Explorations in Olfactory Receptor Structure and Function

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Neurobiology in the Graduate School of Duke University

2014
ABSTRACT

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Abstract

Olfaction is one of the most primitive of our senses, and the olfactory receptors that mediate this very important chemical sense comprise the largest family of genes in the mammalian genome. It is therefore surprising that we understand so little of how olfactory receptors work. In particular we have a poor idea of what chemicals are detected by most of the olfactory receptors in the genome, and for those receptors which we have paired with ligands, we know relatively little about how the structure of these ligands can either activate or inhibit the activation of these receptors. Furthermore the large repertoire of olfactory receptors, which belong to the G protein coupled receptor (GPCR) superfamily, can serve as a model to contribute to our broader understanding of GPCR-ligand binding, especially since GPCRs are important pharmaceutical targets.

In this dissertation, I explore the relationship between olfactory receptors and their ligands, both by manipulating the ligands presented to the olfactory receptors, as well as by altering the structure of the receptor itself by mutagenesis. Here we report the probable requirement of a hydrated germinal-diol form of octanal for activation of the rodent OR-I7 receptor by ligand manipulation, and the successful in vitro modeling and manipulation of ketamine binding to MOR136-1. We also report the results of a large-scale screen of 1190 human and mouse olfactory receptors for receptors activated by volatile general anesthetics, which has lead to the identification of 34 olfactory receptor-volatile general anesthetic pairs.
To the memory of my Gong Gong and Ah Po
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1. Introduction

1.1 What is Olfaction

Olfaction, put simply, is the sense of smell – the ability of organisms to detect chemical cues in the environment around them, so that they can respond appropriately. Tracing a sketch of the evolutionary origins of olfaction, we go from the terrestrial organisms smelling odors in the air, to aquatic organisms detecting aqueous compounds in the water they live in, all the way to the single-celled bacteria moving in response to sensing sugar derivatives (Adler, Hazelbauer, & Dahl, 1973). In this way, olfaction is one of the most primitive, and arguably one of the most important of the senses, since it is vital to a diverse range of fundamental biological processes, which include recognition and appropriate responses to food sources, predators, noxious/dangerous chemicals, and even identification of kin. Its relevance to the human condition cannot be understated, since among other things olfaction is critical to the ability of disease-carrying mosquitoes to sense and track humans, in our ability to associate smells with memories and places, and in our perception and enjoyment of our food.

1.2 Mammalian Main Olfactory System

The peripheral components of the mammalian main olfactory system are illustrated in Figure 1. The main olfactory system is made up of ~6-10 million olfactory sensory neurons (OSNs) distributed throughout the olfactory epithelium (OE), usually located in the dorsal regions of the nasal cavity (Firestein, 2001). Each of these neurons typically express only one kind of olfactory receptor (OR) in a monallelic manner (Chess, Simon, Cedar, & Axel, 1994), and their axons pass through the skull to reach the main olfactory bulb.
(MOB), where axons of OSNs expressing the same OR converge into discrete glomeruli (Mombaerts et al., 1996). The circuitry of the MOB is still not well understood, but in general each glomerulus will signal to higher brain regions via excitatory projection neurons called mitral/tufted cells, which appear wired to one glomerulus (Firestein, 2001; Mori, Nagao, & Yoshihara, 1999).

Figure 1: Odorant Receptors and the Organization of the Olfactory System
Small, volatile odor molecules are drawn into the nasal cavity by sniffing, where they reach the (OE). Here they bind to and activate ORs on the cilia of OSNs (1) and depolarize them, sending electrical signals (2) that converge in glomeruli (3) and are transmitted to higher brain regions (4) such as the olfactory cortex. Illustration adapted from the press release for the 2004 Nobel Prize in Physiology or Medicine, Nobel Media AB, 2004.
Odors and mixtures of odors activate distinct subsets of these glomeruli, forming a stereotyped odor map that appears stable and repeatable for each odor (Leon & Johnson, 2003; Mori, Takahashi, Igarashi, & Yamaguchi, 2006; Takeuchi & Sakano, 2014; Xu, Greer, & Shepherd, 2000), but are variable between individuals and which appears to change when odor concentrations are varied (Oka et al., 2006).

1.3 Olfactory Receptors (ORs)

While higher regions of the brain are important in processing incoming odor information, it would appear that one of the keys to understanding olfaction lies with decoding the initial step of olfaction, specifically the function of ORs and how these receptors detect and discriminate between odor compounds, in order to understand the formation of an olfactory percept.

Early attempts to explain the molecular mechanisms underlying this complex system of olfactory detection came in the form of the stereochemical theory of olfaction (Amoore, 1963), which, in a fashion similar to the lock-and-key model of enzymatic action, postulated that there existed many receptor sites for odorants, and that odor detection happens only when odorant molecules and the binding site structure matched. Support for this theory was strengthened in 1991, when Buck & Axel (Buck & Axel, 1991) discovered and cloned a large multigene family of receptor proteins that encode odorant receptors in rat, and estimated the number of OR genes to be ~1000. Since then, as information about more mammalian genomes has been made available, this has been proven to be correct. The most recent estimates of the numbers of functional and pseudogenized ORs are listed in Table 1, with data taken from Niimura, Matsui, & Touhara, 2014. The sheer astounding number of OR genes in the mammalian genome
render it the largest class of G protein coupled receptors (GPCRs, discussed below), and likely the largest gene family in the mammalian genome.

Table 1: Recent Estimates of Olfactory Receptor Numbers in Mammalian Genomes

<table>
<thead>
<tr>
<th>Animal</th>
<th>Intact ORs</th>
<th>Pseudogenized ORs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elephant</td>
<td>1948</td>
<td>2230</td>
</tr>
<tr>
<td>Cow</td>
<td>1186</td>
<td>1057</td>
</tr>
<tr>
<td>Dog</td>
<td>811</td>
<td>278</td>
</tr>
<tr>
<td>Horse</td>
<td>1066</td>
<td>1569</td>
</tr>
<tr>
<td>Rabbit</td>
<td>768</td>
<td>256</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>796</td>
<td>1340</td>
</tr>
<tr>
<td>Rat</td>
<td>1207</td>
<td>508</td>
</tr>
<tr>
<td>Mouse</td>
<td>1130</td>
<td>236</td>
</tr>
<tr>
<td>Marmoset</td>
<td>366</td>
<td>231</td>
</tr>
<tr>
<td>Macaque</td>
<td>309</td>
<td>280</td>
</tr>
<tr>
<td>Orangutan</td>
<td>296</td>
<td>488</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>380</td>
<td>414</td>
</tr>
<tr>
<td>Human</td>
<td>396</td>
<td>425</td>
</tr>
</tbody>
</table>

As more information about ORs and their cognate ligands have been published, it seems likely that odors are recognized by a combinatorial coding strategy (Malnic, Hirono, Sato, & Buck, 1999; Saito, Chi, Zhuang, Matsunami, & Mainland, 2009). In essence, the combinatorial coding strategy rests on the hypothesis that an OR detects one (or a limited subset) of a variety of physiochemical features of any particular odor molecule, such as functional groups, conformation, polarity, hydrophobicity, rather than the entire set of features specific to one particular molecule. This is conceptually shown in simplified form in Figure 2, the significance of which is that one OR can recognize multiple odorants, and one odorant may activate several ORs, although indeed there exist some ORs which are more narrowly-tuned specialist receptors and others which are more broadly-tuned generalist receptors (Saito et al., 2009).
Figure 2: Combinatorial Code for Odorant Recognition
Odors (left) are recognized by receptors (right) that recognize different features of the odorants. Here the features of the odorants and the receptors that are activated by them are color coded in grey, yellow, cyan and red. In this manner, the pattern of activated receptors forms a combination code that can discriminate between a larger number of odorants than are receptors. Illustration after Malnic et al., 1999.

This many-to-many relationship between odorants and receptors therefore suggests that the olfactory system is able to detect compounds that had not been previously encountered, and that it can discriminate between a much larger number of odorants than there exist receptors in the system. Indeed, the human olfactory system has most recently been calculated to be able to discriminate at least 1 trillion olfactory stimuli (Bushdid, Magnasco, Vosshall, & Keller, 2014), even though by latest estimate there are just 396 functional human ORs in our genome (Table 1, Niimura et al., 2014).
1.3.1 Olfactory Receptor Signaling

GPCRs mediate external signals in a metabotropic manner, acting thorough second messengers by interacting with a heterotrimeric G protein (guanine nucleotide-binding protein). There are three canonical G protein effector cascades, which act through three different heterotrimeric G proteins: $G_{\alpha}$, $G_{q}$, and $G_{i}$, and ORs signal through $G_{\alpha}$. A brief summary of the OR signal cascade in an OSN is shown in Figure 3.

![Figure 3: Olfactory Receptor Signaling Cascade](image)

Once an odor binds to and activates an OR, it activates the G protein with the help of RIC8 guanine nucleotide exchange factor B (Ric8b). The activated $G_{\alpha_{olf}}$ dissociates from the $\beta\gamma$ subunits and activates adenyl cyclase III (ACIII), which converts adenosine triphosphate (ATP) into the second messenger cyclic adenosine monophosphate (cAMP). In the OSN, the cAMP then binds to cyclic nucleotide gated channel alpha 2 (CNGA2) channels, which open to allow the entry of Na$^+$ and Ca$^{2+}$ ions to depolarize the OSB (Firestein, 2001; Mombaerts, 1999, 2004).
1.3.2 Structural Overview of Olfactory Receptors

ORs are members of the protein superfamily of G protein-coupled receptors (GPCRs), which all share a seven transmembrane (7TM) domain topology and whose signal transduction is mediated by a heterotrimeric G protein. In particular, ORs belong to the rhodopsin-like (Class A) GPCR family, which include light-sensing opsins, chemokine receptors, opiate receptors, lipid receptors, hormone receptors, and receptors for ubiquitous neurotransmitters like adenosine, dopamine and acetylcholine (Katritch, Cherezov, & Stevens, 2013). OR genes are typically intronless coding sequences of ~1 kilobase in length encoding proteins ~300-350 amino acids in length. ORs contain structural features that are common to GPCRs (Gat, Nekrasova, Lancet, & Natochin, 1994; Pierce, Premont, & Lefkowitz, 2002; Strader, Fong, Tota, Underwood, & Dixon, 1994; Vaidehi et al., 2002), including the 7TM domains, an n-terminal NXS/T n-terminal glycosylation site, conserved cysteines for a disulphide bridge in the first two extracellular loops, the putative G protein-binding E/DRY motif in transmembrane domain 3 (TM3), and the NPXXY motif at the cytosolic end of TM7 (Figure 4, pink circles). There are also a number of OR-specific conserved motifs, labeled in blue circles in Figure 3.

Despite the recent renaissance in the determination of GPCR structures (Katritch et al., 2013), including those of the G protein-bound β-adrenergic receptor in its active state (Rasmussen, Choi, et al., 2011; Rasmussen, DeVree, et al., 2011), crystal structures of ORs have yet to be successfully determined, thus most studies examining the relationship between OR and ligand, including this one, rely on comparative homology models to interrogate OR structure.
Figure 4: Topology of a Typical Olfactory Receptor
ORs are ~300-350 amino acid long proteins with 7 TM domains. Amino acid residues typically conserved in GPCRs are highlighted in pink, OR-specific conserved residues are highlighted in blue with their single letter amino acid codes labeled.

1.3.3 OR Classification & Nomenclature

There are a number of systems in concurrent usage for the classification and nomenclature of ORs, most of which attempt to classify ORs according to their inferred phylogenetic relationships through sequence comparisons (Dayhoff, Barker, & Hunt, 1983). These classification systems vary for different species, but generally take the hierarchical order of families, subfamilies and members. The nomenclature used in this dissertation for human ORs follows the ORnXm format, where n is the Arabic numeral of the 18 human OR families, X being the alphabetical designation for the 300 subfamilies,
and the final m being the Arabic numeral for the member number (Olender, Lancet, & Nebert, 2008). For example, OR51E2 is an OR that belongs to family 51, subfamily E and it is the second member of that family. The nomenclature for mouse ORs used here follows the Zhang & Firestein, 2002 classification and nomenclature system, where the mouse ORs are referred to in the MORA-B format, where A is the Arabic numeral of the 228 mouse OR (mOR) families, and B is the Arabic numeral for the family member number. Hence MOR136-1 belongs to family 136 and is the first member.

1.3.4 Expanding Domains of Olfactory Receptor Expression

When ORs were cloned in 1991 (Buck & Axel, 1991), the authors worked under the hypothesis that ORs are exclusively expressed in the OE. However, it has been shown that receptors encoded by OR genes may not be restricted to neurons in the OE. As early as 1992, ORs had been reported to be expressed in the non-olfactory tissue of the testis (Parmentier et al., 1992), and it was later found that the activation of OR1D2 in spermatozoa was functionally implicated in the swimming speed and direction of spermatozoa (Spehr et al., 2003). That ORs could be expressed and may have functions outside olfaction is further bolstered by the finding that OR genes are expressed normally in the testes of a number of mammalian species (Vanderhaeghen, Schurmans, Vassart, & Parmentier, 1997a, 1997b), and that prostate-specific G protein-coupled receptor (PSGR), a marker overexpressed in certain types of prostate cancer (L. L. Xu et al., 2000), was actually encoded by a functional OR, OR51E2 (Cunha, Weigle, Kiessling, Bachmann, & Rieber, 2006; Xia, Ma, Wang, Hua, & Liu, 2001).

Transcriptional investigations into OR expression have revealed OR transcription in many different tissues, including gut (Braun, Voland, Kunz, Prinz, & Gratzl, 2007), kidney
(Pluznick et al., 2009), erythroid cells (Feingold, Penny, Nienhuis, & Forget, 1999), brain (Conzelmann et al., 2000; Otaki, Yamamoto, & Firestein, 2004; Raming, Konzelmann, & Breer, 1998), nervous system (Weber, Pehl, Breer, & Strotmann, 2002), tongue (Durzynski et al., 2005; Gaudin, Breuils, & Haertle, 2001) and placenta (Itakura, Ohno, Ueki, Sato, & Kanayama, 2006). The increasing availability of next generation sequencing (NGS) data profiling tissue transcriptomes is beginning to allow the comprehensive analysis of “ectopic” OR expression in the tissues of human and other species, and in a recent analysis of human RNASeq data it was reported that 11 of ~400 human ORs are expressed in tissues other than the OE, with 73% of these ectopically expressed ORs also showing expression in the OE, where they presumably participate in their canonical function of olfaction (Flegel, Manteniotis, Osthold, Hatt, & Gisselmann, 2013).

The ubiquity and apparent consistency of ectopic expression of ORs suggest that ORs could function as chemosensory receptors in various tissues outside the OE, and the large overlap between the OE-expressed and ectopically expressed OR genes indicates that they function in a manner consistent with the canonical function of ORs as chemosensory receptors and do not behave dissimilarly to ORs expressed for olfaction. While this has some clinical implications in the case of PSGR/OR51E2 (Neuhaus et al., 2009; Rigau et al., 2010; Rodriguez et al., 2014), there is still much unclear about the functions of these ORs, especially in light of the lack of functional information on OR-ligand pairs.
1.4 Approaches to Understanding Olfactory Receptor Function

1.4.1 Deorphanizing Olfactory Receptors

An important step in understanding OR function is the pairing of an OR to its cognate ligand(s), which are ligands that can activate the receptor in a dose-dependent manner. While the molecular mechanisms involved in signal transduction have been well defined for some time, it remains the case that the majority of ORs remain “orphan” receptors – receptors with unknown ligands (Peterlin, Firestein, & Rogers, 2014; Saito et al., 2009). There are two main driving factors in the slow progress of OR deorphanization, the first of which is the stimulus problem (Herrnstein, 1982), which is the complexity in understanding the stimulus under question. In olfaction, this question is particularly important, as there is a relatively poor understanding of the stimulus in terms of the relationship between chemical structure and activity as it relates to OR activation. This is best illustrated when comparing to other senses such as vision and hearing, where the stimulus in question is a function of wavelength and frequency respectively (referred to here separately even though they are related physical properties). In olfaction, this stimulus problem is still not well understood, but it is generally regarded to be a multidimensional problem, where 1664 or more physiochemical descriptors can be used to represent odors in “odor space” based on similarities based on this multidimensional matrix of properties (Haddad et al., 2008).

The other major impediment in the progress of OR deorphanization is the poor performance of ORs in cell-based functional assays compared to other GPCRs. The earliest deorphanization efforts therefore relied on OSNs as the expression system, since OSNs would already contain the necessary cellular machinery for proper OR expression. The first
example of this was the use of adenovirus-mediated overexpression of the rat OR-17 (cloned by Buck & Axel, 1991) in rat OSNs and the matching to its cognate ligand, octanal (Zhao et al., 1998). An alternative to this method was the gene targeting strategy employed in Bozza, Feinstein, Zheng, & Mombaerts, 2002, where OSNs expressing the M71 mouse OR were tagged with the green fluorescent protein (GFP) and subsequently matched to its ligand acetophenone. A third, single cell reverse transcriptase polymerase chain reaction (RT-PCR) strategy was employed in the identification and cloning of OR-EG from single dissociated mouse OSNs that were activated by eugenol (Kajiya et al., 2001). All of these cases, like other OSN-mediated deorphanization strategies, are fairly low throughput, since a limited number of ORs and ligands could be screened at one time.

1.4.2 High Throughput Functional Assays for Measuring Activation of ORs in Heterologous Cells

Given the large numbers of ORs making up the receptor repertoires of mammals (Table 1), there would be a clear advantage to the use of heterologous cell systems to enable high-throughput screening of large numbers of ORs and ligands. This required the overcoming of a few technical hurdles, since ORs were poorly expressed and trafficked to the cell surface, and coupled poorly to components of the downstream signaling cascade (Gimelbrant, Haley, & McClintock, 2001; McClintock et al., 1997; McClintock & Sammeta, 2003), and ORs were found to be retained and degraded in the endoplasmic reticulum (ER) (Lu, Echeverri, & Moyer, 2003; Lu, Staszewski, Echeverri, Xu, & Moyer, 2004).

Initial efforts to circumvent this trafficking block centered around adding N-terminal sequence tags from proteins known to be trafficked to the cell surface (Hatt,
Gisselmann, & Wetzel, 1999; Krautwurst, Yau, & Reed, 1998), though none of the attempts assisted in the proper tracking of all ORs, leading to the suggestion that there could be additional molecules involved in OR trafficking (Matsunami, 2005; McClintock & Sammata, 2003; Saito, Kubota, Roberts, Chi, & Matsunami, 2004). This seemed to be the case in other chemosensory systems such as OR93b in *Drosophila* (Larsson et al., 2004) and ODR-4 in *C. elegans* (Dwyer, Troemel, Sengupta, & Bargmann, 1998). This proved to be the case, when receptor transport protein 1 and 2 (RTP1 and RTP2), along with receptor expression enhancing protein (REEP) were found to be expressed in OSNs, coimmunoprecipitate with ORs, and when coexpressed with ORs in HEK293T cells, promote cell surface expression of ORs (Saito et al., 2004). A shorter isoform of RTP1, RTP1S, was subsequently also identified and showed to promote better OR surface expression, and the coexpression of RTP1S, Ric8b and $G_{\alpha_{olf}}$ served to enable functional expression of mammalian ORs (Zhuang & Matsunami, 2007). It was also found that the coexpression of the type 3 muscarinic acetylcholine GPCR (M3) could enhance the OR response without affecting OR surface expression (Li & Matsunami, 2011).

Hana3A, a HEK293T-derived cell line that stably expressed RTP1, RTP2, REEP1, Ric8b and $G_{\alpha_{olf}}$ was established (Zhuang & Matsunami, 2008), and enabled the high throughput screening of 245 human and 219 mouse ORs against a library of 93 odorants (Saito et al., 2009). This study lead to the largest-scale deorphanization of ORs to date, and matched 63 diverse odorants to 10 human and 52 mouse ORs. It is this expression system that will be used in this study, with some modifications as detailed in Chapter 7.
1.4.3 Understanding OR-Ligand Interactions

Once ORs have been paired with ligands, we can then begin to understand how these ligands interact with and activate ORs. Two of the earliest ORs to be deorphanized, OR-I7 and OR-EG, have been the most well studied, and are instructive in the approaches that can be employed in the deciphering of OR-ligand interactions.

