water, air, and food quality; and in basic science, to perform benchtop sample diagnostics. Sensors based on the olfactory system attempt to replicate the breadth and sensitivity of olfactory receptors using technologies including gas chromatography, quartz crystal microbalances, surface plasmon resonance, and antibody detection (Du, Wu, Liu, & Wang, 2012a; Du, Wu, Peng, Zhao, & Wang, 2012b; S. H. Lee, Jun, Ko, Kim, & Park, 2009; Q. Liu et al., 2012; Sankaran, Panigrahi, & Mallik, 2011; C. Wu, Du, Di Wang, Zhao, & Wang, 2011). However, these techniques are complex and can be difficult to use outside of a laboratory setting, or without substantial machinery outside of the sensing device. Additionally, scaling the responses of these devices ultimately depends on building a library of sensors that can provide combinatorial responses, much like the olfactory system.

Sensors that use olfactory receptors as sensing elements have been built to address the detection of individual odorants of interest (Far, Flitti, Guo, & Bermak, n.d.; Mastromatteo & Villa, 2012; J. Park et al., 2012; Wilson & Baietto, 2011). As the majority of olfactory receptor-ligand interactions have not yet been characterized, it has been difficult to produce a sensor capable of mimicking even a fraction of native olfactory response. The work presented in the main chapters of this thesis detail a process that can be used to de-orphan an olfactory receptor and tune its responsiveness for better distinguishability and coverage of a larger set of identifiable odorants. The next step for a set of receptors with distinguishable responses to a group of odorants of interest is installation of the receptors in a platform from which their responses can be easily detected and interpreted. Such a platform should be easy to use without external equipment or microscopes. It should use a small sample volume, and require minimal assay time. It should be specific and sensitive, and ideally would provide visible light output that can be interpreted by the human eye. The device itself should be capable of cell and receptor containment, ligand flowthrough, and visual readout upon ligand binding. (See Table 4-2).

A number of assays exist that use visible light or fluorescence to report activation of components involved in the olfactory receptor signaling cascade. A Cre-luciferase construct uses a cAMP responsive element to drive production of luciferase within a cell, with increases in luciferase production driven by the increase in soluble cAMP observed after ligand binding and activation of an olfactory receptor (Katada et al., 2003). A beta-arrestin GFP protein conjugate can also be transfected into cells and activated when the cell recycles an activated receptor (Barak, Ferguson, Zhang, & Caron, 1997). Arrestin binds to a receptor after it has been activated, blocking G protein signaling and targeting the receptor for internalization (Atwood, Lopez, Wager-Miller, Mackie, & Straiker, 2011). Increased GFP fluorescence at the cell membrane would indicate arrestin localization to an activated receptor, a means of determining receptor activation in real-time. Either of these assays is compatible with a device holding single cells transfected.
with a receptor of interest and retaining their native machinery to respond to receptor activation through a cAMP-mediated signal transduction cascade.

Results

A microfluidic device suitable for maintaining live cells expressing olfactory receptor variants was designed and fabricated. (See Figure 4-1). The device was poly-D-lysine coated and seeded with HEK 293 and A549 cells (an epithelial cell line) as proof of principle for their ability to contain cells. The device was fabricated such that eight individual populations of cells with eight separate olfactory receptors could be maintained separately, allowing for combinatorial responses from eight receptors to be measured. (See Table 4-1). The device is transparent and can be imaged on a microscope or observed by eye to measure either bioluminescent or fluorescent output from one of the assays mentioned previously.

The size parameters of the device fabricated and pictured herein included eight wells 2 mm in diameter, baffles between wells spaced 10 microns apart, channels between wells 3.5 mm in length, and a linear design (where ligand must be flowed to each well independently and simultaneously. (See Figure 4-1). These parameters were justified by practical considerations including the size of HEK cells and the development of laminar flow. An average HEK cell has a 375 square micron footprint, meaning that each well can accommodate 8,000 cells, with approximately 100 copies of receptor expressed per cell (Atwood et al., 2011). Baffles were chosen to prevent HEK cells from moving between baffles during washing or flow-through. Channels were chosen to be much longer than the length required to establish laminar flow, and could be shortened for multiplexing, but also establish a density filter such that contaminating particulates of an input solution will sink to the bottom of the channel before reaching a well. A linear design was chosen as opposed to the more popular “fan” designs that permit many more wells per chip because fan designs create an artificial size gradient for ligands and would increase the assay time (K. Liu, Wu, Chuang, Khoo, & Huang, 2010).