From the first identification of octanal as the cognate ligand of I7 (Zhao et al., 1998), it had been appreciated that the OR-ligand interaction can be probed by measuring the differential activation caused by exposing related odorants. Zhao et al. varied the length of the alkyl chain from C3 to C12, and found that while octanal (C8) elicited the best response, they were able to measure significant responses from saturated heptyl (C7), nonyl (C9) and decyl (C10) aldehydes. The basic concept, borrowed from the pharmaceutical concept of rational drug design, proved useful, and this work was subsequently extended by Araneda, Kini, & Firestein, 2000) where they measured the responses of rat I7 to a large variety of octanal analogs, and concluded that the aldehyde functional group was absolutely required for I7 activation, but had more general steric requirements for the remaining alkyl chain.

Apart from enabling high throughput identification of OR-ligand pairs heterologous cell systems also provide an alternate approach to looking at OR-ligand interactions. This approach relies on the mutation of receptors to probe the structural basis for ligand recognition by the OR rather than altering the ligand. OR-EG, another early OR to be deorphanized, and its cognate ligand, eugenol is just such an example (Katada, Hirokawa, Oka, Suwa, & Touhara, 2005). While OR-EG was identified as a eugenol ligand via single cell RT-PCR of eugenol-activated OSNs, it was fortuitous that it is one of the relatively few
examples where the OR could be expressed on the surface of HEK293T cells without complication (Kajiya et al., 2001; Katada, Nakagawa, Kataoka, & Touhara, 2003). In addition to testing a series of odorants structurally related to eugenol, Katada et al., 2005 performed single site-directed mutagenesis to map the odorant-binding site by mutating single amino acid residues, and found that 9 of them had a significant effect on OR-EG response to eugenol.

### 1.4.4 What Understanding Olfactory Receptor Function Tells Us

The pairing of ORs and odorants, and the understanding of OR-ligand interactions can have significance beyond academic understanding of how the olfactory system works – it can also have implications on day-to-day life. In the instance of the association between PSGR overexpression in prostate cancer, groups have varyingly reported that activation of PSGR/OR51E2 with its cognate ligand β-ionone could either inhibit prostate cancer cell growth (Neuhaus et al., 2009) or promote cancer cell invasiveness and metastasis (Sanz et al., 2014), with implications for the possibility of pharmacological interventions via perturbation of PSGR/OR51E2 activity.

On a perceptual level, understanding OR function can give us insights into how and perhaps why odor perception differs between individuals, a topic of particular importance to the food and fragrance industries. Androstenone is an odorous testosterone-derived steroid that is present in pork, and has been described by different individuals as either offensive, pleasant or as having no odor (1-3). Combining perceptual testing, genetics and testing in heterologous cells, Keller, Zhuang, Chi, Vosshall, & Matsunami, 2007 showed that two genetically-linked non-synonymous single nucleotide polymorphisms (SNPs) in the OR7D4 receptor gene accounted for a significant part of the
sensitivity and pleasantness (or unpleasantness) of androstenone and the structurally related steroid androstadienone. Individuals with the OR7D4 RT/RT genotype (named for the amino acid substitutions) were the most sensitive to these steroids, and described them more frequently as unpleasant, compared to individuals with the RT/WM or WM/WM genotypes, who were less sensitive to these odors, and found them less unpleasant. This correlation was confirmed by \textit{in vitro} assay that showed the WM allele severely impaired the activation of OR7D4 by both androstenone and androstadienone – the first reported link between a genetic polymorphism in an OR gene and altered percept of its cognate ligand.

The potential implications of the growing recognition of “ectopic” OR expression in various mammalian tissues are also significant for a broad range of diseases beyond prostate cancer. GPCRs are involved in most aspects of human physiology (Pierce et al., 2002), and over 30\% of FDA-approved therapeutics target GPCRs (Overington, Al-Lazikani, & Hopkins, 2006). With more knowledge of ORs expressed outside the olfactory system, along with discoveries about their ligand receptive ranges and activation requirements, there could be potential for interventions and therapeutics that take advantage of these ORs as a more targeted means of modulating cell function that could mean fewer pharmacological side effects.

\textbf{1.5 Focus of this Dissertation}

Thus far, we have been introduced to the olfactory system, its peripheral components, and the challenges and significance in understanding the olfactory system. This dissertation attempts to explore olfactory receptor structure and function by examining
the OR-ligand interaction by a) examining the OR binding site with novel ligands designed
to probe the ligand-binding pocket of OR-I7 in Chapter 2 and b) by identifying novel
anesthetic-activated ORs and mapping their binding sites by *in silico* comparative
homology modeling and site directed mutagenesis in Chapters 3-5.
2. Probing the OR-I7 Binding Pocket with Custom Compounds

2.1 Introduction

Ketones and aldehydes play important roles in both natural and synthetic odorant compounds (Robert, Heritier, Quiquerez, Simian, & Blank, 2004). Despite the relatively simple structure of octanal, there are a number of different molecular features that can determine its recognition by OR-I7. As detailed in the introduction, previous investigations identified the ideal carbon chain length of 8 carbons (Araneda et al., 2000; Zhao et al., 1998), along with the critical requirement for an aldehyde functional group on the first carbon C-1 (Araneda et al., 2000), along with other features of OR-I7’s receptive range (Araneda et al., 2000). In general, while we refer to these receptors generally as OR-I7, in the mouse mOR-I7 is also known as MOR103-15 or Olfr2, and the rat rOR-I7 is also known as Olr226.

From these previous studies, we know that an 8-carbon chain is the most ideal chain length; the aldehyde functional group is essential for receptor activation, and the likely carbon chain conformation of octanal responsible for receptor activation (Figure 5).

Figure 5: Schematic Depiction of Octanal Configuration in OR-I7 Activation
Octanal appears to be able to bind to the OR-I7 receptor binding pocket in varying conformations as either an agonist or an antagonist. Adapted from Peterlin et al., 2008.
These results suggest that while ORs can bind multiple conformations of their odors, it is likely that only certain conformations of the many rotatable bonds are acceptable for receptor activation as shown in Figure 5.

2.1.1 Investigating the Hydration State of Octanal in OR-I7 activation

Despite this wealth of information about the properties and conformation of octanal required to activate OR-I7, one distinct possibility had not been previously considered, specifically, the requirement for octanal to dissolve in the aqueous olfactory mucosa before detection by OR-I7-expressing ORs, and the subsequent tendency for aldehydes to be hydrated under aqueous conditions, shown in the top half of Figure 6, where a molecule of water hydrates octanal to form the 1,1-geminal-diol or gem-diol form, with 4 H-bond acceptors and 2 H-bond donors, which is a significant increase of possible hydrogen bonding compared to the aldehyde form of octanal.

Figure 6: Hydration of Octanal and Difluoroocanal in Aqueous Solutions
(Above) In aqueous solutions, octanal is partially hydrated to octan-1,1-diol. (Below) The addition of two fluorines at the second carbon alters the equilibrium such that the solubilized compound is almost entirely hydrated. Adapted from Ho et al., 2014.

It is possible to test this hypothesis, by manipulating the structure of the ligand presented to the OR-I7. By substituting the hydrogens on the C2 carbon of octanal, we can alter the hydration equilibrium shown in Figure 6 (above). Substitution of hydrogens by a
strongly electron-withdrawing group such as fluorine would shift the equilibrium in favor of hydration, and substitution with an electron-donating group such as methy groups would shift the equilibrium in favor of the aldehyde. Octanal and structural analogs we investigated are detailed in Figure 7 below, and are referred to subsequently by their labeled numbers.

Figure 7: Octanal and Structural Analogs Screened Against OR-I7
Octanal 1, Difluorooctanal 2, Dimethyloctanal 3, Difluorooctanol 4, Octanol 5.

Perhaps of particular note is our choice to substitute both hydrogens on C-2 carbon in the case of compounds 2, 3 and 4, rather than substituting just one of the two hydrogens for either a fluorine or methyl group, which would arguably diminish the stearic effects of both the relatively small F and somewhat larger CH3, though both substituents are large compared to the H in the unsubstituted compounds. This is due to the relative difficulty of synthesis for mono-substituted variants, and due to reported instability in some of these variants (Steiner, Mase, & Barbas, 2005). Further this avoids the complications that a chiral
C-2 would create, both synthetically and experimentally, since ORs and OSNs are known to be able to discriminate stereoisomers (Yoshii & Turin, 2003).

We elected to also test compounds 4 and 5 as controls, to ensure that OR-17 activation was due to the presence of a second hydroxyl at C-1 but not the substituted fluorines at C-2 or by octanal 5, which has only a single –OH on C-1.

2.1.2 Synthesis Scheme

Synthesis of all novel compounds, as well as purification of commercially available octanal and octanal, were performed by our collaborator Yadi Li at The City College of New York, NY.
Figure 8: Synthesis Scheme of Difluoroctanal and Dimethyloctanal

a) Synthesis scheme for difluoro substituted analogs. b) Synthesis scheme for dimethyl substituted analog. Adapted from Ho et al., 2014

2.2 Results

To verify the substituted forms of octanal were indeed altering the hydration equilibrium as predicted, our collaborators performed $^1$H-NMR spectroscopy with the shorter and more soluble heptanal and its structural analogs, and were able to show that 43% of the aldehyde is hydrated in D$_2$O, while the difluoroaldehyde was 100% hydrated.
and the dimethylaldehyde was not hydrated. Results are shown in Table 1 below, adapted from Ho et al., 2014.

**Table 2: Hydration Equilibrium of Aldehydes Measured by ¹H-NMR in D₂O at 23 °C**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Aldehyde</th>
<th>Diffuoroaldehyde</th>
<th>Dimethylaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectrum</td>
<td>9.67 ppm (ALD)</td>
<td>5.02 ppm (HYD)</td>
<td>9.20 ppm (ALD)</td>
</tr>
<tr>
<td>Chemical shift</td>
<td>9.67 ppm (ALD)</td>
<td>5.2 ppm (HYD)</td>
<td>9.1 ppm (ALD)</td>
</tr>
<tr>
<td>In D₂O</td>
<td>57%</td>
<td>43%</td>
<td>0%</td>
</tr>
</tbody>
</table>

**Octanal Analog Screening in OSNs.** In experiments performed by our collaborator Zita Peterlin at Columbia University, NY, we used calcium imaging recordings to profile 1053 functional OSNs following dissociation of the cells from the rat olfactory epithelium and mucus (Araneda et al., 2000; Araneda, Peterlin, Zhang, Chesler, & Firestein, 2004). Since OSNs express a single OR family member (Chess et al., 1994; Malnic et al., 1999), single-cell activity can be taken to represent a single OR’s response to each of compounds 1–5. In this technique, the OSNs are first loaded with the calcium sensitive fluorescent dye Fura-2-AM and then exposed to 30 µM ligand solutions in a flow-through perfusion chamber fitted onto a fluorescence microscope. The short lifetime of the dissociated OSNs limits the number of tests that can be done on dissociated OSNs, so we relied on a single concentration (30µM) that was previously found to be conducive for detecting low and high affinity ORs and for detecting functional group selectivity in OSNs (Araneda et al.,
Compounds functioning as agonists activate signal transduction within the cells, leading rapidly to depolarization-driven calcium influx and a reduction of fluorescence at the monitored 380 nm excitation and 510 nm emission wavelengths. Thus, optical monitoring of the dispersed cells permits the screening of many OSNs while retaining single-cell, and therefore single OR family member, resolution.

**Figure 9: OSN Responses to Octanal Analogs**

a) Representative calcium imaging trace depicting the response of cell c35. Broken line shows the octanal trend-line over the course of the experiment which were used to normalize responses. Small squares summarize the fluorescence response normalized to that of octanal, according to color scheme shown in panel b. The tick mark below each compound number marks the start of the 4s injection of odorant solution into buffer stream flowing over cells.

b) Summary of responses for all octanal-activated cells to compounds 1–5 at 30 μM. (na, no data). Fluorescence changes are normalized to each cell’s response.
to compound 1, which is set to 100%. Adapted from Ho et al., 2014 with data from Z. Peterlin.

The fluorescence trace of a representative octanal-activated cell is shown in Figure 9a, and a summary of the responses of all octanal-activated cells to the screening compounds is shown in Figure 9b. Responses for each compound are expressed as fractional changes (ΔF/F) and are normalized to the octanal response generated by that cell, which is set to 100% (red in color scale), and the cells are grouped according to similarity of response. Out of 1053 cells tested, 87 cells (8%, Figure 9b, c1–c87) were activated by octanal and then observed for their response to compounds 2–5. Substitution at C-2 was generally unfavorable for octanal OR activation. Only 28% of octanal-activated cells were activated by 3, and 52% were activated by 2. This trend argues that the loss of activation of these ORs is more steric than electronic, as the smaller fluorine substituent was better tolerated. This experimentally verified bias against C-2 substitution increased our expectation that there would be some false negatives, that is, aldehyde-specific ORs that our approach would not be able to identify as either carbonyl- or gem-diol-specific.

Octanal and octanol are natural products that differ only by the oxidation state at C-1. Of the 87 cells activated by octanal, 59 cells (68%; Figure 9B, c13–c26, c41–c70, c73–c87) were also activated by octanol. The ORs expressed in these cells failed to distinguish between octanal and octanol and are therefore not aldehyde group-specific octanal ORs. In contrast, 24 cells (28%, c1–12, c27–37, c71) were activated by octanal but not by alcohols 4 or 5. These cells express ORs appearing to require the aldehyde group for activation. The remaining ≈4% of cells (c38–40, c72) was activated by difluoro
alcohol 4 but not by octanol 5. Of these cells, c38–39 appear to have some affinity for the fluorine substituents or their dipoles, and thus, we do not assign them to the gem-diol specific category even though they are strongly activated by gem-diol 2.

The 24 cells appearing to require the aldehyde for activation by octanal fell into four subgroups: those stringently specific for octanal and responding to no other analog (50%, c1–12); those producing the pharmacologic pattern consistent with a requirement for the gem-diol (42%, c27–36; 11% of all octanal-activated cells); one cell producing the pharmacologic pattern consistent with a requirement for the carbonyl form (4%, c71); and one indeterminate cell appearing to require the gem-diol, but also activated by 2,2-dimethyloctanal (4%, c37). Assuming the aldehyde is recognized as either the carbonyl or gem-diol, cells c1–12 could be false negatives for either carbonyl- or gem-diol-specific ORs, but we cannot assign them to either category. The data from cells c27–36 support the surprising conclusion that, among aldehyde-specific cells, about 42% (10/24) appeared to require the gem-diol. Thus, recognition of the gem-diol may be a common means to discriminate the aldehyde functional group from other H-bond accepting functional groups such as the corresponding alcohol. We note that the actual percentages found here apply only to our sampling of 1053 cells, which approaches nominal 1× coverage of the ≈1100 rat ORs. At this low level of coverage, some ORs were likely not present, and some may occur more than once. The time- and labor-intensive nature of live neuron screening makes a higher sampling coverage impractical using current methods, and the limited lifetime of the dissociated OSNs precludes the testing of a larger group of compounds on a given OSN.
**Dose–Response Curves in the Aldehyde-Specific Receptor OR-I7.** Though it is not possible to identify which OR family member is expressed in each of the cells profiled in Figure 9b, the data suggest that gem-diol recognition is common among ORs specific for the aldehyde functional group. Pharmacologically, the rodent OR-I7 is one of the most thoroughly characterized ORs and has been found to have a strict requirement for the aldehyde group in the context of aliphatic chains with 6 to 11 carbons (Araneda et al., 2000; Bozza et al., 2002; Krautwurst et al., 1998; Peterlin et al., 2008; Zhao et al., 1998). We first attempted to measure the activation of mouse and rat OR-I7 using our regular cAMP-mediated luciferase transcriptional reporter assay. However, we obtained confusing results from this assay, which we discovered was due to a large difference in the volatility of octanal 1 compared to difluoroctanal 2 (data not shown). We therefore switched to a GloSensor assay for a more real-time measurement of OR-I7 activation that excluded differential volatility as a factor. GloSensor allows a cell-intact real-time kinetic measurement of receptor activation by a genetically encoded cAMP-activated luciferase sensor, which allows us to examine the excitation kinetics for each compound at different concentrations, and 1.5min intervals. Figure 10 to Figure 14 show the real-time GloSensor results for each compound with mouse OR-I7. To minimize the effects of differential activation while sampling the maximum amount of data, it was determined that we would bin the 3-7.5 minute. This would allow us to capture the maximum amount of the initial activation of OR-I7 by each compound.
Figure 10: Real-Time GloSensor Measurements for mOR-I7 and Octanal

Figure 11: Real-Time GloSensor Measurements for mOR-I7 and Difluorooctanal
Figure 12: Real-Time GloSensor Measurements for mOR-I7 and Dimethyloctanal

Figure 13: Real-Time GloSensor Measurements for mOR-I7 and Difluoroctanol
The results of the GloSensor experiments are shown below in Figure 15, with accompanying calcium imaging data of OR-I7-expressing rat OSNs from our collaborator Zita Peterlin at Columbia University, NY. As mentioned before, we binned the 3-7.5min readings for the GloSensor experiment. All data was then baselined them to the blank control, then normalized to the maximum response of each respective OR-I7, which is set to 1.0.
Figure 15: Dose-Response Curves for Octanal and Structural Analogs and rodent OR-I7
Figure adapted from Ho et al., 2014. a) GloSensor assay results for mOR-I7. n=4 real-time kinetic results were binned from 3-7.5 min and fitted to a dose-response model. b) Calcium imaging in OR-I7-expressing rat OSNs shows a similar result to the in vitro responses. Calcium imaging data courtesy Z. Peterlin.

Table 3: Curve Fit Values for GloSensor Experiments with Octanal and Difluorooctanal

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log$<em>{10}$EC$</em>{50}$</th>
<th>S.E.M.</th>
<th>EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Octanal</td>
<td>-5.8</td>
<td>0.06</td>
<td>$1.54 \times 10^{-6}$</td>
</tr>
<tr>
<td>2 Difluorooctanal</td>
<td>-4.9</td>
<td>0.05</td>
<td>$1.25 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

2.3 Discussion

The data show that, as expected, neither the dimethylated aldehyde 3 nor the alcohol 5 activate either rodent OR-I7 in a dose-dependent manner. It also demonstrates that contrary to our hypothesis, the difluoro aldehyde 2 does not activate rodent OR-I7 as well as the same concentration of octanal 1, which should contain <50% of the hydrated gem-diol form, and we would expect a 2-3 fold increase in detection sensitivity for compound 2 form due to the predominance of the gem-diol. Despite this, evidence that 2, containing two hydroxyl groups, is able to activate rodent OR-I7 would preclude the exclusive activation of OR-I7 by the carbonyl of 1. It is therefore possible that the gem-diol form is preferred or even required for OR-I7 activation, especially since neither primary
alcohol 4 nor 5 was able to activate OR-I7, suggesting that the second –OH functional group is important to the activation of OR-I7. One explanation for the ~7-fold decrease in sensitivity to difluoro 2 is that the difluoro substitutions, while being electronically favorable in enabling additional hydrogen bonds, is sterically unfavorable due to the addition of the larger fluorine molecules, which have a Van der Waals radius of 1.47Å compared to the 1.20Å of Hydrogen (Bondi, 1964).

To further evaluate the possibility that rat and mouse OR-I7 might be activated by the gem-diol, our collaborator Tali Yarnitzky at The Hebrew University, Israel, modeled both orthologues with this form of the aldehyde functional group (Levit, Barak, Behrens, Meyerhof, & Niv, 2012). We modeled the structure in silico of rat OR-I7 using recently-solved crystal structures of the activated, ligand-, and G protein-bound β2-adrenergic receptor (β2AR) (Pdb 3SN6) (Rasmussen, DeVree, et al., 2011), and docked into the model the gem-diol of a conformationally restricted analog of octanal shown in Figure 5 and evaluated its accommodation in the binding site for the best scored poses. The more flexible octane-1,1-diol was then superposed on and replaced the optimal pose of this ligand. In its most favorable position (Figure 16a, rat OR-I7 model), the gem-diol ligand was found tipping slightly down toward the intracellular side and aiming the gem-diol at TM2 and TM7. The geminal hydroxyls appear well oriented to interact through hydrogen bonds with Y742.53 and Y2576.48 (Figure 16c). Superscripts on residues indicate the location within the GPCR structure, following the Ballesteros-Weinstein indexing system. Both gem-diol and aldehyde were well accommodated in the binding pocket of the receptors (data not shown), and the values of interaction energy with the rOR-I7 (-18.12 kcal/mol for the
gem-diol, -12.05 kcal/mol for the carbonyl form), predict that the gem-diol is superior to the aldehyde by ≈6 kcal/mol.

Figure 16: Homology Model of rOR-I7 Bound to Octan-1,1-diol
Figure adapted from Ho et al., 2014. a) Overall structure showing rOR-I7 and octane-1,1-diol with the polar diol pointed downwards in the binding pocket. b) A hydrophobic pocket formed by TM3, 5 and 6 accommodate the nonpolar carbon chain of octanal. c) Possible H-bonding between both hydroxyls of the gem-diol and Y74 and Y257. Models courtesy of T. Yarnitzky.

Since the most of an aldehyde sample reaching the nose through the air will initially be in the un-hydrated carbonyl form, there is also a question of the conversion to the gem-diol. Aldehydes undergo rapid acid- and base- catalyzed hydration (Buschmann, Dutkiewicz, & Knoche, 1982; Gruen & McTigue, 1963), but at the slightly acidic pH of the nasal epithelium (Washington et al., 2000), the uncatalyzed rate of hydration is expected to be slow \( k \approx 3.5 \times 10^{-3} \text{ s}^{-1}, t_{1/2} = 3.3 \text{ min} \) (Buschmann et al., 1982). Although some gem-diol will have formed within the time it takes to perceive an aldehyde, without catalysis the equilibrium concentration will not be achieved within that time. In our in vitro and ex vivo experiments, where mucus is not present and stimulation was carried out in
aqueous solutions, equilibrating compounds 1–5 in aqueous buffer allowed equilibrium to be reached prior to testing. However, in live animals, an aldehyde hydratase activity might be necessary to meet a gem-diol threshold concentration for some aldehyde ORs, and would be in line with previous observations of enzymatic conversion of odorants in the olfactory mucosa (Nagashima & Touhara, 2010). Alternatively other odorant binding proteins could serve this function as well (Heydel et al., 2013; Pelosi, Mastrogiacomo, Iovinella, Tuccori, & Persaud, 2014). Since rhodopsin, the prototypical Class A GPCR, can harbor significant numbers of ordered water molecules in the vicinity of highly conserved residues and in the retinal pocket (Okada et al., 2002) and are predicted to contain even more water (Grossfield, Pitman, Feller, Soubias, & Gawrisch, 2008) during activation, some aldehyde ORs might mediate aldehyde hydration themselves upon ligand binding, enabling gem-diol formation and detection on the short time scale of olfactory detection.

These data suggest that aldehydes may be detected via their gem-diol form, where the additional hydrogen-bonding possibilities could allow aldehyde-specific discrimination from other H-bond accepting functional groups.
3. ORs, GPCRs and Anesthetics

Beyond ligand manipulation as discussed in Chapter 2, a complimentary strategy to approach understanding of receptor-ligand interactions is by understanding the structural determinants of ligand binding and activation in the receptor itself. Here, we have chosen to explore the binding and activation of ORs by general anesthetic compounds, a previously unconsidered class of molecules that are of great medical importance, and yet which has been little studied in the context of interactions with metabotropic receptors such as GPCRs, which are the largest class of small molecule pharmaceuticals (Overington et al., 2006).

3.1 Introduction

Anesthesiology is a crucial component of modern surgical medicine – its widespread adoption has allowed painful surgical procedures to be undertaken relatively routinely. The properties of general anesthetics include desirable effects of analgesia, amnesia, immobilization and loss of consciousness, along with other undesirable effects such as nausea, autonomic instability, hypotension, hypoventilation and changes in heart rate (Campagna, Miller, & Forman, 2003).

Anesthetics are commonly categorized into the volatile inhalational anesthetics and nonvolatile intravenous anesthetics, with examples shown in Figure 17. Well-known anesthetics include prototypical anesthetics like chloroform and other halogenated hydrocarbons, and diethyl ether, to which many modern clinically important anesthetics are similar. This category also includes unusual and unexpected molecules like commonly-used nitrous oxide, and noble gasses like Xenon, which due to its fast action and relatively
minor side effects (Sanders, Ma, & Maze, 2004) appears to be an excellent general anesthetic, albeit one which is extremely expensive to obtain and this impractical to use. Intravenous injectable anesthetics include barbiturates, the dissociative drugs, benzodiazepines, barbiturates and alkylphenols.

Figure 17: Overview of Clinically Important Anesthetics

Anesthetics are commonly categorized into the volatile inhalational anesthetics (above) and nonvolatile intravenous anesthetics (below). Years listed below the names of the molecules are the year of first clinical use. (above) Common inhalational anesthetics include ethers such as diethyl ether, halogenated hydrocarbons similar to chloroform and halothane, small molecules like nitrous oxide and noble gas xenon. (below) Intravenous anesthetics include barbituates thiopental and methohexital, dissociative agents such as ketamine, benzodiazepines such as midazolam, the nonbarbiturate hypnotic etomidate and the alkylphenol propofol. Figure adapted from data taken from Kopp Lugli, Yost, & Kindler, 2009.
Indeed, because presence of a structurally diverse range of anesthetic agents, and the observation by Meyer and Overton of the strong correlation of anesthetic potency and lipid solubility (Meyer, 1901; Overton, 1901), it had been theorized that anesthetics acted through a unified nonspecific mechanism of lipid membrane disruption (Tang, Yan, & Xu, 1997). However, exceptions to this correlation, along with lipid perturbations that are unable to produce anesthetic effects (Franks, 2008) have largely caused the theory to be disregarded in modern research, even though recent work has demonstrated an influence of lipid membranes on ion channels (Andersen, 2013). Further, prior work demonstrating that anesthetic-induced immobility is mediated by the spinal cord (Antognini & Carstens, 2002) while amnesia and hypnosis are mediated by the brain (Eger et al., 1997), points at the minimum a diversity of tissue targets, and the possibility of a diverse range of molecular targets.

3.1.1 Anesthetics and their Targets

Despite widespread use, the targets underlying the actions of general anesthetics remain poorly understood, both for small, relatively featureless inhaled anesthetics but also for more potent injectables like barbiturates and alkylphenols. Though the precise molecular mechanisms of general anesthetic agents have yet to be defined, it is likely that they act through multi-transmembrane proteins such as ionotropic cys-loop ligand-gated ion channels (LGICs) like GABA$_\lambda$ and glycine receptors (Campagna et al., 2003; Franks, 2008). However, these receptors do not appear either necessary or sufficient for the general anesthetic action or differing side effect profile of these drugs (Franks, 2008), implicating the requirement the presence of other targets.
3.1.2 GPCRs as Anesthetic Targets

The possibility of other molecular targets for anesthetics is exemplified by ketamine in particular, since it does not appear to affect LGICs, but is widely recognized to be an N-methyl-D-aspartate (NMDA) receptor antagonist (Browne & Lucki, 2013; Franks, 2008), though other, perhaps more specific NMDA antagonists do not share the anesthetic function. That ketamine been gaining prominence for its long lasting antidepressant properties (Browne & Lucki, 2013; Caddy, Giaroli, White, Shergill, & Tracy, 2014) raises the possibility that it interacts with GPCRs in the CNS, which are one of the major receptor target classes for antidepressant and other psychopharmaceuticals (Nickols & Conn, 2014; Thompson, Burnham, & Cole, 2005; Urs, Nicholls, & Caron, 2014), and which is also consistent with previous studies showing ketamine interacts with the Gq coupled opioid and muscarinic receptors (Minami & Uezono, 2013). Previous work has also shown similar interactions between the smaller inhalational anesthetic halothane and the prototypical class A GPCR rhodopsin, which binds to rhodopsin and competitively inhibits retinal binding (Ishizawa, Sharp, Liebman, & Eckenhoff, 2000).

3.2 ORs as Anesthetic Targets

Thus far, studying interactions between general anesthetics and Gs-coupled receptors has been challenging and under-studied (Minami & Uezono, 2013). Since ORs are the largest group of GPCRs (Buck & Axel, 1991) each tuned to recognized an overlapping set of generally volatile molecules (Saito et al., 2009), and offer a diverse set of ~1000 murine and ~300 human receptors (Buck & Axel, 1991) that are highly similar in
sequence (and therefore structure). We hypothesized that it would be advantageous to study the structure-function interaction between ORs and general anesthetic compounds, as the sequence similarity and compound specificity can then be used to elucidate the structural determinants of class A GPCR binding and activation by anesthetics. Prior work has also demonstrated the ability of different volatile anesthetics to activate distinct subsets of OSNs in an *ex vivo* preparation, suggesting that ORs can be activated by anesthetic compounds, and that information on patterns of OR activation by different anesthetics can be combined with structural and computational approaches to determine the structural determinants for anesthetic binding and activation of other anesthetic GPCR targets.

This information, combined with the increasing awareness of OR expression outside of the olfactory epithelium (see Chapter 1.3) favors our unorthodox approach of examining general anesthetic targets by *in vitro* screening of ORs.

### 3.3 Experimental Approach

To achieve our aims, we used an iterative approach for examining OR-anesthetic interactions. To find initial anesthetic-activated ORs, we screened ORs against anesthetics in an *in vitro* luciferase assay screen. Once reliable receptor targets were found, our collaborators made *in silico* models of OR structure based on other published GPCR structures, which were then used in docking experiments to determine favorable binding conformations of the anesthetic. These docking poses were analyzed to predict putative receptor-ligand interacting residues, which were mutated in the receptors and empirically tested for their effect on anesthetic response. This response was then iteratively fed back into our molecular model to refine the model for anesthetic action. We achieved particular
success with ketamine, which is presented here along with initial screening results for other anesthetic-receptor pairs.
4. MOR136-1 and Ketamine

4.1 Initial Screen for Anesthetic-Responsive ORs

Since ligands for the majority of ORs remain unknown, and therefore it is impossible to determine whether a receptor is functional and trafficked to the cell surface by primary sequence, our initial screen involved screening 94 broadly-distributed human and mouse ORs previously found to function in our in vitro functional assay (Saito et al., 2009), in order to ensure only functional receptors were screened in this smaller scale experiment. The full list of ORs screened can be found in Appendix A. In this primary screen, we exposed Hana3A cells to 25µL of 9 anesthetics and analogs at 100µM concentration (shown in Table 4, experimental details found in Chapter 7.2.5).

We chose to screen the halogenated alkanes chloroform, halothane and F3 cyclobutane and its nonanesthetic analog F6 cyclobutane, the esters enflurane and isoflurane, the dissociative agent ketamine, and the alkylphenol propofol and a photoactive analog previously used to study propofol binding (Weiser, Kelz, & Eckenhoff, 2013). From the results of the initial screen, we selected 33 candidate ORs for a secondary screen where the experiment was repeated in triplicate and compared to solvent only control.
Table 4: Anesthetics and Analogs Screened in Chapter 4

<table>
<thead>
<tr>
<th></th>
<th>Chloroform</th>
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<th>Enflurane</th>
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<tr>
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<td>Cl</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>MOR136-2</td>
<td>Cl</td>
<td>Cl</td>
<td>Cl</td>
</tr>
<tr>
<td>F3 Cyclobutane</td>
<td>F</td>
<td>Cl</td>
<td>Cl</td>
</tr>
<tr>
<td>F6 Cyclobutane</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Propofol</td>
<td>F</td>
<td>Cl</td>
<td>Cl</td>
</tr>
<tr>
<td>mAzi-Propofol</td>
<td>F</td>
<td>Cl</td>
<td>Cl</td>
</tr>
<tr>
<td>Ketamine</td>
<td>F</td>
<td>Cl</td>
<td>Cl</td>
</tr>
</tbody>
</table>

Among the tested anesthetics, we found MOR136-1 and MOR136-1 to respond significantly \((p \leq 0.05)\) and specifically to ketamine (Figure 18). This screen was subsequently repeated with 21 other ORs that are members of the MOR136, MOR139 or related OR families (Figure 19) and MOR136-3 was identified that also responded significantly and specifically to ketamine.
Figure 18: MOR136-1, MOR136-3 and MOR139-1 Respond Specifically to Ketamine

MOR136-1, MOR136-3 and MOR136-9 respond specifically to ketamine and not other anesthetics and analogs screened in Table 4. Response was normalized to the MOR136-1 response to ketamine, n=4, significance at p≤0.05. pCI is the vector only control.

Figure 19: Response of MOR136, MOR139 and related family ORs to Ketamine

Among related ORs, only MOR136-1, MOR136-3 and MOR139-1 respond to ketamine. pCI is the vector-only control. Response was normalized to the MOR136-1 response to ketamine, n=4, significance at p≤0.05. pCI is the vector only control.
We then constructed dose-response curves for the three responders to ketamine, and confirmed that all three respond in a dose-dependent manner with EC$_{50}$ values that approximate steady-state plasma values during ketamine anesthesia (White et al., 1985).

**Figure 20: Dose Response of MOR136-1, MOR136-3 and MOR139-1 to Ketamine**

Dose-Response Curves for MOR136-1 (red solid line, circles), MOR136-3 (dotted lines, triangle) and MOR139-1 (dashed line, square). MOR136-1 responds most strongly to ketamine followed by MOR136-3 and MOR139-1. The respective EC$_{50}$ values for each of the ketamine responders are indicated. Data shown are normalized to the maximal responder (MOR136-1 and ketamine, see Materials and Methods), pCI is the vector only control.

**4.2 Molecular Modeling and Predictions for MOR136-1/Ketamine Interactions**

**4.2.1 Comparative Homology Modeling of MOR136-1**

To understand the structural basis for specificity of ketamine recognition by MOR136-1, MOR136-3 and MOR139-1, comparative homology models of MOR136-1 and other receptors were created (Figure 22a). All molecular modeling and docking studies
were performed by our collaborator Jose Manuel Perez-Aguilar and Lu Gao at University of Pennsylvania, PA, and all figures and illustrations were collaboratively created. Models were constructed based on available GPCR structures: bovine rhodopsin (Okada et al., 2004), human β2 adrenergic receptor (Cherezov et al., 2007), turkey β1 adrenergic receptor (Warne et al., 2008), human A2A adenosine receptor (Jaakola et al., 2008) and human D3 dopamine receptor (Chien et al., 2010). In order to generate a homology model, we first performed multiple sequences alignment, shown for MOR136-1 in Figure 21, which was then used in the subsequent modeling steps using all four GPCR crystal structures as guides for the creation of the homology model. For each of the ORs, one hundred models were generated using Modeller (Eswar et al., 2006; Sali & Blundell, 1993). The best structure based on Modeller’s scoring function was selected in each case. The structure of the ketamine responder MOR136-1 is displayed in Figure 22a. Intracellular (IC) and extracellular (EC) loops connecting the helices were also included in the models along with a small helical segment at the C-terminus nearly perpendicular to the seven-helix bundle (Figure 22a). Each model structure contains the canonical seven transmembrane helices (Hanson & Stevens, 2009; Pierce et al., 2002; Rosenbaum, Rasmussen, & Kobilka, 2009). Using computational docking studies, a ligand-binding site was identified that is consistent with that previously identified for ORs (Man, Gilad, & Lancet, 2004).
Figure 21: Multiple Sequences Alignments Used to Generate MOR136-1 Homology Model

Multiple sequences alignment of MOR136-1 against model crystal structure templates. Conserved residues are highlighted, and the disulfide bond is marked.
Figure 22: MOR136-1 Model Structure & Ketamine Docking Poses

a) MOR136-1 model structure obtained via comparative modeling. The 16 positions that were identified to form the ketamine binding site (within 5.0 Å of docked ketamine molecules) are displayed as space-filling representations (yellow). In this model, the residues forming the ketamine binding pocket are located mainly in TM3, TM5 and TM6 with just one residue in TM2 and one in TM7.

b) The top scoring pose of ketamine (blue) in MOR136-1 obtained from the docking calculation. Possible interactions of the positively charged nitrogen of ketamine with S105 and with D109 are indicated.

c) The top second pose of ketamine (orange) in MOR136-1 shows the interactions of the positively charged nitrogen of ketamine with S112 and the keto moiety with S105.

d) The third ketamine pose (magenta) from docking calculations suggests interaction between the nitrogen in ketamine and S112 but also a possible interaction of the Cl atom with residue T279.

e) The complete sequence of MOR136-1. The 16 positions predicted to form the putative binding site for ketamine are highlighted in yellow. The conserved residues (highly conserved in GPCRs) at each of the TM helices are colored in blue. The three positions considered to play a major role in the interaction with ketamine are colored in red. Data courtesy J.M. Perez-Aguilar.
In addition, models of MOR136-1 based on the structures of two published GPCRs known to be in activated state, bovine rhodopsin (Choe et al., 2011) and human \( \beta_2 \) adrenergic receptor (Rasmussen, Choi, et al., 2011; Rasmussen, DeVree, et al., 2011) were also generated. The representative models from both protocols were compared and displayed a minimal overall backbone RMSD of 1.7 Å (Figure 23). Furthermore, the ligand docking poses were similar (not shown).

![Figure 23: Comparison of Active and Inactive Models of MOR136-1](image)

The active state model is represented in green, while inactive state model is represented in magenta. (A) Side view with TM1, TM2, TM3, TM4, and TM5 facing front. (B) Side view with TM6, TM7, and TM1 facing front. (C) Top view. Data courtesy L. Gao.

### 4.2.2 Mutations Predicted by Molecular Modeling & Sequence Comparison

Using the representative comparative models, docking calculations for R-ketamine were carried out with AutoDock4 (Morris et al., 2009) and a binding site for R-ketamine with MOR136-1 was identified. In the case of MOR136-1, the three best-scoring docking results are located at the same binding site, which largely comprised side chains from...
helices TM3, TM5 and TM6 (Figure 22b, c, d). Using three ketamine poses, residues having an atom within 5.0 Å of any atom of ketamine were identified as potential lining residues of the ketamine binding pocket in MOR136-1, yielding 16 lining residues (Figure 22a, e). In addition, camphor, an odorant containing the same cyclohexanone moiety as ketamine, and which was previously shown to activate the ketamine responder MOR136-1 (Saito et al., 2009), was also docked to the model of MOR136-1 (Figure 24).

Comparison of the amino acid residues at the 16 binding site positions suggests possible key interactions relevant to the activation of MOR136-1 by ketamine. The top-scoring ketamine pose (Figure 22b) has the positively charged ammonium nitrogen in close proximity to S105$^{3.33}$ (3.9 Å to hydroxyl oxygen) and D109$^{3.37}$ (4.5 Å to carboxyl oxygen). Superscripts on residues indicate the location within the GPCR structure, following the Ballesteros-Weinstein indexing system (Ballesteros & Weinstein, 1995). In the second highest scoring ketamine pose, the proximity to S105$^{3.33}$ is again observed between ketamine ketone oxygen atom and hydroxyl oxygen atom of S105$^{3.33}$ with a distance of 3.2
Å, and the structure also suggests a possible role of residue S112<sup>3.40</sup> because of the proximity of ketamine ammonium nitrogen to hydroxyl oxygen of S112<sup>3.40</sup> with a distance of 3.8 Å (Figure 22c). Lastly in the third highest scoring pose, ketone oxygen atom of ketamine is close to the hydroxyl oxygen atom of residue S112<sup>3.40</sup> with a distance of 4.6 Å and its Cl atom is 4.5 Å from the hydroxyl oxygen atom of residue T279<sup>7.42</sup> (Figure 22d).

The potential importance of position S112<sup>3.40</sup> has been suggested from studies in the murine eugenol olfactory receptor, mOR-EG, where the equivalent position S113<sup>3.40</sup>, forms a hydrogen bond with the oxygen of the serine hydroxyl and the eugenol keto functional group (Baud et al., 2011). Camphor elicits a response in heterologous cells expressing MOR136-1 (Saito et al., 2009). In our model structure, the camphor ketone oxygen atom is positioned 3.4 Å from the hydroxyl oxygen atom of S112<sup>3.40</sup>, similar to the interaction observed in one of docked poses of ketamine (Figure 22 & Figure 24).
Table 5: Sequence Comparison of Binding Site Residues for MOR136 family Receptors

<table>
<thead>
<tr>
<th>Residue</th>
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<th>105</th>
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<th>109</th>
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</table>

The first three receptors (MOR136-1, MOR136-3, and MOR136-5) displayed response for ketamine. Displayed are the 16 positions identified as ketamine binding residues shown in Figure 22a, where the first and second row shows the corresponding residue number of MOR136-1 and the Ballesteros-Weinstein numbering positions in the structure Ballesteros & Weinstein, 1995, respectively. The three responders only differ at position 109\textsuperscript{3.37} (bolded blue). For each of the non-responders, the residue identities not present in any of the three responders at the corresponding position are indicated in bolded red.