A flow-rate of 1 uL/min did not disturb cells that had been seeded in the wells of the device, indicating that cells could be fed within the device and that ligands could be added and removed without dissociating cells already seeded onto the device. The baffles prevented cells from leaving the well, but did not impede the flow of media, and should permit the introduction of ligands smaller than 10 microns in diameter. The hydraulic resistance of the channels between inlet ports and between wells was calculated by \( \frac{(12*\mu*L)}{(w*h^3*(1.630(h/w)))} \), which resulted in Poiselle flow. The Reynold’s number for cells in the device was .001; cells experienced a characteristic settling time of time \( \tau = 4.5\eta / (\rho a^2) \); where \( a = 10\mu m \) and \( \rho = 5, \tau = 5 \times 10^{-6} \) seconds. The cells experienced .9pN of drag force (Stokes’ drag given as \( F = -6\pi\eta vr \)), and shear stress of .01N/m\(^2\) to .001N/m\(^2\) (Shear stress given as \( \mu (du/dy) \)). These stresses are negligible to the cell and would not result in shearing of extracellular domains of receptors. The entrance length required to develop a laminar flow profile for any ligand entering the device is given as \( L_e = \frac{D}{0.379e^{-0.148Re} + 0.0550Re + 0.260} \) resulting in a length for this device of 66.1 microns. All measurement wells are at least 1 mm away from inlets and separated by at least 1 mm from one another, indicating that laminar flow can be established.
throughout the device. The device has 3.9 cubic microliters of flow-through room for a ligand solution \((9 \times 1 \times .150) + (\pi \times 8 \times .150)\). In each .5 cubic microliter of sample, the device would require at least 1ppm analyte = 1mg/L = 1x10^{-3} ug to be effective. If a receptor can detect micromolar concentrations, then a .5 picomolar concentration of ligand per well would be necessary for detection, which in the case of an amino acid is approximately 1x10^{-4} ug. This represents the detection limit for the device described, with its size parameters as given.

**Discussion**

The device described in this section is theoretically sufficient for use as a bioluminescent sensing system capable of detecting responses from up to eight olfactory receptor variants. Using the olfactory receptor mutations described in Chapter 3 and previously identified amino acid-sensing receptors such as 5.24, Z06, and mGluR, the device should be capable of identifying and discriminating between sixteen amino acids. Further experiments are required to determine the signal-to-noise ratio of the luciferase reporting system, and the amount of time necessary for the assay to provide distinguishable responses that can be read by the human eye. Previous work using the same assay has shown that times of 8 to 24 hours are sufficient for a visible signal, and 24 to 48 hours for the development of a complete response. To be useful in a field setting, these responses are sufficient, but further work focused on shortening the responses while maintaining their accuracy would be of great interest.

The general utility of an olfactory receptor-based microfluidic sensing device with output that can be read visually spans medical and scientific fields that are interested in olfactory or GPCR-based signaling. The particular utility of an amino acid-sensing device is in the fields of environmental sensing and medical sensing. Elevated levels of amino acids in natural water samples can be indicative of the presence and relative quantities of bacterial and algal populations (Henderson & Snell, 1948), (Crawford, Hobbie, & Webb, 1974). In the healthcare setting, amino acid screens are routinely performed on newborns to test for healthy levels of amino acids in blood. Amino acidopathies can be detected through this test, which is currently performed by chromatography, as well as specific diseases such as eclampsia, kidney failure, ketoacidosis, Huntington’s chorea, nephrotic syndrome, and rheumatoid arthritis, among others (Britz-McKibbin, 2013; C. Wang et al., 2013; Zytkovicz et al., 2001). Of particular interest for the receptor studied herein, elevated levels of branched chain amino acids leucine and valine are indicative of a condition called maple syrup urine disorder, and testing for levels of these amino acids is used to diagnose this disorder in newborns. The disorder is very dangerous and can lead to toxic encephalopathy and death within seven days of birth if not properly treated (Knerr, Weinhold, Vockley, & Gibson, 2012). Currently, blood is used to detect plasma amino acid levels in newborns, but urinary testing of amino acids is also used in adults to detect metabolic, nutritional, and neurological disorders (Zytkovicz et al., 2001). The use of a microfluidic device to perform such a test would decrease the sample volume needed, increase the number of tests that could be performed in a 24-hour time period, and move the testing procedure from the clinical laboratory bench to the bedside, where a trained clinician could read test results directly from the device. Further investigation in these areas and in the development of such a diagnostic tool is of great interest.
Materials and Methods