In initial screens, among the MOR136 family of receptors only MOR136-1 and MOR136-3 responded to ketamine. Among the identified binding site residues, the only position that differs between MOR136-1 and MOR136-3 is position 3.37 (D109\textsuperscript{3.37} in MOR136-1) (Table 1). This observation suggests that the different responses of these two receptors to ketamine may be due to the different residues at this position (Henceforth residue numbers corresponding to those of MOR136-1 are used unless otherwise indicated).

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Receptor sequence comparisons, particularly at the proposed binding site positions, can potentially be useful in understanding the features that distinguish responders from non-responders (Table 5). Focusing on positions in the putative binding pocket, a combination of residues can be identified that could potentially distinguish responders from non-responders. Sites 3.33, 3.36, and 3.40 (S105$^{3.33}$, A108$^{3.36}$, and S112$^{3.40}$ in MOR136-1, respectively) were identified as key positions. At position 105$^{3.33}$, the responders contained either Ser or Thr, but these polar residues were much less frequent at this position among the non-responders; all but one of which have hydrophobic residues at this position. All of the responders have Ala at site 108$^{3.36}$ and Ser at site 112$^{3.40}$. Thus, informed by the screening results, molecular models and sequence comparison, two classes of mutations were considered to further explore details of OR activation by ketamine: mutations were identified that were (i) expected to decrease the ketamine response of MOR136-1 and (ii) expected to introduce ketamine response to non-responders MOR136-4.

4.3 Probing Predicted Interactions by Mutagenesis

4.3.1 Decreasing ketamine response of MOR136-1

From inspection of the model structures and the comparison of the sequences of responders and non-responders, three residues were identified as potentially playing significant roles in ketamine binding by MOR136-1: S105$^{3.33}$, D109$^{3.37}$, and S112$^{3.40}$. Mutations to these residues were expected to decrease ketamine response of the receptor. The hydroxyl group at position 105$^{3.33}$ (S105$^{3.33}$) may coordinate the ammonium group of ketamine, and the mutation S105A$^{3.33}$ would delete this interaction. The negatively
charged D109$^{3.37}$ may electrostatically associate with the positively charged ketamine ammonium group; D109$^{3.37}$ is expected to abrogate this interaction. S112$^{3.40}$ is present in all the responders and may form a hydrogen bond to the keto group of ketamine; S112$^{3.40}$ would eliminate this hydrogen bonding interaction. To explore the impact of multiple such mutations, the double mutant (S105$^{3.37}$/S112$^{3.40}$) and triple mutant (S105$^{3.33}$/D109$^{3.37}$/S112$^{3.40}$) were also considered. Further, we proposed the mutation D109$^{3.37}$ in MOR136-1, to introduce the amino acid present in MOR136-3 (one of the other responders); the mutation was expected to decrease the response to ketamine, consistent with the initial screen (Figure 19). Additional point mutations D109$^{3.37}$, D109L$^{3.37}$, D109N$^{3.37}$, D109V$^{3.37}$, and D109K$^{3.37}$ were also proposed to explore the sensitivity of the response to the amino acid size and hydrophobicity present at this site associated with the binding pocket.
Figure 25: Dose-Response Curves for MOR136-1 Mutations

Responses are normalized to WT MOR136-1 (red circles, solid line in both panels). a) The removal of the hydroxyl group in the single mutant, S105A (orange squares, dot-dash line) decreases the affinity and response of MOR136-1 to ketamine. Similarly, the S112A (green upward arrowhead, solid line) mutant drastically reduces the ability of ketamine to activate MOR136-1. Moreover, the S105A/S112A (cyan downward arrowhead, solid line) double mutant virtually abolishes the ability of ketamine to activate MOR136-1. The D109A (blue diamonds, dashed line) mutation increases the affinity and response of MOR136-1 to ketamine. The enhancement of the response due to the mutation D109A (blue) is also observed in the triple mutant S105A/D109A/S112A (purple empty symbol).
circle, dotted line). All of the listed EC50 values are significantly different compared to MOR136-1 (P≤0.05). **(b)** Dose-response curves for a series of MOR136-1 point mutations at position D109. As before, D109A (blue squares, dashed line) increases the affinity and response of MOR136-1 to ketamine, along with the mutations D109L (orange triangle, dotted line), D109S (black empty circle, dot dash line). D109N (purple upward arrowhead) was indistinguishable from WT, while D109V (green diamonds, solid line) and D109K (cyan circle, no line) virtually abolishes ketamine activation of the mutant receptor. All of the listed EC50 values are significantly different compared to MOR136-1 (P≤0.05) except for D109N.

We performed site directed mutagenesis of MOR136-1, measured response to ketamine, and constructed dose-response curves for each mutant (Figure 25a). The first mutation proposed was the removal of the hydroxyl group at position 105 (S105A). As predicted, the S105A mutant decreased the response to ketamine. Similarly, S112A was expected to attenuate the ketamine response, and this mutation completely eliminated response. Consistent with the single point mutation results, the double mutant S105A/S112A also completely eliminated the response to ketamine. The mutation D109A, however, unexpectedly enhanced the response to ketamine when compared to the wild-type OR. Interestingly, the triple mutant S105A/D109A/S112A displays ketamine response comparable with that of the single mutant S105A. D109A therefore appears to be an “enhancing” mutation that is able to rescue some of the depression introduced by S105A and S112A (Figure 25a). We further explored the impact of mutations at position 109 by producing the single-site mutants D109N, D109L, D109S, D109K and D109V. Despite their different chemical structures, D109N, D109L, D109S and D109K exhibit similar response to ketamine, while introducing the β-branched side chain with D109V or the large side chain with D109K eliminates the response (Figure 25b). Given that Asn, Leu, and Asp have similar
molecular volumes and flexible side chains, these observations are consistent with steric (van der Waals) interactions between the protein and ketamine determining the response, with smaller amino acids yielding a greater normalized response.

4.3.2 Introducing ketamine response to MOR136-4

Introducing a response via mutation is perhaps a more stringent test of our understanding of ketamine recognition than reducing or eliminating a response, as described above. Among the 16 identified binding pocket residues, MOR136-4 has high sequence similarity compared with MOR136-1 (Table 5; 12 out of the 16 residues are identical to the responder MOR136-1. The docking calculation and the sequence comparison analysis suggested that positions 104\textsuperscript{3.32}, 105\textsuperscript{3.33}, 112\textsuperscript{3.40} could be responsible for the difference in ketamine response between MOR136-1 and MOR136-4. Position 104\textsuperscript{3.32} is located in TM3 and near the extracellular entrance to the binding pocket. MOR136-4 is the only receptor in the MOR136 family that has a Tyr instead of Phe at this position. MOR136-4 also does not possess a polar residue at position 105\textsuperscript{3.33}. At position 112\textsuperscript{3.40}, the conserved Ser of the responders is proximate to the ketone oxygen of ketamine in the docking results (Figure 16d and Figure 24), but Asn is present at this position in MOR136-4. Additionally, position 207\textsuperscript{5.47} located in TM5 differs from that in MOR136-1 (I207\textsuperscript{5.47}), with MOR136-4 the sole receptor in the MOR136 family with a polar Thr instead of Ile at this position. For MOR136-4, we therefore proposed and generated the mutations Y104F\textsuperscript{3.32}, I105S\textsuperscript{3.33}, N112S\textsuperscript{3.40} and T207I\textsuperscript{5.47} to test our hypotheses that each of these 4 residues play important roles in ketamine binding, and if involved, that these proposed mutations would cause an increase in ketamine response in the mutant MOR136-4 receptor.
Figure 26: Dose-Response Curves for MOR136-4 Mutations
Responses are normalized to WT MOR136-1 (red circles, solid line). WT MOR136-4 does not respond to ketamine (orange squares), but N112S (green downward arrowhead, dotted line) and Y104F (blue diamonds, dashed line) mutations cause these receptors to respond to ketamine, especially in the case of the Y104F mutant, where both affinity and response magnitude to ketamine are markedly higher than MOR136-1.

As above, we used site directed mutagenesis, and measured receptor responses to varying amounts of ketamine in order to assess whether responsiveness to ketamine could be introduced to a normally unresponsive MOR136-4 (Figure 26). As expected, N112S, designed to mimic the identity of the binding site of MOR136-1, introduced ketamine responsiveness. But the other two mutations I105S and T207I failed to introduce responses to ketamine. Remarkably, the mutation Y104F at the entry to the binding pocket dramatically introduced ketamine responsiveness, at a level that was even greater than observed for MOR136-1 (Figure 24).
4.4 Receptor Cell Surface Expression Studies

For mutations producing a decreased response, the possibility that the mutation caused misfolding and trafficking dysfunction was also considered. We used Fluorescence-Activated Cell Sorting (FACS) to query surface expression and found that both the S112A\textsuperscript{3.40} and S105A\textsuperscript{3.33}/S112A\textsuperscript{3.40} mutants were expressed at the surface of HEK cells (Figure 27), demonstrating that the proteins had passed cellular quality control mechanisms, an indication of correct folding, hence the diminished responses are likely due to direct modulation of the receptor/ketamine interactions.

We also performed FACS analysis for MOR136-4 mutants (Figure 28), and found that the hyper-active MOR136-4 Y104F\textsuperscript{3.32} mutant had the lowest surface expression staining of all mutants, demonstrating that all the mutants were expressed on the cell surface to some degree, and that there is a lack of correlation between amount of surface expression and response to ketamine.
Figure 27: Receptor Surface Expression of MOR136-1 and Mutations
All point mutations were expressed at the surface of the cells when compared to vector only control Rho-pCI (red, shaded curve) and WT MOR136-1 (grey curve).
Figure 28: Receptor Surface Expression of MOR136-4 and Mutations

All point mutations had geometric means of surface stain intensity higher than the superfunctional Y104F mutant, indicating that functional receptors were expressed at the surface for all the mutants. All point mutations were expressed at the surface of the cells when compared to vector only control Rho-pCI (red, shaded curve).
4.5 Receptor Baseline Responses

The data shown thus far have been presented as values baselined to the media only, no agonist condition (i.e. stimulation media without ketamine). However, it is possible that our site-directed mutations can introduce changes in the basal activation state of the new mutated receptor. In other words, a mutated receptor could be constitutively more active than the wild-type receptor upon which it was based. This would not normally be observed with our baselining approach. Presented below are the basal activation levels of each of the ORs previously discussed, after normalizing to control only for plate-to-plate variation.

**Figure 29: Basal Responses of MOR136-1 Mutations**
Basal responses of the MOR136-1 mutants shown in Figure 25. Statistical significance was determined by one way ANOVA followed by bonferroni corrected multiple comparisons at a p≤0.05 level.
Figure 30: Basal Responses of MOR136-4 Mutations
Basal responses of MOR136-4 mutants shown in Figure 26. Statistical significance was determined by one way ANOVA followed by bonferroni corrected multiple comparisons at a p≤0.05 level.

Most of the point mutations appear to be well-behaved, and statistically indistinguishable from the wild-type receptor on which they are based, except for D109V. This suggests that the bulky valine residue at this position affects the basal activation of the receptor alongside its detrimental effect on ketamine activation of this mutant.

4.6 Discussion
Ketamine, a relatively potent general anesthetic, analgesic and antidepressant, has generally been considered to transduce its hypnotic properties via interactions with NMDA receptors (Franks, 2008). Here, we provide evidence for a highly specific interaction of ketamine with ORs, which suggests that a component of ketamine’s pleiotropic action might result from interactions with these or other CNS GPCRs, and which we believe is the
first demonstration of a GPCR being strongly activated by an anesthetic compound. Ketamine’s analgesic action, for example, might result from specific actions on opioid GPCRs, and its antidepressant effects could arise from interactions with monoaminergic GPCRs. Beyond providing evidence of other potential GPCR actions, it is possible that specific interactions with CNS ORs may be responsible for some ketamine actions. Analyses of human RNASeq data from the Illumina Bodymap project as well as other database resources have provided evidence that ORs may be expressed in various tissues throughout the body, and are not simply limited to the olfactory epithelium (Feldmesser et al., 2006; Flegel et al., 2013). Though it is unclear whether these ORs are functionally expressed in these tissues, or what their function might be, it may indicate a broader function for ORs in chemosensation not limited to smell.

**Elucidating a signature binding pocket for ketamine.** In addition to suggesting relevant molecular targets, this combined experimental and modeling study provides a molecular-level glimpse of GPCR elements involved in ketamine binding and their spatial distribution in the binding pocket. The location of the putative binding site of ketamine based on the docking results is in agreement with binding site residues identified in other ORs (Abaffy, Malhotra, & Luetje, 2007; Baud et al., 2011; Katada et al., 2005; Man et al., 2004; Schmiedeberg et al., 2007).
Figure 31: Representation of the Ketamine Binding Site

Positions 104^{3.32}, 105^{3.33}, 108^{3.36}, 109^{3.37}, 112^{3.40} and 259^{5.47} (showing here as red spheres) were investigated by mutagenesis studies in the responder MOR136-1 and in the non-responder MOR136-4. A representative ketamine pose is depicted as blue sticks while different TMs are shown as ribbons. At positions 105^{3.33} and 112^{3.40} the hydroxyl group from the small polar residues Ser and Thr was required for ketamine binding. The small apolar side chains of Ala or Val are preferred at position 108^{3.36}. At position 104^{3.32} the Phe residues was required to provide ketamine response. At position 109^{3.37}, the small residues Ala or Gly improve ligand response but the wild-type receptor, with an Asp at this position, also displayed significant ketamine binding capabilities. Lastly, position 259^{5.47} showed a minor role in ketamine recognition. From this figure, the importance of TM3 as the ketamine binding hub in the investigated ORs, is evident. A summary of the investigated positions for the three wild-type ketamine responders, MOR136-1, MOR136-3 and MOR136-5, is shown. The preferred residue type at each site based on residue identity is also displayed.

<table>
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<td>S</td>
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<tr>
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</table>

The general behavior of the binding site positions investigated to dissect the different interactions involved in ketamine binding displayed an interesting and particular fingerprint (Figure 31). At positions 105^{3.33} and 112^{3.40} in TM3, the small polar residues Ser
and Thr that contain a hydroxyl group were found to be important in the ketamine response. At position $108^{3.36}$, the small apolar side chains of Ala or Val are preferred. Remarkably, we found that the presence of a Phe residue at position $104^{3.32}$ is necessary to confer ketamine response to our tested ORs (F104$^{3.32}$ in MOR136-1). Position $104^{3.32}$ is located at the extracellular entrance of the binding pocket. At position $109^{3.37}$, the presence of the negatively charged Asp residue was not essential and replacement by the small residues Ala or Gly significantly improves ketamine response (Figure 31). By comparing the sequences of the identified binding positions between responders and non-responders (Table 5), specific preferred residue identities at each of the positions can be inferred, although the specific roles and interactions of each position need further investigation.

Particularly important for ligand binding is positions $105^{3.33}$ and $112^{3.40}$ (S105$^{3.33}$ and S112$^{3.40}$ in MOR136-1) since, from our computational models, they establish polar interactions with the ammonium group and the ketone oxygen (Figure 22b, c, d) of ketamine. Also, their residue identities are conserved in the responders but rare among non-responders (Table 5). Position $112^{3.40}$ is also likely a key interaction locus not only for ketamine response but also in odor response, since we found interaction of the hydroxyl group with the cyclohexanone oxygen atom of ketamine and camphor, respectively (Figure 22d and Figure 24). Moreover, this particular position has been identified to play an important role in odorant binding by ORs (Baud et al., 2011).

Since position $D109^{3.37}$ is the only position among the 16 candidate binding pocket residues where the two responders MOR136-1 and MOR136-3 differ (Table 5), mutations at this position can potentially explain the differences in the response to ketamine of these two ORs. We attempted to decrease the ketamine response of MOR136-1 by mimicking
the weakly responding MOR136-3 with the D109S\textsuperscript{3.37} mutation, but it marginally increased the ketamine sensitivity of the mutant receptor. Similarly, mutating D109\textsuperscript{3.37} to the smaller nonpolar Ala residue, D109A\textsuperscript{3.37}, increased the MOR136-1 response to ketamine. Thus, it appears that a small apolar residue is preferred at this position. In this regard, we subsequently cloned and measured the response to ketamine of a MOR136 family member which was previously absent from our OR library. This OR, MOR136-5, contains the even smaller residue Gly at the 109\textsuperscript{3.37} position (Table 5), and is the best overall responder (Figure 32), providing further evidence that a small apolar residue at this position increases ketamine response.

![Figure 32: Dose-Response Curves for MOR136-5 and Ketamine](image)

Responses are normalized to WT MOR136-1 (red circles, solid line). MOR136-5 (orange squares, dashed line) has an increased affinity for ketamine.
Furthermore, from our extensive investigation of the role of position 109\textsuperscript{3.37} in MOR136-1 (Figure 25b), three categories of mutations have been identified: 1) mutations D109A\textsuperscript{3.37} and D109N\textsuperscript{3.37} increased its response to ketamine; 2) mutations D109L\textsuperscript{3.37}, D109S\textsuperscript{3.37} produced a marginally increased sensitivity to ketamine while maintaining similar magnitude to wild-type receptor; 3) D109V\textsuperscript{3.37} and D109K\textsuperscript{3.37} eliminated completely its response to ketamine. We interpret these results by suggesting that a small non-polar residue better accommodates the relatively large ketamine ligand, increasing the response. On the other hand, D109V\textsuperscript{3.37} may disrupt the transmembrane helix segment (TM3), and D109K\textsuperscript{3.37} could form a salt bridge to E111\textsuperscript{3.39} on TM3 or simply bring the bulky positively charged K109\textsuperscript{3.37} into the binding pocket, which in either case makes the fit to ketamine less favorable.

![Figure 33: Behavior of Residues D109\textsuperscript{3.37} and K109\textsuperscript{3.37} in the Putative Ketamine Binding Pocket based on Constrained MD Simulations and Molecular Modeling](image)

**Figure 33:** Behavior of Residues D109\textsuperscript{3.37} and K109\textsuperscript{3.37} in the Putative Ketamine Binding Pocket based on Constrained MD Simulations and Molecular Modeling

a) Superposition of 50 conformations taken every 2 ns from the entire simulation is shown, where the S105\textsuperscript{3.33}, D109\textsuperscript{3.37} and S112\textsuperscript{3.40} side chains are displayed; b) D109K\textsuperscript{3.37} mutant of MOR136-1 overlaps with ketamine binding site. Here possible interactions between K109\textsuperscript{3.37} and E111\textsuperscript{3.39} are depicted. Data courtesy L. Gao.
To understand why a smaller apolar residue is preferred at position 109\textsuperscript{3.37}, constrained molecular dynamics simulations were performed with MOR136-1 receptor. The variability of the conformations adopted by the D109\textsuperscript{3.37} side chain is clearly reflected in the superposition of different snapshots from the entire 100 ns trajectory as indicated in Figure 33a. The D109\textsuperscript{3.37} side chain is observed to take on a variety of side-chain conformational states, a fraction of which overlap with the putative ketamine binding site. Based on the results from MD simulations of MOR136-1 and the D109A\textsuperscript{3.37} mutation, we speculate that this dynamic partial occlusion of the ketamine binding site is responsible for the decrease in ketamine response (relative to Gly and Ala) observed among variants having larger amino acids (Asp, Asn, Lys, Val) at position 109\textsuperscript{3.37}.

We also explored whether the connection between residue character of position 109\textsuperscript{3.37} and ketamine binding was through modulation of cavity volume. To address this, we used CASTp (Dundas et al., 2006) with a 1.4 Å radius probe to calculate the cavity volume of the binding pocket in our homology model structures. The solvent accessible cavity volume of the putative binding pocket decreases from 896 Å\textsuperscript{3} to 471 Å\textsuperscript{3} in MOR136-1 D109K\textsuperscript{3.37}, but maintains about the same volume with D109A\textsuperscript{3.37} (896 Å\textsuperscript{3}). This is because Lys at this position (Figure 33b) effectively divides the cavity into two smaller ones, neither of which would be accessible to the ketamine molecule.
The non-responder MOR136-4 was converted to a responder by introducing the mutation Y104F\textsuperscript{3.32}, which is located at the extracellular entry to the binding pocket (Figure 22b and Figure 34). Similarly, the responder MOR136-1 is converted to a non-responder by introducing the opposite mutation F104Y\textsuperscript{3.32} (Figure 26). This indicates that
Phe is necessary at this position to confer ketamine response to the ORs investigated here. To understand the molecular basis for this, we also performed constrained molecular dynamics (MD) simulations of both wild-type (WT) MOR136-4 and its Y104F \textsuperscript{3.32} mutant. The trajectory of the WT showed that Y104\textsuperscript{3.32} and T279\textsuperscript{7.42} formed a hydrogen bond (Figure 34a, c), highly restricting the Tyr side-chain conformations. In contrast, Phe at this position, not being able to participate in a hydrogen bond, was less constrained, explored a wider range of side-chain conformations, and could better accommodate ketamine (Figure 34b). From the distributions of side-chain dihedral angles of both Tyr and Phe at this position, the Tyr was confined to a more restricted region of dihedral angle space (Figure 34a), while the Phe explored more broad range of states (Figure 34b). In addition, upon forming a hydrogen bond with the Y104\textsuperscript{3.32} hydroxyl group, the T279\textsuperscript{7.42} residue on TM7 is not available for interacting with the Cl atom of ketamine as suggested by the docking calculations (Figure 22d and Figure 24). CASTp was also used to explore the size effects of mutation Y104F \textsuperscript{3.32} in MOR136-4. The solvent accessible cavity volume of the putative binding pocket increases from 555 Å\textsuperscript{3} to 951 Å\textsuperscript{3} in MOR136-4 with the mutation Y104F\textsuperscript{3.32}. Thus, consistent with the experimental data, this mutation should make the pocket below the phenyl ring more accessible to ketamine (Figure 34d).

Presently, there are no reported crystallographic structures of ORs, therefore our structural analyses necessarily employ homology models, which are based on previously published structures of other GPCRs (Cherezov et al., 2007; Chien et al., 2010; Jaakola et al., 2008; Okada et al., 2004; Warne et al., 2008). The broad tertiary structures of GPCRs are often recovered using comparative modeling, but the models may potentially still not capture details of the structure that significantly impact ketamine binding. Nevertheless,
the striking success of the predicted mutations in modulating ketamine activation, both positive and negative, suggests such models can indeed be useful. Another limitation is that the structures used all correspond to an inactive state of the corresponding GPCR. One would anticipate that the active state would yield better results for an activating ligand. We attempted to determine the magnitude of this limitation by comparing the structures of inactive and active structures (Figure 23), and found the RMSD between the backbone atoms of the superimposed models of MOR136-1 to be only 1.7 Å and little difference in the structures of binding site residues and docking of ketamine. These small structural variations make it difficult to rigorously compare the differences in structures, so the experimental functional studies presented herein provide the more critical assessment of the models and our molecular understanding of ketamine binding and response in ORs.

In summary, we provide evidence for highly specific and selective ketamine activation of discrete ORs, suggesting at the minimum that GPCRs could serve as functional targets of this unique general anesthetic, but perhaps more intriguingly that CNS ORs may be anesthetic/psychotropic targets. Further, using a combination of molecular modeling, sequence comparison and mutagenesis, we characterize the important structural determinants of ketamine activation by both removing and introducing responsiveness in specific receptors. The optimized combination of key interaction loci in the binding pocket for ketamine can potentially be used as a signature binding pocket to explore protein structural databases to search for other candidates as ketamine responders.
5. In Search of Volatile Anesthetic-Responsive ORs

With the success of our approach to the ketamine-responsive ORs in Chapter 6, we extended our search in a larger scale to examine more ORs and in particular, sought to find ORs that responded to the relatively featureless volatile anesthetics.

5.1 Screening Methodology

From the absence of ORs that responded to volatile anesthetics in Chapter 3, along with insights gained from the effects of octanal evaporation in experiments detailed in Chapter 2, we inferred that our original experimental methodology could have resulted in the loss of volatile anesthetics through evaporation, which would increase the probability of false negatives. To reduce the effects of evaporation, we therefore increased the volume of media used to stimulate the OR-expressing cells. In this screen we chose a slightly altered set of anesthetics (shown in Table 6). We also screened for the nonanesthetic analogue F6 Cyclobutane (shown in Table 4).

Table 6: Anesthetics Screened in Chapter 5

<table>
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<th>Enflurane</th>
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</table>
5.2 Results

In the primary screen, we exposed Hana3A cells to 200µL of 6 anesthetics at 200µM concentrations to 790 mouse ORs and 400 human ORs, all of which were screened in duplicate. The full list of 1190 ORs screened can be found in Appendix B. From the results of the primary screen, we selected 59 candidate ORs for re-screening in quadruplicate against the same panel of anesthetics at 200µM. There were 43 OR-anesthetic pairs tested for activity at varying anesthetic concentrations, of which 34 OR-anesthetic pairs showed dose-dependent responses with ≥1.5 fold maximal response compared to baseline. Shown here are the dose response curves of all 34 OR-anesthetic pairs, along with the associated values from the dose-response curve fits, which are the maximal activation value (fold change over baseline), the EC\textsubscript{50} values from these fits, alongside the standard error for each of those values. The full CCDS sequences of the 17 volatile anesthetic-activated ORs are listed in Appendix E – Amino Acid Sequences of Volatile Anesthetic-Responsive ORs.
Figure 35: Dose-Response Curves of Chloroform-Activated ORs

Table 7: Curve Fit Values for Chloroform-Activated ORs

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Figure 36: Dose-Response Curves of Halothane-Activated ORs

Table 8: Curve Fit Values for Halothane-Activated ORs

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Figure 37: Dose-Response Curves of Enflurane-Activated ORs

Table 9: Curve Fit Values for Enflurane-Activated ORs

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<td>-3.2</td>
<td>0.1</td>
</tr>
<tr>
<td>MOR253-9</td>
<td>0.446</td>
<td>0.084</td>
<td>-4.1</td>
<td>0.3</td>
</tr>
<tr>
<td>MOR182-5</td>
<td>13.2</td>
<td>0.78</td>
<td>-3.7</td>
<td>0.07</td>
</tr>
<tr>
<td>MOR184-5</td>
<td>3.68</td>
<td>0.16</td>
<td>-3.8</td>
<td>0.06</td>
</tr>
<tr>
<td>MOR184-1</td>
<td>13.5</td>
<td>0.74</td>
<td>-3.4</td>
<td>0.06</td>
</tr>
<tr>
<td>MOR202-8</td>
<td>18.4</td>
<td>1.4</td>
<td>-3.6</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Figure 38: Dose-Response Curves of Isoflurane-Activated ORs

Table 10: Curve Fit Values for Isoflurane-Activated ORs

<table>
<thead>
<tr>
<th>OR</th>
<th>Max. Response</th>
<th>S.E.M.</th>
<th>LogEC$_{50}$</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR253-4</td>
<td>11.8</td>
<td>0.43</td>
<td>-4.3</td>
<td>0.06</td>
</tr>
<tr>
<td>MOR253-5</td>
<td>7.63</td>
<td>0.31</td>
<td>-4.2</td>
<td>0.07</td>
</tr>
<tr>
<td>MOR253-9</td>
<td>1.12</td>
<td>0.072</td>
<td>-4.1</td>
<td>0.1</td>
</tr>
<tr>
<td>MOR185-1</td>
<td>16.1</td>
<td>0.48</td>
<td>-3.9</td>
<td>0.04</td>
</tr>
<tr>
<td>MOR252-2</td>
<td>24.9</td>
<td>1.5</td>
<td>-3.7</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Figure 39: Dose-Response Curves of Sevoflurane-Activated ORs

Table 11: Curve Fit Values for Sevoflurane-Activated ORs

<table>
<thead>
<tr>
<th>OR</th>
<th>Max. Response</th>
<th>S.E.M.</th>
<th>LogEC(_{50})</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR253-4</td>
<td>16.2</td>
<td>0.45</td>
<td>-4.9</td>
<td>0.06</td>
</tr>
<tr>
<td>MOR253-5</td>
<td>10.8</td>
<td>0.61</td>
<td>-4.5</td>
<td>0.1</td>
</tr>
<tr>
<td>MOR253-9</td>
<td>2.26</td>
<td>0.12</td>
<td>-4.3</td>
<td>0.09</td>
</tr>
<tr>
<td>MOR185-1</td>
<td>15.0</td>
<td>0.55</td>
<td>-4.2</td>
<td>0.06</td>
</tr>
<tr>
<td>MOR189-1</td>
<td>28.2</td>
<td>0.97</td>
<td>-4.1</td>
<td>0.06</td>
</tr>
<tr>
<td>MOR251-5</td>
<td>1.39</td>
<td>0.15</td>
<td>-3.9</td>
<td>0.2</td>
</tr>
<tr>
<td>MOR253-2</td>
<td>22.8</td>
<td>1.5</td>
<td>-3.5</td>
<td>0.07</td>
</tr>
<tr>
<td>MOR252-2</td>
<td>25.7</td>
<td>1.4</td>
<td>-3.6</td>
<td>0.06</td>
</tr>
<tr>
<td>MOR253-3</td>
<td>21.1</td>
<td>1.6</td>
<td>-3.4</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Figure 40: Dose-Response Curves of F3 Cyclobutane-Activated ORs

Table 12: Curve Fit Values for F3-Cyclobutane-Activated ORs

<table>
<thead>
<tr>
<th>OR</th>
<th>Max. Response</th>
<th>S.E.M.</th>
<th>LogEC₅₀</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR253-4</td>
<td>16.4</td>
<td>1.1</td>
<td>-3.6</td>
<td>0.08</td>
</tr>
<tr>
<td>MOR253-5</td>
<td>10.9</td>
<td>2.4</td>
<td>-3.1</td>
<td>0.2</td>
</tr>
<tr>
<td>MOR253-9</td>
<td>2.12</td>
<td>0.088</td>
<td>-4.1</td>
<td>0.07</td>
</tr>
<tr>
<td>MOR136-3</td>
<td>9.22</td>
<td>0.54</td>
<td>-3.5</td>
<td>0.06</td>
</tr>
<tr>
<td>MOR170-4</td>
<td>6.15</td>
<td>0.8113</td>
<td>-3.3</td>
<td>0.1</td>
</tr>
<tr>
<td>OR2B11</td>
<td>35.3</td>
<td>54</td>
<td>-2.1</td>
<td>0.7</td>
</tr>
<tr>
<td>MOR212-3</td>
<td>1.31</td>
<td>0.24</td>
<td>-3.5</td>
<td>0.2</td>
</tr>
<tr>
<td>MOR251-5</td>
<td>0.855</td>
<td>0.15</td>
<td>-4.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Figure 41: Dose-Response Curves of F6 Cyclobutane-Activated ORs

Table 13: Curve Fit Values for F6-Cyclobutane-Activated ORs

<table>
<thead>
<tr>
<th>OR</th>
<th>Max. Response</th>
<th>S.E.M.</th>
<th>LogEC$_{50}$</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR253-4</td>
<td>No Response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR253-5</td>
<td>No Response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR253-9</td>
<td>0.498</td>
<td>0.16</td>
<td>-3.5</td>
<td>0.4</td>
</tr>
<tr>
<td>MOR136-3</td>
<td>12.3</td>
<td>0.41</td>
<td>-4.2</td>
<td>0.06</td>
</tr>
<tr>
<td>MOR170-4</td>
<td>8.07</td>
<td>0.43</td>
<td>-3.6</td>
<td>0.06</td>
</tr>
<tr>
<td>OR2B11</td>
<td>18.2</td>
<td>6.8</td>
<td>-2.6</td>
<td>0.2</td>
</tr>
<tr>
<td>MOR212-3</td>
<td>0.804</td>
<td>0.11</td>
<td>-4.0</td>
<td>0.2</td>
</tr>
<tr>
<td>MOR251-5</td>
<td>No Response</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3 Discussion

For the clinically used anesthetics, we can compare the in vitro EC$_{50}$ values to ED$_{50}$ values used in the clinic, which gives a useful estimate of how relevant the response profiles of these receptors are to clinical use. For volatile anesthetics, this number is measured as the minimum alveolar concentration (MAC), which is defined as the concentration of inhalational anesthetic required to blunt the muscular response to surgical skin incision of 50% of a population of unparalyzed patients (Dilger, 2000), expressed as % atmospheres, and can be converted into millimolar values for comparison to values we have experimentally determined.

Table 14: Table of Clinical ED$_{50}$ Values Compared to in vitro measured EC$_{50}$ Values

<table>
<thead>
<tr>
<th>Anesthetic</th>
<th>MAC</th>
<th>ED$_{50}$ (mM)</th>
<th>Best Responder</th>
<th>Measured EC$_{50}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halothane</td>
<td>0.77</td>
<td>0.19</td>
<td>MOR252-2</td>
<td>0.15</td>
</tr>
<tr>
<td>Enflurane</td>
<td>1.7</td>
<td>0.52</td>
<td>MOR202-8</td>
<td>0.25</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>1.2</td>
<td>0.24</td>
<td>MOR252-2</td>
<td>0.21</td>
</tr>
<tr>
<td>Sevoflurane</td>
<td>2.0</td>
<td>0.29</td>
<td>MOR252-2</td>
<td>0.24</td>
</tr>
</tbody>
</table>

The empirically measured EC$_{50}$ values seem to agree fairly well with ED$_{50}$ values in the literature (Dilger, 2000) as shown in Table 14. Apart from these values, it is interesting that there appear to be a select few ORs that seem to be activated by more than one anesthetic, none more so obvious than MOR252-2, which is the top responder in terms of maximal activation for three of the volatile anesthetics, halothane, enflurane and sevoflurane. If we superimpose all three dose-response curves as shown in Figure 42, there appears to be very close overlap between the dose-response curves for all three compounds.
More interestingly, if we examine the protein sequence by BLAST, the most closely related human OR to MOR252-2 is OR13A1, which has been shown to be expressed in the RNASeq libraries derived from human adrenal, brain, lung and prostate tissues (Flegel et al., 2013). If indeed it is verified to be functionally expressed in some of these tissues, it could be an interesting candidate receptor for the mediation of anesthetic effects and side effects in these tissues.

Additionally, there appears to be three MOR253 family members, which are activated to varying degrees by most of the anesthetic compounds that were tested. These are shown in Figure 43 (MOR253-4), Figure 44 (MOR253-5) and Figure 45 (MOR253-9), with the corresponding tables curve fit values of the maximal response and EC50 values underneath.
Figure 43: Dose-Response Curves of MOR253-4

Table 15: Curve Fit Values for MOR253-4 and 6 Anesthetics

<table>
<thead>
<tr>
<th>Anesthetic</th>
<th>Max. Response</th>
<th>S.E.M.</th>
<th>LogEC₅₀</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>19.7</td>
<td>0.99</td>
<td>-3.4</td>
<td>0.05</td>
</tr>
<tr>
<td>Halothane</td>
<td>9.92</td>
<td>0.36</td>
<td>-4.4</td>
<td>0.06</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>11.8</td>
<td>0.43</td>
<td>-4.3</td>
<td>0.06</td>
</tr>
<tr>
<td>Sevoflurane</td>
<td>16.2</td>
<td>0.45</td>
<td>-4.9</td>
<td>0.06</td>
</tr>
<tr>
<td>Enflurane</td>
<td>10.1</td>
<td>1.2</td>
<td>-3.2</td>
<td>0.1</td>
</tr>
<tr>
<td>F3 Cyclobutane</td>
<td>13.4</td>
<td>1.2</td>
<td>-3.8</td>
<td>0.1</td>
</tr>
<tr>
<td>F6 Cyclobutane</td>
<td>No Response</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

83
Table 16: Curve Fit Values for MOR253-5 and 6 Anesthetics

<table>
<thead>
<tr>
<th>Anesthetic</th>
<th>Max. Response</th>
<th>S.E.M.</th>
<th>LogEC$_{50}$</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>7.78</td>
<td>2.33</td>
<td>-3.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Halothane</td>
<td>6.81</td>
<td>0.62</td>
<td>-4.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>7.63</td>
<td>0.31</td>
<td>-4.2</td>
<td>0.07</td>
</tr>
<tr>
<td>Sevoflurane</td>
<td>10.8</td>
<td>0.61</td>
<td>-4.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Enflurane</td>
<td>5.29</td>
<td>0.65</td>
<td>-3.2</td>
<td>0.1</td>
</tr>
<tr>
<td>F3 Cyclobutane</td>
<td>10.9</td>
<td>2.4</td>
<td>-3.1</td>
<td>0.2</td>
</tr>
<tr>
<td>F6 Cyclobutane</td>
<td>No Response</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.1 Activation Patterns of Anesthetic-Responsive ORs

As detailed above, there are a number of anesthetic-responsive ORs that respond to more than one anesthetic. While many of the anesthetics tested are similar in chemical
structure, one important further question is whether these anesthetic-responsive ORs are actually broadly-tuned receptors that can be activated by a variety of odorous stimuli, or if they are more narrowly activated by specific kinds of odorants. One way to examine this is to measure the response of these receptors to a panel of odorous compounds that are evenly-distributed in odor space (Saito et al., 2009). We therefore selected 12 odors from a library of 2718 odorants that are evenly distributed in odor space (according to the strategy in Saito et al., 2009), shown below in Figure 46.

Figure 46: 12 Evenly Distributed Odors
We then tested these odors against 18 anesthetic-responsive ORs in a screen similar to our secondary screen (as detailed in Chapter 5.2 Results), with all compounds screened in quadruplicate at a concentration of 200µM.

**Figure 47: Response of MOR136-1 to 12 Evenly Distributed Odors**

**Figure 48: Response of MOR136-3 to 12 Evenly Distributed Odors**
Figure 49: Response of MOR170-4 to 12 Evenly Distributed Odors

Figure 50: Response of MOR182-5 to 12 Evenly Distributed Odors
Figure 51: Response of MOR184-1 to 12 Evenly Distributed Odors

Figure 52: Response of MOR184-5 to 12 Evenly Distributed Odors
Figure 53: Response of MOR185-1 to 12 Evenly Distributed Odors

Figure 54: Response of MOR189-1 to 12 Evenly Distributed Odors
Figure 55: Response of MOR202-8 to 12 Evenly Distributed Odors

Figure 56: Response of MOR251-5 to 12 Evenly Distributed Odors
Figure 57: Response of MOR252-1 to 12 Evenly Distributed Odors

Figure 58: Response of MOR252-2 to 12 Evenly Distributed Odors
Figure 59: Response of MOR253-2 to 12 Evenly Distributed Odors

Figure 60: Response of MOR253-3 to 12 Evenly Distributed Odors
Figure 61: Response of MOR253-4 to 12 Evenly Distributed Odors

Figure 62: Response of MOR253-5 to 12 Evenly Distributed Odors
Figure 63: Response of MOR253-9 to 12 Evenly Distributed Odors

Figure 64: Response of OR2B11 to 12 Evenly Distributed Odors
As shown in Figure 58, MOR252-2, the top responding OR to halothane, isoflurane and sevoflurane appears to be specific for these anesthetics, and while we cannot rule out the possibility that it responds to other odors we have not tested, we can conclude that MOR252-2 is likely not to be a broadly-tuned receptor. In other words, it likely responds to a relatively small number of compounds.

This is in contrast to the other MOR253-4, -5 and -9 receptors (dose responses shown in Figure 43, Figure 44 and Figure 45 respectively, and responses to the 12 evenly distributed odors in Figure 61, Figure 62 and Figure 63). MOR253-4 (Figure 61) and MOR253-5 (Figure 62) in particular respond strongly to >2 of the 12 odors, and also show significant responses to a common set of 6 of the odors. MOR253-9 responds to an even broader range of 8 of the 12 tested odors albeit with reduced response magnitude. This indicates that these three MOR253 family receptors appear to be fairly broadly tuned.
Though this screen of 12 broadly distributed odors can give us some clues about the breadth of tuning of our receptors of interest, it is important to note that these were tested at a fairly high concentration, and only a single concentration was tested. A comparison that would potentially be more useful would be the measurement of the dose-dependent responses for all of these odor-receptor combinations. This would verify that they truly activate these receptors in a dose-dependent manner, and to be able to compare their EC\textsubscript{50} values to establish the relative biological relevance of each OR’s odor repertoire.
6. Future Directions

6.1 OR-I7

The exact structural and conformational differences between activators and competitive inhibitors of the OR-I7 binding pocket remain unknown. We know that there are a range of molecular lengths which are activating and inhibitory to OR-I7 (Figure 5, Peterlin et al., 2008), which depend on the conformation of the alkyl chain distal to the aldehyde functional group of octanal. We intend to examine this by generating aldehydes with different terminal carbon conformations and assay their effectiveness at competitively inhibiting OR-I7 activation by octanal.