AutoCAD Design

A mask design for the device was drawn in AutoCAD and printed at Fineline Imaging (Colorado). The mask design is 30 mm long by 15 mm tall, and contains 8 wells 2 mm in diameter separated by 1 mm long channels between each well. The channels are connected to inlet and outlet ports by 4 mm long channels. Baffles within the channels are 10 microns apart to prevent cells from detaching and leaving their wells, as the average cell diameter for either HEK 293 cells or A549 cells is 15-20 microns. The smallest feature size for the AutoCAD document was 10 microns.

Soft Lithography

Traditional soft lithography was performed with PDMS as described previously. Briefly, photoresist was spin-coated onto silicon wafers, which were heat-treated and then exposed to UV radiation through the mask design to cross-link photo resist in unmasked regions. Non-cross-linked photoresist was washed off, and cross-linked resist was developed to form a relief pattern of the mask design. PDMS (Sylgard 184) (10:1, pre-polymer:curing agent) was degassed and dispensed onto the master, and cured for 1 hour at 80 °C. The device was then removed from the wafer, cut to size, and adhered to a plasma ionized and cleaned glass coverslip. Holes for the inlet and outlet ports and the cell loading ports were drilled with a 16 G syringe needle tip.

Cell Culture and Imaging

A549 epithelial cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum. Cells were dissociated with PBS and injected into the PDMS device via gravity-driven flow. Cell loading and settling in the device were imaged on an inverted light microscope (Nikon) at 10x magnification.
**Figure 4-1**: AutoCAD design of the proposed device with selected features, and fabricated device with cells loaded into each of the wells. On the left are the AutoCAD drawings of the device design, showing the location of cell wells, the flow of analyte into the wells, and the baffles designed to trap cells within the wells. On the right is a fabricated PDMS device with A549 cells loaded into each of the wells.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>(NMDA: N-methyl-D-aspartic acid receptor)</td>
</tr>
<tr>
<td>Arginine, Histidine, Lysine, Glutamine, Glutamate</td>
<td>5.24 and mutant 5.24 (basic/neutral amino acids)</td>
</tr>
<tr>
<td>Serine</td>
<td>(GlyR: glycine receptor)</td>
</tr>
<tr>
<td>Glutamate</td>
<td>MGluR: metabotropic glutamate receptor</td>
</tr>
<tr>
<td>Cysteine</td>
<td>CCR7</td>
</tr>
<tr>
<td>Glycine</td>
<td>(GlyR: glycine receptor), mutated 5.24</td>
</tr>
<tr>
<td>Alanine</td>
<td>L-alanine Taste Receptor</td>
</tr>
<tr>
<td>Isoleucine, Leucine, Valine</td>
<td>35/V2R2</td>
</tr>
<tr>
<td>Methionine</td>
<td>Muscarinic receptor</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>(TRP-R)</td>
</tr>
</tbody>
</table>

**Table 4-1**: Amino acids recognized by currently available receptors. This table demonstrates the feasibility of detecting amino acids by a subset of receptors whose functionalities and responsive characteristics are known.
Table 4-2

<table>
<thead>
<tr>
<th>Olfactory Receptors</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection threshold at 1ppt (part per trillion); average on the order of .01 ppm.</td>
<td>Detection threshold at .1ppm (part per million)</td>
</tr>
<tr>
<td>Specificity allows resolution of enantiomers (R- and L-carvone)</td>
<td>Specificity allows resolution of enantiomers (alpha- and beta-estradiol)</td>
</tr>
<tr>
<td>Can report binding automatically intracellularly</td>
<td>Requires secondary treatment to determine binding (fluorophore conjugation)</td>
</tr>
<tr>
<td>Binding specificity built-in, but can be fine-tuned.</td>
<td>Binding specificity assured, tunable</td>
</tr>
</tbody>
</table>

Table 4-2: Comparison of the properties of olfactory receptors and antibodies with respect to their suitability for a microfluidic detection device. Olfactory receptor detection thresholds surpass those of antibodies generally; olfactory receptors and antibodies have comparable specificities, and olfactory receptors are capable of reporting binding without secondary treatment in a properly established cell system containing a bioluminescent reporter gene.


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doi:10.1186/1471-2164-7-309


doi:10.1186/1471-2164-12-14


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