6.2 Anesthetic-Responsive ORs

With these new OR-anesthetic pairs, we expect to extend our understanding of how ORs, and GPCRs in general, recognize these molecules with relatively few distinguishing features. Our work on MOR136-1 and ketamine has shown that our analysis pipeline is effective for determining important binding residues for anesthetic molecules, and comprehensive work in defining a signature binding pocket in ORs can be extended to other GPCRs as well. The intriguing possibility that MOR252-2 and its best human match OR13A1 could be expressed outside of the olfactory epithelium and potentially function in a non-olfactory capacity is also an excellent prospect for pursuing the idea that olfactory GPCRs have a function that is outside of the domain of olfaction.
7. Materials & Methods

7.1 Materials

7.1.1 Odors Tested

Structural variants of octanal and octanol used in Chapter 2 were synthesized by Yadi Li at The City College of New York, NY and purified by chromatography. All compounds used in Chapter 2 were sent under argon in individual glass vials as a 1M stock in DMSO. A fresh vial was used for each experiment to preclude aldehyde oxidation once the vial had been opened.

Anesthetic compounds were gifted by Roderick Eckenhoff at University of Pennsylvania, PA, either as a 1M stock in DMSO (propofol, F3 cyclobutane, F6 cyclobutane, ketamine) or pure compound (chloroform, halothane, isoflurane, enflurane, sevoflurane).

All other odors were obtained from commercially available sources.

7.1.2 Olfactory Receptors Tested

All olfactory receptors were obtained from the library of olfactory receptors generated for Saito et al., 2009. For the cloning of MOR136-5, genomic DNA was obtained from C56BL/6J mice.

7.1.3 Cell Line

HEK293T-derived Hana3A cells stably expressing RTP1L, RTP2, REEP1 and Ga_{olf} (Zhuang & Matsunami, 2008) were used for all mammalian cell culture assays.
7.2 Media & Solution Recipes

7.2.1 Bacterial Culture

2XYT + Amp bacterial growth media
16g Bacto-tryptone (BD 211705)
10g Bacto-yeast extract (BD 212750)
5g NaCl
up to 1L volume with dH2O
100mg/mL Ampicillin

7.2.2 Cell Culture

M10 cell maintenance media
45mL Eagle’s Minimum Essential Medium (MEM) with L-glutamine, Earle’s salt and bicarbonate (Sigma M4655)
5mL Fetal Bovine Serum

M10-PSF cell maintenance media with penicillin/streptomycin/amphotericin
45mL Eagle’s Minimum Essential Medium (MEM) with L-glutamine, Earle’s salt and bicarbonate (Sigma M4655)
5mL Fetal Bovine Serum
250µL Penicillin (10,000 units/mL)-Streptomycin (10mg/mL) (Sigma P4333)
250µL Amphotericin B solution (250µg/mL, Gibco 15290)

Freezing Media
9mL Fetal Bovine Serum
1mL DMSO

7.2.3 Live Cell Surface Staining

PBS Staining Buffer
500mL Phosphate Buffered Saline (Corning Cellgro 21-040-CV)
10mL Fetal Bovine Serum
5mL 1.5M NaN₃

7.2.4 GloSensor Assay

HBSS Stimulating Medium
500mL HBSS (Gibco 14025)
5mL 1M HEPES (Gibco 15630, 10mM final)
200µL 45% Glucose (Sigma G8769, 1mM final)
**GloSensor cAMP Assay (Promega E1291)**

2% GloSensor cAMP Reagent was prepared according to manufacturer’s instructions by mixing 75µL GloSensor reagent with 3.75mL HBSS stimulating medium.

### 7.2.5 Luciferase Assay

**CD293 Stimulating Medium**

- 1000mL CD293 (Gibco 11913)
- 10mL 200mM L-Glutamine (Gibco 25030)
- 30µL 1M CuCl₂

**Dual-Glo Luciferase Assay System (Promega E2980)**

Dual-Glo Luciferase Reagent and Dual-Glo Stop & Glo Reagent prepared according to manufacturer’s instructions.

### 7.3 OR Cloning, Mutagenesis & Sequencing

#### 7.3.1 rho-pCI Mammalian Expression Vector

The mammalian expression vector, pCI (Promega E1731), was modified to express the first 20 amino acids of bovine rhodopsin (N-MNGTEGPNFYVPFSNATGVVR.C), referred to as the rho-tag, between the Nhel and EcoRI restriction sites. All ORs used were cloned into this expression vector, referred to as rho-pCI.
7.3.2 Site Directed Mutagenesis

Site directed mutagenesis of MOR136-1 and MOR136-4 in rho-pCI vector was accomplished by chimeric PCR with Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo # F-549L) Briefly in a first phusion PCR reaction, reverse and forward “chimeric” primers containing the desired mutation(s) were combined with pCI forward (5’, 5’ CTCCACAGGTGTCCACTC 3’) and reverse (3’, 5’ CACTGCATTCTAGTTG 3’) sequencing primers respectively in separate PCR reactions to generate separate amplified 5’ and 3’ fragments of the OR containing the desired mutation(s). These reaction products were then diluted and mixed together, and a second phusion PCR reaction was performed to generate the full-length OR containing the desired mutations. The amplicon was then purified, digested with restriction enzymes, ligated into the rho-pCI vector and prepared as described below in 7.3.4-7.

**First Phusion PCR Reaction**

1µL  rho-pCI-OR template DNA (1ng/µL)  
2µL  Phusion buffer (5x)
1µL dNTP mix (Roche 040 638 956, final 2mM)
0.1µL Phusion Hot Start polymerase (Thermo F549L)
1µL Forward primer (5µM, pCI forward or forward chimeric primer)
1µL Reverse primer (5µM, reverse chimeric or pCI reverse primer)
5µL deionized water

**Second Phusion PCR Reaction**
1.25µL 1:10 dilution of first PCR reaction
1.25µL 1:10 dilution of second PCR reaction
5µL Phusion Buffer (5x)
2.5µL dNTP mix (2mM)
0.25µL Phusion Hot Start polymerase
2.5µL Forward primer (5µM, pCI forward or forward chimeric primer)
2.5µL Reverse primer (5µM, reverse chimeric or pCI reverse primer)
11µL deionized water

**Thermocycler Program**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>98°C</td>
<td>30s</td>
</tr>
<tr>
<td>98°C</td>
<td>5s</td>
</tr>
<tr>
<td>55°C</td>
<td>15s</td>
</tr>
<tr>
<td>72°C</td>
<td>30s</td>
</tr>
</tbody>
</table>

for 20 cycles

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>72°C</td>
<td>5 min</td>
</tr>
<tr>
<td>4°C</td>
<td>soak</td>
</tr>
</tbody>
</table>

**7.3.3 MOR136-5 Cloning**

MOR136-5 was amplified from genomic DNA obtained from C57BL/6J mice using Phusion Hot Start II High-Fidelity DNA Polymerase (as above). Forward and reverse primers were designed were designed against the 5' and 3' regions of the open reading frame (ORF) of MOR136-5, and contained a 5'-MluI (5' AAACGCGT 3') and a 3'-NotI (5' AAGCGGCCGC 3') restriction site linker. The sequences of the MOR136-5 primers are as follows:

**Forward Primer**  
5' AAA CGC GTA TGG ATA ATG AGA GCA CTG T 3'

**Reverse Primer**  
5' AAG CGG CCG CTT ATT TTC TGC TGA GGA TAT TT 3'
The amplified ORF from the PCR reaction was then purified, digested with restriction enzymes, ligated into the rho-pCI vector and prepared as described below in 7.3.4-7.

**PCR Reaction**

- 2.5µL C57BL/6J genomic DNA (10ng/µL)
- 5µL Phusion buffer (5x)
- 2.5µL dNTP mix (2mM)
- 0.25µL Phusion Hot Start polymerase
- 2.5µL Forward primer (5µM, pCI forward or forward chimeric primer)
- 2.5µL Reverse primer (5µM, reverse chimeric or pCI reverse primer)
- 11µL deionized water

**Thermocycler Program**

- 98°C 30s
- 98°C 5s
- 55°C 15s
- 72°C 30s
- 72°C 5 min
- 4°C soak

**7.3.4 PCR Amplicon Purification, Digestion, Ligation & Transformation**

Once the PCR reaction is complete, 1µL of the reaction is run on a 1.2% agarose gel to verify amplicon size. The product is then purified using Qiagen minelute columns, digested with restriction enzymes MluI and NotI (along with rho-pCI plasmid), then the insert is ligated into the digested plasmid and transformed into competent *E. coli* bacteria.

**Qiagen PB Purification**

1. Add 200µl of Buffer PB to PCR product
2. Centrifuge for 30s in Qiagen minelute column
3. Add 750µl of Buffer PE
4. Centrifuge for 30s, discard flow-through
5. Centrifuge for 2 min
6. Add 11µl of Buffer EB
7. Centrifuge for 2 min into fresh collection tube
**Restriction Enzyme Digestion**

9µL PCR product, or rho-pCI (100ng/µL)
2µL Buffer 3 (NEB)
0.5µL MluI (NEB R0198L)
0.5µL NotI (NEB R0189L)
0.2µL BSA (NEB)
8µL deionized water

Incubate at 37°C for 2h

**Gel Purification (Qiagen)**

1. Add 4µl of 6x loading dye
2. Run on 1.2% agarose gel
3. Cut the band and place into fresh eppendorf tube
4. Add 500 µl of QG
5. 50°C for 10 min. Mix occasionally
6. Centrifuge for 1s
7. Add 150 µl of isopropanol, confirm the volume is <750 µl. (If your gel is bigger than 150ul, add QG to 3x gel volume)
8. Add mixture to Qiagen minelute column
9. Centrifuge for 30s
10. Add 500 µl of QG
11. Centrifuge for 30s
12. Add 750 µl of PE
13. Centrifuge for 30s, discard flow-through
14. Centrifuge for 2 min
15. Add 10 µl of EB (30 µl for rho-pCI)
16. Centrifuge for 2 min into fresh collection tube

**Ligation**

2.5µL Insert
1µL Digested rho-pCI
0.5µL T4 DNA ligase (NEB M0202L)
0.5µL T4 DNA ligase Buffer (NEB)
0.5µL deionized water

Incubate at room temperature for >1h

**Transformation**

1. Thaw competent cells
2. Add 2.5 µl of ligation product
3. On ice >10 min
4. Heat shock for 30s at 42°C
5. Plate on LB-amp plate
6. Incubate at 37°C overnight
7.3.5 Colony PCR

In order to verify the presence of the insert in transformed cells, colony PCR was performed on selected single bacterial colonies from each transformation, which were picked and soaked in 20µL of deionized water, and amplified using the pCI forward and reverse primers as detailed in 7.3.2, and amplified with Qiagen HotStarTaq. PCR products were then run on 1.2% agarose gel to verify presence of a ~1kb OR band, and these colonies were then grown overnight in 5mL 2XYT-Amp media for plasmid preparation and sequencing.

**Colony PCR Reaction**

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>2µL</td>
<td>Bacteria soaked in deionized water</td>
</tr>
<tr>
<td>1µL</td>
<td>HotStarTaq buffer (10x)</td>
</tr>
<tr>
<td>1µL</td>
<td>dNTP mix (2mM)</td>
</tr>
<tr>
<td>0.05µL</td>
<td>HotStarTaq polymerase (Qiagen 203205)</td>
</tr>
<tr>
<td>1µL</td>
<td>pCI forward primer (5µM)</td>
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<tr>
<td>1µL</td>
<td>pCI reverse primer (5µM)</td>
</tr>
<tr>
<td>5µL</td>
<td>deionized water</td>
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</table>

**Thermocycler Program**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95ºC</td>
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</tr>
<tr>
<td>95ºC</td>
<td>15s</td>
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<tr>
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<td>15s</td>
</tr>
<tr>
<td>72ºC</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>for 25 cycles</td>
</tr>
<tr>
<td>72ºC</td>
<td>1 min</td>
</tr>
<tr>
<td>4ºC</td>
<td>soak</td>
</tr>
</tbody>
</table>

7.3.6 Plasmid Preparation

Plasmid DNA for all DNAs used in functional assays was prepared using a modified miniprep DNA protocol (Denville Scientific) with an additional phenol-
chloroform extraction step to ensure purity of DNA for clean transfection. All centrifugation is performed at maximum speed of \(~13,000-15,000\) RPM. Wash solution was prepared according to manufacturer instructions. The steps are as follows:

1. Pellet bacteria grown overnight in 5mL 2XYT0-Amp in a 2mL Eppendorf tube
2. Resuspend in 150µL resuspension solution
3. Add 300µL lysis buffer and mix gently by inversion >10 times until solution becomes viscous and slightly clear
4. Add 525µL of neutralization solution within 5min of lysis buffer addition, and mix gently by inversion >20 times. Visible precipitate should form
5. Centrifuge 5 min to pellet precipitate
6. Transfer supernatant to a fresh Eppendorf tube containing 500µL of phenol/chloroform/isoamyl alcohol (25:24:1 ratio)
7. Centrifuge 5 min to separate phases
8. Carefully transfer the upper aqueous phase to a miniprep column
9. Centrifuge 30s, discard flow-through
10. Add 750µL wash solution
11. Centrifuge 30s, discard flow-through
12. Centrifuge additional 2 min to dry column
13. Transfer the column to a 1.5 ml Eppendorf tube
14. Add 104 µl of elution
15. Centrifuge 2 min to elute plasmid.
16. Take 4ul of DNA and measure OD.
17. Adjust the concentration to 100ng/µl, store at 4°C or lower

7.3.7 Sequencing

Clones were sequenced to verify the identity of the insert and the presence of the desired mutations, again using the pCI forward and reverse primers from above. Sequencing was conducted using either a 3100 or a 3730 Genetic Analyzer (ABI Biosystems). A Whatman 96-well filter plate was filled with 40µL sephadex G-50 resin (GE/Amersham) and hydrated for >3 hours with 300µL of deionized water in each well while the sequencing reaction was in progress.

Sequencing Reaction
2µL    DNA template (100ng/µL)
1µL Sequencing primer (either pCI forward or reverse)
1.5µL BigDye v1.1
2µL ABI 5x sequencing buffer
5.5µL deionized water

**Thermocycler Program**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<td>5s</td>
</tr>
<tr>
<td>60ºC</td>
<td>2 min</td>
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</tbody>
</table>

for 25 cycles

**Sephadex G-50 Sequencing Product Clean-Up**

1. Add 40µL sephadex G-50 resin to each well of a Whatman 96-well filter plate to be used
2. Add 300µL deionized water to each well to hydrate resin
3. Soak for >3h at room temperature
4. Pre-spin for 5 min at 2000 RPM into used 96-well semi-skirted plate (Neptune) and discard flow-through
5. Add 10µL of deionized water to each well used
6. Add 10µL of sequencing reaction to each well used
7. Spin for 5 min at 2000RPM into a fresh 96-well semi-skirted plate
8. Fit 96-well semi-skirted plate into holder and read with sequencer

Sequences were analyzed with ApE (Davis, 2011) version 2 for MacOS X.

### 7.4 Cell Culture

#### 7.4.1 Cell Maintenance & Passaging

Hana3A cells were cultured in a 100mm cell culture dish (Greiner BioOne 664160) at 37ºC, 5% CO₂ and saturating humidity. These adherent cells were maintained in 10mL of M10-PSF as described above, and were passaged at ~85-95% confluency with a split ratio of 1:4 or 1:8.

**Cell Passaging/Splitting**

1. Aspirate media and wash cells gently with 7.5mL Ca²⁺-free & Mg²⁺-free PBS (Corning Cellgro 21-040-CV)
2. Aspirate PBS and trypsinize with 3mL Trypsin-EDTA (0.05%, Sigma)
3. Incubate at 37ºC for 2 min
4. Add 5mL M10 to quench trypsin
5. Triturate cells
6. Centrifuge desired amount of cells for 5 min at 1000 RPM
7. Resuspend in 10mL of M10-PSF per 100mm dish and plate
8. Incubate at 37°C, 5% CO₂, saturating humidity

For assays, cells were resuspended in M10-PSF and plated at the appropriate density for 96-well poly-D-lysine (PDL) coated clear bottom plates (NUNC) or 35mm dishes (Corning)

7.4.2 Freezing & Thawing Cells

Cells were maintained as described above. To generate frozen stock, each 100mm dish was trypsinized and centrifuged as above, resuspended in 1mL freezing media, and transferred into a 1.5mL cryovial (VWR Scientific). Cryovials were frozen overnight in a -80°C freezer in an isopropanol bath to ensure ~1°C/min cooling rate for ideal preservation. Cryovials were stored at -80°C.

As required, one cryovial was rapidly thawed in a 37°C bath, resuspended in 14mL M10, and centrifuged for 5 min at 1000 RPM, and the supernatant discarded. The cell pellet was resuspended in 10mL M10 and plated on a 100mm cell culture dish, and allowed to adhere overnight, and were passaged when confluent.

7.5 GloSensor Assay

The GloSensor cAMP Assay (Promega) was used to measure the activation of OR-I7 by structural variants of octanal in Chapter 2. Briefly, the assay uses a cAMP-activated split luciferase strategy to measure close to real-time Gₛ coupled GPCR activation using a
genetically encoded “GloSensor” with cAMP binding domains fused to mutant forms of 
_PhotoBerlinus pyralis_ luciferase (Binkowski, Fan, & Wood, 2009, 2011; Fan et al., 2008). Upon 
receptor activation and cAMP production, binding of cAMP to the GloSensor causes 
conformational changes that increase light output with a pre-loaded substrate, which 
allows the kinetic measurement of receptor activation.

**Plating and transfection.** Hana3a cells were plated into 96-well PDL coated clear 
bottom cell culture plates (NUNC) at ~25% density in 50µL of M10-PSF on the first day of 
the experiment. On the second day, the media each well was aspirated and replaced with 
50µL of M10 transfection media containing 80ng rho-tagged OR, 10ng M3-R, 10ng RTP1S, 
10ng pGloSensor-22F and 0.2µL of lipopectamine 2000.

**Stimulation and measurement.** 18-24h following transfection, the cells were 
washed with 37µL HBSS stimulating medium and loaded with 37µL 2% GloSensor reagent 
for 2h at room temperature in the dark. At the end of 2h, the baseline luminescence of 
each well was read using a Polarstar Optima plate reader (BMG). Immediately following 
that, 37µL of odor diluted in HBSS stimulating medium (at 2x concentration) was added to 
each well to achieve 74µL total volume at the desired concentration, and each well was 
read with the same plate reader with a time interval of 90s per well for 30 minutes.

**Data analysis and interpretation.** Since the GloSensor assay allows a kinetic 
measurement of receptor activation, we were able to select and bin measurements that 
would better represent the initial activation of OR-I7. Therefore responses over t = 3-7.5 
minutes were binned and analyzed in Graphpad Prism where dose-response curves were 
fitted to determine the EC₅₀ values of each stimulating compound.
7.6 Luciferase Assay

The Dual-Glo Luciferase Assay System (Promega) was used to screen for and measure OR responses to anesthetics. Here, the experimental paradigm takes advantage of cAMP-mediated protein kinase A (PKA) activation, which phosphorylates the transcription factor cAMP response element binding protein (CREB), which causes transcriptional activation of a cAMP response element (CRE) driven firefly luciferase roughly proportional to the level of receptor activation. Combined with a constitutively-transcribed Renilla luciferase, the ratiometric measurement of both luciferases allow the quantitative measurement of OR activation. Further, since this is a lytic measurement process and not a kinetic measurement of activation, the longer stimulation durations (allowing for more luciferase accumulation) possible with this experimental approach increase the signal to noise ratio, and the relatively short read time for each plate enables a much higher throughput workflow compared to the GloSensor assay, which made a large-scale screen of anesthetic-activated ORs possible.

**Plating and transfection.** Hana3a cells were plated into 96-well PDL coated clear bottom cell culture plates (NUNC) at ~25% density in 50µL of M10-PSF on the first day of the experiment. On the second day, the media each well was aspirated and replaced with 50µL of M10 transfection media containing 5ng rho-tagged OR, 2.5ng M3-R, 5ng RTP1S, 10ng CRE-Luciferase, 5ng pSV40-Renilla and 0.2µL of lipofectamine 2000. Each plate contained a positive normalizing control of OR-S6 on the 12th column.

**Stimulation and measurement.** 18-24h following transfection, the cells were washed with 37µL CD293 stimulating medium and replaced with 25µL (ketamine) or 200µL (all other anesthetics) of CD293 stimulating medium containing the desired
concentration of test odor. Cells were incubated at 37°C, 5% CO2 and saturating humidity for 4h. At the end of 4h, 174µL of CD293 stimulating medium was carefully aspirated from wells containing 200µL media, and loaded into a Polarstar Optima plate reader (BMG) programmed to inject and measure in the following sequence via the top optic, without filter:

**Luciferase Injection and Measurement Protocol**
1. Read baseline and inject 10µL Dual-Glo luciferase reagent in each of the 96 wells
2. Shake 30s
3. Read luciferase values and inject 10µL Dual-Glo stop & glo reagent in each of the 96 wells
4. Shake 30s
5. Read Renilla luciferase value

**Data analysis and interpretation.** Data was output as raw values in a text file, imported into Microsoft Excel, then normalized and baselined. Data points were then imported into Graphpad Prism. Significance was determined by ANOVA analysis with Bonferroni post-tests at p ≤ 0.05 for comparisons at a single odorant concentration. For dose-response measurements, data was fit to a dose-response model in a similar fashion to GloSensor Data. Significance was calculated by comparing the fitted EC50 values with an extra sum-of-squares F test at p ≤ 0.05 with Prism.

### 7.7 Cell Surface Staining & Fluorescence-Activated Cell Sorting

Live cell surface staining of ORs was used to determine whether mutations that showed a decreased response relative to unmutated receptors was due to translation, folding, or trafficking dysfunction caused by the mutation. Here, the staining of live,
unpermeabilized Hana3A cells expressing rho-tagged mutant ORs would demonstrate the presence of the desired OR at the cell surface and show that they are indeed properly trafficked. Primary antibody anti-rho 4D2 was a gift from R. Molday (Laird & Molday, 1988).

**Plating and transfection.** Hana3a cells were plated into 35mm cell culture plates (Corning) at ~25% density in 2mL M10-PSF on the first day of the experiment. On the second day, the media each well was aspirated and replaced with 1mL of M10 transfection media containing 1.2µg rho-tagged OR, 0.3µg RTP1S, 20ng GFP, and 4µL of lipofectamine 2000 and incubated for 18-24h at 37°C, 5% CO₂ and saturating humidity.

**Live Cell Staining**
1. Aspirate transfection media and gently wash with 1mL PBS staining buffer
2. All subsequent steps are performed on ice
3. Aspirate PBS and add 1mL Cellstripper (Corning Cellgro 25-056-CI)
4. Triturate cells and transfer to 5mL round bottom polystyrene (PS) tubes (Falcon 2052)
5. Wash 35mm dish with 1mL PBS staining buffer and transfer to PS tube
6. Centrifuge at low speed for 2 min in cold room and aspirate liquid
7. Resuspend cells in 50µL PBS staining buffer containing 1:50 dilution of anti-Rho 4D2 primary antibody
8. Incubate >30 min on ice
9. Add 2mL PBS staining buffer
10. Centrifuge at low speed for 2 min in cold room and aspirate liquid
11. Resuspend cells in 50µL PBS staining buffer containing 1:100 dilution of PE-conjugated anti-mouse secondary antibody
12. Incubate >30 min on ice in the dark
13. Add 2mL PBS staining buffer
14. Centrifuge at low speed for 2 min in cold room and aspirate liquid
15. Resuspend in 2mL PBS staining buffer
16. Centrifuge at low speed for 2 min in cold room and aspirate liquid
17. Resuspend in 500µL PBS staining buffer containing 1µL 7-Aminoactinomycin D (7AAD)
18. Vortex PS tube before reading with BD FACSCanto II FACS.

**Data analysis and interpretation.** Data was collected with BD's FACSDiva software on a BD FACSCanto II cell sorter and results were analyzed with FlowJo software package.
Gating was adjusted such that only roughly spherical singlet cells were gated, and a total of 10,000 GFP-positive cells were counted. Results were plotted in a histogram format and the geometric average of each OR was calculated.
Appendix A – Table of ORs Screened in Chapter 4

Listed here are the ORs screened in Chapter 4

<table>
<thead>
<tr>
<th>MOR1-1</th>
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Appendix B – Amino Acid Sequences MOR136 family ORs

Alignment courtesy J.M. Perez-Aguilar
Appendix C – Amino Acid Sequence of MOR139-1

MOR139-1 Length: 930

1 ATGGAGCCAA GAAACAATAC TCATATTTTA GAATTTTTTC TATTGGGATT
51 TTCACAGGAC CCAAACCTGC AGCCTGTCTT ATGTGGCCTT TTCCTCTCCA
101 TGTACTTTGAT CACTGTAGTT GGAACCTTGC TCATTATCT CACCAATAC
151 TCAGATGCCA ACTTGCAATC ACCCATGTAC TTTTTTCTCT CTAACCTGTC
201 ATTTGTGGAT ATCTGCTTTT TTTCCACCAC TGTCCCAAAA ATGCTTGGA
251 ATATTCAAC ACAGAAAAT TCCATTAGCT ATGGAGATTG CATCACCAG
301 ATGTACTTTT TTCTGATTTT TGTGGAATG GACAACCTTC TCTTGCGCTG
351 ATGGGCCCTAT GACCGTTATG TGGCCATCTG CCATCCACTG CATTATCTG
401 GTATCATGAA TCAGAGACTC TGTGGATTTT TGGTTCTTGT ATGCTTGATT
451 GTGAGTGTTT TGCGAGCCTT GTCACAAATG ATGATGTAG TGCGGCTGTC
501 CTTCTGCACA GACATTGAAC TCCCACACTT CTTCTGGAAT TTGAATCAGG
551 TGCCACAGCT AACCTGGTTCT GACACCTTAC TCAATGATGT GTCATGGAT
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701 AGTATAAAGC ATTTTCCACC TGTGCATCTC ATCTCCTTCT GTTCTCGCTA
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801 CTCACAAGCA AGGTCAACAG CCTCAGATG GCTACAGATG TCGACTCCCA
851 TGCTAAACCC TTTCTACTAC AGCCTGAGGA ATAAAGATGT AAAAGGAGCC
901 CTGAAAGAGGC TCTTAGGCTT AAAACTATGA
Appendix D – Table of ORs Screened in Chapter 5

Listed here are the 791 mouse and 398 human ORs screened in Chapter 5.

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<thead>
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<th>MOR0-6</th>
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Appendix E - Amino Acid Sequences of Volatile Anesthetic-Responsive ORs

MOR136-3 Length: 930

1 ATGAGGATGG ATAATGACAG CGCTCTGTCT GAGTTCTACC TCTTGGGGCT
51 CCCCATTCGA GCAGAAGACC AAGCTTTGTA CTCTGTCTTG ATCCTGGCCA
101 TGTACCTGAC AACTGTGCTT GGGAACCTGC TCATCATCC ATACAGGGTG TGGTCTCCAG
151 CTGGACTCTC ACCTCCACAC CCCCATGTAC TTCTTCCTCA GCCACTTGGGCT
201 CTTCACAGAC ATCTCTTTTT CATCAGTCAC AGCTCCTAAG ATGCTCGTGA
251 ATATGCTGAC ACACAGTAAAG TCCATCCCAT ATACAGGGTG TGGTCTCCAG
301 GTGTATTGTT TCACCTTTTC TCAACGAGTT GCATGTTTTTC TTCTCAAACTC
351 CATGGCATAT GACAGGTATG TGGCCATCTG CCATCCTCTG CATTATAACA
401 TCATCATGAA TCTGAGGCTC TGTGTTCTTC TAGTAGTAAT ATCATGGGCC
451 TATCTTTTAA CCAATGCTCT GGCACACCCT CTTCTCTTTG CTGGGCTAC
501 TCACCTTTGA AACAATACCA TCCCCACTA TTTCTGTGAC CTCTCCTACCT
551 TGCTGAAGCT GTCTAGCTCA GACACCACCA TCAATGAGCT GGTACCTTTT
601 GTTTTAGTGA ATGCTGTGTAT TTCAATGCTCT GGGCACACCA TCTCCCTACCT
651 TTATGGGCTAC ATTTGTTGTA CACCTCTGAA AACTCTCCCT ATCAAGGGAA
701 TCCACAAAGC CTTGCACCAG GTGTGGCTCTC ACCTCTGTGT GGTACCTTTT
751 TACTATGGGG CCATCATTGG ACTATATTTT GTCCCCTCAT CTAATAACAC
801 TAATGACAAA GATGCTTGTG TGGCTGTGAT GTACACTGTC GTGCTACCCA
851 TGCTGAAATCC CTTTATTTAT AGTCTGAGGA ATCGGGATAT GAAAGAACA
901 CTGAAATAA TCCTCAGCAG AACAATAGA

MOR170-4 Length: 942

1 ATGAAACAAA TGGCAACAAA AAATGACTCT TCAGTGCTGT GTTTATTCTT
51 TATGGGACTG ACAGATCAAC CTGAGCTCCA GTTGCCCCTG TTTTTCCTGT
101 TCTTGCTGAA CCATACTGTT ATAGTGGTGG GTAATTTGAG CTTAATGAGT
151 CTTATTATCT TAAACTCCAA TCTCCACACT CCTATGTACT TTTTCCTTTT
201 AATATGACAA GATGCTTGTG TGGCTGTGAT GTACACTGTC GTGCTACCCA
251 TGCTGAAATCC CTTTATTTAT AGTCTGAGGA ATCGGGATAT GAAAGAACA
301 CTGAAATAA TCCTCAGCAG AACAATAGA
351 GCTGACAGTC ATGGCCTATG ATCGCTATGT GGCCATTTGT AAGCCTTTAC
401 TGTACAAGGC TATCATGGTA CCTAGGATCT GTTGTCTGCT GATGTGTGAC
451 TCATATTTGA TAGGGTTTGC TAGTGCCATG GTTCTGGCAG GTTTAATGAT
501 TAGGCTCAAC TTTTGTAATA ACAACATCAT CAATCCTAC ATGTGTGACA
551 TCTTCCCTGT CTCCTGGGATC TCTTCGGATC TCCTGCAGTA ACACCTATCT CAATGAGGCT
601 GTGAGTACTG CTGAGTGTC GGTCAGCTATC ATTTTATGTA GTCTGATTAT
651 CTTCATCTCA TATGCTATGA TCCCTTTCAA TATCGTTCAT ATGTCATCAG
701 GAA GGTTTGTC GTTCAAAGCC GGAAGTTCG AACCATGCAAA ATGTATTTT
751 GGT AGTTTTTT CTTAGATGAC TGGGCTCCTT GTGTATTG CTATGACAA
801 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGA
851 TAAGTTTGCTG TGAAGAAAAC CATAAAGAGA ATCAGTAAAC ACAAGGTTA
901 AAAGTTGCTG TGAAGAAAAC CATAAAGAGA ATCAGTAAAC ACAAGGTTA

MOR182-5 Length: 921

1 ATGGGAGTGA CACAGACCCG GGTGACTGCG TTTATTCTCA GAGGAA
51 AGATCTCTCCA GAGCTGCAAG TTCCCTGTT CTTGGTGTTC TTCTTCATCT
101 ATGTCGATCTG CATGGTGGGC AACCTTGGCT TGATCGTTCT CATCTGGAAG
151 GACCCTCGTC TTCACACACC CATGTACTTT TTCCTTGGAA GGTGGATAT
201 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
251 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
301 ATGTCGATCTG CATGGTGGGC AACCTTGGCT TGATCGTTCT CATCTGGAAG
351 GACCCTCGTC TTCACACACC CATGTACTTT TTCCTTGGAA GGTGGATAT
401 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
451 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
501 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
551 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
601 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
651 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
701 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
751 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
801 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
851 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
901 TGGTGCCCAT GCTGATGGTC ATGTTTGT GATTTCCTTC TGGGAGGAGAT
MOR184-1 Length: 927

1 ATGACTGAGG ACAACTACTC CTTGACAACA GAGTTCATCC TCATAGGATT
51 CTCAGACCAC CCAGACTTAA AGATACTTCT ATTCCTGGTG TTCTCTACCA
101 TCTATCTGGT CACCACTGTT GGGAACTTGG GGCTGTCGGC TCTGATCTAC
151 ATGGAGCCTC GTCTCCACAC ACCCATGTAC ATCTTTCTGG GCAACCTGCG
201 TCTCATGGAC TCCTGCTGCT CCTGTGCCAT CACTCCCAAG ATGCTAGAGA
251 ACTTTTTTTC TGTAAAACCA AGGATTCTCT TCTATGAATG CATGGCAGC
301 TCTATTTTTC TCTGTCTTGC TGAACCTGCA GACTGCTTTT TCTGGCAGC
351 CATGGGCTATG GACCGCTATG TGGCCATATG CAACCCACTG CAGTACCACA
401 CCATGATGTC CAAGAAGCTC TGCCCTGAAA TGACCACAGG AGCCCTACATA
451 GCAGGAAAAAC TCAGGCTCAT GATCCACATA GGTTCTGTG TCAGGTTCAC
501 TTCTGCTAGG TCTCATGTGA TCAAGCAGTG AGTCTGTTCT ATTTTATTCC
551 TATACAGACT TCTATGGTC CACCCCTTATA TCAATGAACT GATGATCTC
601 ATCTTTTTCGG ATCACTTTTCC AACCCTTCC ATTTATTAG TCTGATTTGC
651 TTATTTTCTGC ATCATTTTTA CTATATTTAG AGAGAGGAA
701 GAAGCAAGAC TCTATCTCAG TGCCCTGAAA TGACCACAGG AGCCCTACATA
751 TTCTATGCTG CTTCTGCTGC CACCTGCTCA CACTCCCAAG ATGCTAGAGA
801 GGAAATGAA GACATACCTG TTGCTATTTT ATATTCTCTG GTAATTCCTT
851 TATATAACCC ATTTATTTAT AGTGTGAGAA ACAATATAG GAATTAAGCA
901 ATTAAGAAGA CCATGAACCA AGGATGAG

MOR184-5 Length: 927

1 ATGGCTGAGA ACAACTATTC TGTGACAATA GAATTCATCC TGGGAGGGTT
51 CTCAGACCAC CCAGACTTAA AGATACTTCT ATTCCTGGTG TTCTCTACCA
101 TCTATCTGGT CACCACTGTT GGGAACTTGG GGCTGTCGGC TCTGATCTAC
151 ATGGAGCCTC GTCTCCACAC ACCCATGTAC ATCTTTCTGG GCAACCTGCG
201 TCTCATGGAC TCCTGCTGCT CCTGTGCCAT CACTCCCAAG ATGCTAGAGA
251 ACTTTTTTTC TGTAAAACCA AGGATTCTCT TCTATGAATG CATGGCAGC
301 TCTATTTTTC TCTGTCTTGC TGAACCTGCA GACTGCTTTT TCTGGCAGC
351 CATGGGCTATG GACCGCTATG TGGCCATATG CAACCCACTG CAGTACCACA
401 CCATGATGTC CAAGAAGCTC TGCCCTGAAA TGACCACAGG AGCCCTACATA
451 GCAGGAAAAAC TCAGGCTCAT GATCCACATA GGTTCTGTG TCAGGTTCAC
501 TTCTGCTAGG TCTCATGTGA TCAAGCAGTG AGTCTGTTCT ATTTTATTCC
551 TATACAGACT TCTATGGTC CACCCCTTATA TCAATGAACT GATGATCTC
601 ATCTTTTTCGG ATCACTTTTCC AACCCTTCC ATTTATTAG TCTGATTTGC
651 TTATTTTCTGC ATCATTTTTA CTATATTTAG AGAGAGGAA
701 GAAGCAAGAC TCTATCTCAG TGCCCTGAAA TGACCACAGG AGCCCTACATA
751 TTCTATGCTG CTTCTGCTGC CACCTGCTCA CACTCCCAAG ATGCTAGAGA
801 GGAAATGAA GACATACCTG TTGCTATTTT ATATTCTCTG GTAATTCCTT
851 TATATAACCC ATTTATTTAT AGTGTGAGAA ACAATATAG GAATTAAGCA
901 ATTAAGAAGA CCATGAACCA AGGATGAG
MOR185-1 Length: 957

1 ATGGCAACAG GGAATCTCAC TCATGTCACG GAGTTCATAC TCATGGGGGT
51 AACTGACCAG CCGAGACTGC AGGGCTCCTG GCATCATCAC CCTGACCTCC
101 GTGGACTCAC GCCTGCAAAC TCCCATGTAC TTCTTCCTCA GACACCTGGC
151 GGTCATCAAC TTTGGCAACT CCACGTCATG TGCCTCTAAA ATGCTGCTCA
201 ACTTCTTATG CAGTAAAAAA ACCACCTTAT ACTATGAATG TGCCACGCAG
251 CTGGGAGGAT TTCTGGTTTT CATGGTATCA GAGATCTTCA TGCTGGCTGT
301 GATGGCCTAT GACCGCTATG TGGCCATCTG GAACCCACTG CTCTACATGG
351 TGAGCTTCTT CTGGGAGGAT TTCTGGTTTT CATGGTATCA GAGATCTTCA
401 ATGGCAACAG GGAATCTCAC TCATGTCACG GAGTTCATAC TCATGGGGGT
451 AACTGACCAG CCGAGACTGC AGGGCTCCTG GCATCATCAC CCTGACCTCC
501 GTGGACTCAC GCCTGCAAAC TCCCATGTAC TTCTTCCTCA GACACCTGGC
551 GGTCATCAAC TTTGGCAACT CCACGTCATG TGCCTCTAAA ATGCTGCTCA
601 ACTTCTTATG CAGTAAAAAA ACCACCTTAT ACTATGAATG TGCCACGCAG
651 CTGGGAGGAT TTCTGGTTTT CATGGTATCA GAGATCTTCA TGCTGGCTGT
701 GATGGCCTAT GACCGCTATG TGGCCATCTG GAACCCACTG CTCTACATGG
751 TGAGCTTCTT CTGGGAGGAT TTCTGGTTTT CATGGTATCA GAGATCTTCA
801 ATGGCAACAG GGAATCTCAC TCATGTCACG GAGTTCATAC TCATGGGGGT
851 AACTGACCAG CCGAGACTGC AGGGCTCCTG GCATCATCAC CCTGACCTCC
901 GTGGACTCAC GCCTGCAAAC TCCCATGTAC TTCTTCCTCA GACACCTGGC
951 GGTCATCAAC TTTGGCAACT CCACGTCATG TGCCTCTAAA ATGCTGCTCA

MOR189-1 Length: 948
1 ATGCAGATGG AGAGCCAAAA CCTCACAGTA GTGACTGAAT TTATCCTCAG
51 GGGCATCACT GACCGCCCTG AGCTGCAAGT TCCATTGTTT GGACTGTTTT
101 TCTATGCTTC TATGACGCTG CTGTTTGGCA ACTTGGGCAT GATCATCCTC
151 ACCATAGTGG AATCAAGGCT GCAAACACCT ATGTACTTCT TTCTCAGACA
201 CCTAGCTATC ACAGATCTTG GTTATCAGGC TGCTATTGGA CCTAAAATGT
251 TAGCAAAATTG TGTGCTGAGT AAAAAATAAC TATCTTTTTG TCTTTTGCT
301 ACACAATTAG CCTTCTTCTCT TCTGTTCATT GCTGTTGAAC GTGTCATTCT
351 ATCTGTGATG TCTTACGACC GCTATGTTGCT TATCTGTAAC CCTCTGCTCT
401 ACAATGTCAT CATGTCACAA ACTGTATGTT GGCTACTTTG AGCAATACCA
451 TACCTTTACA GTGTATTTTAT TTCTCTCATA GTCAACATTATA ATATCTTCTC
501 TCTACCTCTTCT GTGTTGTTCTA ATGTCATCCC TCATTTCATC TGGTATAGTC
551 TCCCCCCTATG ATCTCTGCTT TGCACAAATA CAGATAAAAT GGGACTGATA
601 ATTTTAATTT TATCTGTCCAT TAATTTGATGT TCCTCTTCCT TGATCATCCT
651 TGGCTCTTAT CGCTCTCATCT CAGAGAGGAG CCGTCTGTGC TGGCTCTCAG
701 AGGGAGAGAC GAGGCTGTTC TCCACCTGTC GCTCCCATCT GACGGCTGGT
751 TCTGCTTTTT ATGGGACCTTT GATTTTTATG TATGTGCAAC CTAGACCTAG
801 TCCTCTCCTCT GACACTGACA AGGCGGTTCAT CATCTTCTAT ACCCTAGTTA
851 TCCCTATGTT AAATCTTTTGCT TCTCAGACCT AAGAAACACA AGATGGTAAG
901 TATGCTCTTTA GAAAGAGTTCT AAAAATTATA CAAAATAATT TCTCATAG

MOR202-8 Length: 939

1 ATGGAGAACCA GACAGAGGAGCATCTCTAG ATCTTTCTAG TGCTGACCAA
51 TGCCCCAGCA CTGCAGACCC CACTCTTTAT CCTGTCTCAG ATATTCTACT
101 TCACTCAACAT GACCTGAAAC TGGGGATGC TTGGTCTGAT TCTCTGAGAC
151 TCTGCTCTCC AACTCTCTCTTGT CTGTCTGTCTG CAAATCTTTTATG
201 AGACATCTTTT TATCTCTCCTG TCGTACCCCA AACTTTTGTTT GCTGGACTTC
251 TCTGTTGAAAA CCAAAGCTAT TCTCTAAGTA TCTGTGCTGAC CCAGATGGTC
301 TCTTTTTGTAG TCTTCTTGTAC GCGCGAATTAT TCTTCTTGTG CTGTCAATGG
351 CTGTGCTGGTC TGTTTCTGAC TCTGTCTGCTA ACTCTTCTTTG GCTGCAACCTG
401 TGACTCGACG TACATGTTGCA TGCGCTGACCA TGCGCTGCTA TGCTGGTGGT
451 TCTTGTAATT CTCTCATACG TGTCTGCTAG TCTCTTCTCTG GTCTGCTGCTG
501 CAAAAGCAT AATGCTTGCTC AAATTTGTCA CAAATTCTTCGG TGGCTCATAG
551 TCTCTCCTTG TCTCTGACAG CACATCATGAG AGATGGCTCT CTTATATGGA
601 GCAAGTTTTG TCATCTGTTC TGCACTCCTT GTTATTTTGA TATCTTACAT
651 ATTCATTTTT ATCACCATCT TCAAGATGCG CTCAGCTGCA GGATACCAGA
701 AGGCTATGTC TACCTGTGTT TCTCACTTCA CTGCTGTCTC TATTTTCTAT
751 GGCACCTTCA TTTTCTAGTA CTTCGAGCCC AGCTCCAGCC ACTCCATGGA
801 CACTGACAAA ATGTTGCTGT TGTCTACAC CATGTCATT CCCATGCTGA
851 ACCCTGTGGT CTATAGCTTA AGAAACAGAG AGGTGAAAAG TGCATTCAAG
901 AAAGTTGGAAA AAGGACAAA ATATACCTTA GGATTTTGA

MOR251-5 Length: 915
1 ATGAATGGGA CACTGGTCAC TGAGTTCCTC ATCCTGGGAT TCTCAGATAT
51 GCCTCACCTT CGGATACTAC TTTTCCTCAG CTTACATTGC ATATACATGG
101 ACCCTGCATA CCCCTATGTA CTTCTTCCTG GTCAACCTGG CTGTGGTGGA
151 ACCCTGCATA CCCCTATGTA CTTCTTCCTG GTCAACCTGG CTGTGGTGGA
201 CATCCTTTGC ACCTCCACCA TCTCACAAAT GCTACTTGAG ACAGTATGTA
251 CAGGAAAGGAC CATCCTCTTC GGGGTCGCA TGGCCAGACT CTTCCTTCTC
301 ACATGGTCAC TGGAGCGACA CTTTCTGTCT TTTCAGCTTA TGGGCTATGA
351 CCGCTTTGTG GCCATCTGCT GTCCCTCTGCA CTACAGTGCT TGGAATGGGC
401 CCAAGGGTGTG TGCCATTCTG CTGGCATCTAG CAGGCTGACT
451 AACACACAGG TGCACACAGG CCTGATGTGG CGTCTACCAT TCTGACAGCT
501 CAATGGATATA GAGCAGGCTT CTTGTAGACA CACCGGACTT TTGGAAGCTT
551 CCTGCTGCTCC AAGCAGGCA AATGAGACCA TGGAATTCTG TGCCGATAGT
601 TTCCGGCTCC TAGGAACTGT CTCTGGGACT ATGAGCTGTTA
651 TGGTGCCAGC ATCCTGAGAA TCCGTCGAC TGGAGCAGGA CGACGACGCT
701 TCTCCACCTG CTCGACACCT CTATCATGGT GCATTGTGTA CTAATCTACCT
751 GTCTACTACA CATTCTATCG CTTCTCCATC AGCTACTAC TAAAGAAGGA
801 CAAGGTGGTG TCCACGCTCT ACAAACGCTG GGCACCCACC TTGAACCCCC
851 TCTACACAC CCTGAGGAAAC AAGGATGTCA AAGTTGCACCC CCGAGACTT
901 TTCTCTCTGC GCTGA

MOR252-1 Length: 924
1 ATGGCTCACG TGAACGACTG AAGTATAACC ATGGTCATCC TGCAAAAATCT
51 CGTTGATGAC CCCTCCGATCC AGATATGCT CTTCTGACCT CTTTTGCTC
101 TGTTTTATGGG AGCCATAGCT GGCAATGGCC TGATCATCAG CACCATTACAC

127
MOR252-2 Length: 924

1 ATGGCGACTG TAAAACAGTC GGTTTGAGCC ATGTTCATCC TGCAAGGGT
51 TGCTCTGGAC CCGGATCTCC AGGAGTCTCT CTTCTGCCTC TTCTTTGCCT
101 TGCTCTGGAC TAAAACAGTC GGTTTGAGCC ATGTTCATCC TGCAAGGGT
151 AGCCAACGCC AACCCACCAC TCCCACTGAT ACATCACATC TCACTACCTT
201 CGCCATGGAC ATGACATCGC TGAACACACC TACTCATATC TCTGAGATC
251 GCAGCCACAC GGTATCTTTGT GTGTTTCGTGC TATCCAGCCT CAGGGGTAC
301 CTCTGAGATC CTGCTGACCC ACTCTTTATG ATGACATCGC TGAACACACC
351 TGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
401 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
451 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
501 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
551 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
601 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
651 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
701 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
751 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
801 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
851 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
901 TGGGCTGAGCT GGTAGTTTCCT ATGTAAGGGG TGGGCTGTTTT GTGCTGCTT
751 TATTCACCTG TTCTCTATAC TTATGTCCGG CCAGCCCTAG GAACCTGCTGG
801 GTTCCCTGGAC AAACACTTCTG CTGTCTCTGA CACCACTGTA ACCCCCATCTC
851 TGAACCCCTT TATCTATACC CTGAGAAATA AGGAATTAA AATATCTTTT
901 AAAAACTCT TATTTCCCA TTGA

MOR253-2 Length: 936

1 ATGATGTTGA GACTCAACCA GACAGAAGTA ACAGAGTTTG TGCTGGAAGG
51 GTTCTCAGAG CACCCCTGATC TAAGATTTGG TGCTGGAGGG
101 CCCTCTACAT AATGGCTCTG ATGGGCAACA TTTTGATCAT CGCTTTGGTT
151 ACCTCCAGCA CTGGGCTCCA CAATCCCATG TACTTTTTCC TGTGCAACCT
201 GGCTACCACG GATATTTTTG GTGACCTCCTC TGTGATCCCT CAGCTCTTCT
251 TTGGGCAGT GTCTGAGGAA AACACTATCT CCTTCAAGGA ATGTATGCT
301 CAGCTCTTCT TCTTCTGATG GTCAGCATCT TCTGAGCTGC TGCTGCTCAC
351 GGTCATGGCC TATGACCGCT ATGTGGCCAT CTGCTGTCCC CTGCACTACA
401 GCTCTAGGAT GAGCCACACG AGTGTGTTGGG CCTTGGCGCAT GGGTGATAGG
451 TCCATCAGTG CTGTGAATGC ATCTGTGCAC ACTGGCCTGA TGACACCGGT
501 GTCATTCTGT GGACCCAAGG TCATCACCCA CTTCTTCTGT GAGATTCCCC
551 CACCTCCCTCT GCTCTCCTGT AGTCCTACAT ATGTAAATAC CATTATGACT
601 CTTTTGGGAG ATTCCTTTTT TGGAGGGCTC AATTTTGGTC TTAACCTTCT
651 ATCCTATGGC TGCATCATTG CCACGCATCCT GGCGATGCGT TCTGTGAGGG
701 GCACAGAGAA GGCCCTTTCT ACCTGCTCAT CCCACCTCAT TGTGGTCTCT
751 GTGTACTCCT CATCTGGTGT CTGTGCCTCT CGCTGGGCA CTGCTGCTGTA
801 CAGTCCAGAA AGAAGCAAGG TTACCTCAGT GTTATACTCG ATCGTCAGCC
851 CAACCCCTCA CCCCTCCTAC TATAACCTGA GGAAACAGGA TGTCAGCTCT
901 GCCCTGGGCA GAATATTGCC CTCTTTCTCA CATTAA

MOR253-3 Length: 939

1 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
51 AGGGTTCTCA GAGCACCCTG ATCTAGATTGT TCTCTCTATA GGCTTGTCTCC
101 TGTCCTCTCA CATGATGGGC CTAATGGGCA ACATTGTGAT CATCGCTTTTG
151 GTGACCTCCT CAGCTGGGCT CTAATGCTCAT TGTGGCTCTCT GTGTACACT
201 TGTGCCTGGT GTTCTGCTGCTG TCTGCTGGCA
251 CGTGGGCACC ATGGACATTG TGTGGCCTCCT CGTCTGCTGCTG TGTGGCCTCCT
301 TCTCTTCTCA TCTTACTCCT CAGACTAGTA TTTTTTTTTT TTTTTTTTTT
351 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
401 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
451 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
501 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
551 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
601 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
651 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
701 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
751 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
801 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
851 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA
901 ATGATGATGC TAAGACTGAA CCAGACAGAA GTGACAGAGT TTGTGCTGGA

129
301 GCCCAGCTCT TTTTCTGTGT TGGTGCTAGC TCTTCTGAGC TGCTGCTGCT
351 CACGGTCATG GCCATAGACC CAGCTATGTTT CCCCTACTAC
401 ACAGTCTCTAG GTGGACCCCA CAGCTCTGTG GGGCTCTGGC CGTGGGTGTG
451 TGGTCCATCT GTGCTCTGAA TGCATCTGTA CACACTGGTC TGATGACACG
501 GCTGTCATTCC TGGTAGACCA CAGTAATCAC CCACTTCTTC TGTGAGATAC
551 CCACTCCTCC TCTGTTTCC TGGTAGTCAC CATACATTAA TAGTTTTATG
601 ACATCTTGTGGA CAGATGCTCTT TTTAGGAGGC AGCATATTTC TGCTAACCCT
651 GTTGTCCCATG GGGCAGCATT CAGGTCTGTTT TGGTTCTGCTG
701 AGGGGCAAGAG GAAAGCCTTT TCTACCTGTGTC ATCCACCGAC TATGTGATGGTC
751 TCTGTGTACT ACTCATCTGT GTTTCCTGCT CAGATTAGGC ATCAACTTCG
801 GCCACCGGCA AAAGATAGCA AATTCTCTTC TGGTCCATGGGC TACATTTTCT
851 GACAGCTGCTG GCTGTCATTCC TCTGTGCTGAC TTGCTGCTGCT
901 ACCCTGCTCTT CTACCTTCTGT AAGGTGCTGAT GGTGCCTGCTG

MOR253-4 Length: 960

1 ATGGTGGACC TGGTGCTTCTC ATGATGATG CTGAATCTCA ACCAGAGAAG
51 AGTGACAGAG TTTGTGCTGG AAGGGTTCTC AGAGCACCCT TGGCTAAATAT
101 TGGGTTCCATG AGGCTGCCTTC TGGGCTCTTC TGAATGGGCTAG
151 ACCATTTTGA TCACTTCTTC ATGGGTTATC TACCTGCTTC TACCACTGCA
201 CATGTACTTT TTCTGTGCTATG GACCTCAATG GTGTAACCCCT GCACAGGAC
251 TCTTGCTGTGT TCCCTAAGGAC TTTGTGCTGG TGGTTGTCTG GGAAGGCAAG
301 ATCTCGTGTAG CCAGAGGATT CAATGATCAG GAGGTTCTT CAGGTCTGCTG
351 GACAGCTGCTG GCTGTCATTCC TCTGTGCTGAC TTGCTGCTGCT
401 ACCCTGCTCTT CTACCTTCTGT AAGGTGCTGAT GGTGCCTGCTG
451 AGGGGCAAGAG GAAAGCCTTT TCTACCTGTGTC ATCCACCGAC TATGTGATGGTC
501 GACAGCTGCTG GCTGTCATTCC TCTGTGCTGAC TTGCTGCTGCT
551 ACCCTGCTCTT CTACCTTCTGT AAGGTGCTGAT GGTGCCTGCTG
601 ATGGTGGACC TGGTGCTTCTC ATGATGATG CTGAATCTCA ACCAGAGAAG
651 AGTGACAGAG TTTGTGCTGG AAGGGTTCTC AGAGCACCCT TGGCTAAATAT
701 TGGGTTCCATG AGGCTGCCTTC TGGGCTCTTC TGAATGGGCTAG
751 ACCATTTTGA TCACTTCTTC ATGGGTTATC TACCTGCTTC TACCACTGCA
801 CATGTACTTT TTCTGTGCTATG GACCTCAATG GTGTAACCCCT GCACAGGAC
851 GACAGCTGCTG GCTGTCATTCC TCTGTGCTGAC TTGCTGCTGCT
901 ACCCTGCTCTT CTACCTTCTGT AAGGTGCTGAT GGTGCCTGCTG
901 CTGAGGAAACA AGGATGTCAA GCTTGCTTGG GGCAGAATAT TGGCTTCTTT
951 CTCACATTAA

MOR253-5 Length: 939
1 ATGATGGTTG TGAGTCTCAA CCAGACAGGA GTGACAGAGT TTGTGCTTGG
51 AGGGTTCTCA GAGCATCCTG GTCTAAAGACT GTTCTCTCACA GGCTGCTCT
101 TGACCCCTCTA CATGATGGCA CTAATGGGCA ACATTGTGAAC TATGCTTCT
151 GTCACTCCTCA GCACTGGACT CCTCAATCCCA ATGTACTTTT TCCTTGCA
201 CTGATGCTAC GCTGTTATG TGTCACCTTC CCAAGGGAATCG CTATAGCTAG
251 TGATTGGGCTG AGATATCCTG GAAACACTCA TCACCTCTG CAAGATGTATG
301 GCCACGTCTT CTTTCTTGG CAGGACTGGA ATGGAAGAGC TTAGCTGCCT
351 CACGATCTAT GCTATCTTGG CATGATGGTC GGAAGACAGT GAGGATGTATG
401 ATCAGCTGTG AAAGACAGGA GAGGCTGCTG CTGCTGCTTGG CTTGACAGAC
451 GTATGTCTCT CTTTGATCTG TTTCTGATCT GATGTCTCTT TCCTGCACCT
501 GCCATGCTTCT TCCTGATCTG TTTCTGTTGG GTATGTCTCT TTTCTGCTCT
551 CTGAGCTTCT CTTTGATCTG TTTCTGCTCT AGGCTGCTCT GAGGATGTATG
601 ATCAGCTGTG AAAGACAGGA GAGGCTGCTG CTGCTGCTTGG CTTGACAGAC
651 ATCAGCTGTG AAAGACAGGA GAGGCTGCTG CTGCTGCTTGG CTTGACAGAC
701 ATCAGCTGTG AAAGACAGGA GAGGCTGCTG CTGCTGCTTGG CTTGACAGAC
751 ATCAGCTGTG AAAGACAGGA GAGGCTGCTG CTGCTGCTTGG CTTGACAGAC
801 ATCAGCTGTG AAAGACAGGA GAGGCTGCTG CTGCTGCTTGG CTTGACAGAC
851 ATCAGCTGTG AAAGACAGGA GAGGCTGCTG CTGCTGCTTGG CTTGACAGAC
901 ATCAGCTGTG AAAGACAGGA GAGGCTGCTG CTGCTGCTTGG CTTGACAGAC

MOR253-9 Length: 930
1 ATGATGGAGA ACCACACACT GGTAACAGAG TTTCTTGGTC AGGGGCTTCTC
51 TGAGCAACCAA CAGTACCAGC TGTCTCTTC GCTCACTTGC TATGCTTTGC
101 ACTGATGGGC CAGATGACACT TGCATGACAG TGTCTCTTTC TATGCTTTGC
151 AACCTGGGCC CAGGAGGCG CATGATGTTT TTCTGGTATAT ATTTGGCTAG
201 TGAGCATGTC TCTCGCTTAT GCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
251 TGAGCATGTC TCTCGCTTAT GCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
301 TGAGCATGTC TCTCGCTTAT GCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
351 GGCCTACGAC CGTTATGCGA CATGCACTTC CCACTGCTATAC TACAGCAGCA
401 TGATGAGCAA AGCATTCTGT AGCATGTTGG CTGCTGGAGT GTGGGCACTC
451 TGCCCTTTCA ACACAGCCAT TCACACAGGA CTGATGACTC GCTTGAGCTT
501 CTGTGGGCCC AATGTCATTA CACATTTCTT CTGTGAGGTG CCTCCTCTCC
551 TGCTTCTCTC CTGCAGCTCC ACCTACGTGA ACAGTGTCAT GATTGTCTTG
601 GCGATGCTCT TTTATGCACT ATTTGAACCA TTCGAGAGCA AGAGGAAGGA
651 CGGCTTTATC ATTTCCAGCA TCCTGAAGAT GCGACATCA GAAGGGAAGC
701 AGAAAGGCTT CTCAACCTGC TCTTCCCACC TCATTGTGGT GTGCATGTAT
751 TACACCGCTG TCTTCTATGC CTACATAAGC CCTGTCTCCA GCTATAATGC
801 AGGAAAGAGC AAATTGGCTG GTGTGCTGTA TACCATGTTG AGCCCTACGC
851 TCAACCTCTC GTATCTATCT TTGAGAAACA AGGAAATGAA AGCAAGCTCTC
901 AGGAAATTTT TTCTTTCTCT CAGAAATTAA

OR2B11 Length: 954

1 ATGAAAAATG ACAACCATAG CTCTTTAGGG GACTCCCCCTA AAGCCTTCAT
51 CCTTCTGGGT GTGTCGACA GCCGTGGCTG GGAACCTCCT CTCCTTTGGG
101 TCCTCCTGCT GTCTATGCT TGTTGCACTG TGGGGACGT CGCCATCATC
151 CTGGCATCCC GGGTGAGGCT TCGAGAGCCATG TCACCATTTCT AAGCCTGGGT
201 CATGTACCCT GTCTTTCTCG ACCTCTGGCT CACCAACTCC AAGCTCTGCT
251 AGATGCTTGG CAACATGCGG AGTTCCAGGA AGACCACGAG CATATGGGAGC
301 TGCACTCTGC AATATGCGCT CTCTTTAGGG GACTCCCCCTA AAGCCTTCAT
351 CTGTGTGCCT GCCACCCGCT TCGAGAGCCATG TCACCATTTCT AAGCCTGGGT
401 TCAGCCCTCT GACCTGTGCT CACTTGCCCT GCAGGCTCCGT CAGATGCCTG
451 CTGGCTTTCC TCTGCTGGCT CTGGACTCGC TGGCAACCAGT CAGAGCTCCT
501 GGTGCAGGTG CTTCTTTCTC TCTTCTCTCC CTGAGAGCCATG TCACATCGCT
551 AGTGTCGAGC CGTGAGCAGC GCTGAGCGAGT GCTGATGGG GCTGAGCTCC
601 ACCATCTGG CGTGTGCTGG CTGGCTTCTC GCTTTGCTCTG AAATGCCTGG
651 CATCTCCTCT CTATGCTGGT GAGCTCGCTC AATATGCGCT TCCTTTGGGG
701 CCTTCAAGGG ACGACACAGG CCGGTTGGCA GCGATGACCC CGGCGGCACT
751 ATCGTCTCCG TCTTCTACCT ATCTGAGTTA CATATGTGAG GCAGGGCCCG
801 TTCCAGTCTC TCCCAAGAGC AAGGCAAAAT TATTTTCTCT TCTTATTCCA
851 TAAATCCAGCC CACTCTCTAT CCGGTTGAGG CCACCGGAGG AAATAGGAGA
901 ATGAAAGGGG GCCTGAGGAG ACCTCTGGCC AGGATCTGGA GGCTCTGGTG
951 ATGA
References


Biography

Jianghai Ho was born in Johor Bahru, Johor, Malaysia on 21st of November 1985.

In May 2008, he graduated with honors from Duke University, where he majored in biology and music.

First Author Publications:


Other Publications:


Conference Abstracts/Posters:
