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Cristina F. Bush

Olfactory Receptor Dimerization

By
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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
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OLFACTORY RECEPTOR DIMERIZATION

By

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B.S., University of South Florida, 2002

Adviser: Randy A. Hall, Ph.D.

An Abstract of a dissertation submitted to the
Faculty of the Graduate School of Emory University in partial fulfillment of the
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Molecular and Systems Pharmacology Program
Graduate Division of Biological and Biomedical Sciences

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ABSTRACT

Olfactory receptors (ORs) comprise the largest subfamily of G protein-coupled receptors (GPCRs) and are responsible for the initiation of olfactory perception. In the olfactory epithelium, ORs are found at the plasma membrane of olfactory sensory neurons, localized to cilia that extend into the nasal cavity and exposed to the external environment. In this manner, ORs are readily accessible to bind inhaled environmental chemicals that serve as ligands and initiate signaling cascades that result in olfactory perception. Additionally, ORs may be involved in other aspects of chemodetection in the body, as a growing number of non-olfactory tissues including the prostate, spermatids, and developing heart also exhibit OR expression.

Despite intense interest over the past two decades in better understanding OR properties, characterization of OR pharmacology, biochemistry, and signaling mechanisms has been limited. A key obstacle hindering the study of ORs has been difficulty in efficiently expressing these receptors in heterologous cells. When expressed in common cell culture systems, the bulk of OR proteins are retained in the endoplasmic reticulum, with very little of the receptor localizing to the plasma membrane. The central hypothesis of this work is that ORs expressed in heterologous cells lack one or more critical components present in native cells that are required for proper localization. The studies presented here demonstrate that some ORs can heterodimerize with specific non-OR GPCRs, which results in significantly enhanced plasma membrane localization of ORs. Moreover, some of these receptor-receptor interactions can influence the G

protein coupling specificity of ORs. In addition to heterodimerization with non-OR GPCRs, the data presented here demonstrate that ORs also possess the capacity to homodimerize as well as heterodimerize with other ORs. Collectively, these data reveal previously-unappreciated receptor-receptor interactions that can significantly influence OR functionality. The findings presented here provide a means by which certain ORs can be effectively expressed in heterologous cells and shed light on fundamental aspects of OR biology.

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TABLE OF CONTENTS

INDEX OF FIGURES

Page

INDEX OF TABLES

LIST OF ABBREVIATIONS

3-MVa	3-methylvaleric acid
4-MVa	4-methylvaleric acid
4-PB	4-phenylbuturate
A2AR	adenosine A2A receptor
ACP	acetophenone
AR	adrenergic receptor
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CNG	cyclic-nucleotide gated
CPM	counts per minute
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
Fsk	forskolin
GPCR	G protein-coupled receptor
G protein	guanine-nucleotide binding protein
GRK3	G protein-coupled receptor kinase 3
GTP	guanosine triphosphate
HA	hemagglutinin
HEK	human embryonic kidney
HODR-4	human odorant response abnormal-4
HRP	horseradish peroxidase-conjugated

IP_3	inositol (1,4,5)-trisphosphate
ISO	isoproterenol
KO	knockout
MAPK	mitogen-activated protein kinase
M71	mouse 71 olfactory receptor
OE	olfactory epithelium
ODR-4	odorant response abnormal 4
OR	olfactory receptor
OSN	olfactory sensory neuron
P2Y1R	purinergic P2Y1 receptor
P2Y2R	purinergic P2Y2 receptor
PBS	phosphate buffered saline
PDZ	post-synaptic density-95, discs large, zona occludens-1
PIP_2	phosphatidylinositol (4,5)-bisphosphate
POGR	prostate overexpressed GPCR
PSGR	prostate specific GPCR
PTX	pertussis toxin
Rho	rhodopsin
TAAR	trace amine associated receptor
TMD	transmembrane domain
WT	wild-type

CHAPTER 1:

Introduction[1]

[1] A portion of this chapter has been accepted for publication. Bush CF and Hall RA (2008). Olfactory receptor trafficking to the plasma membrane. Cell. Mol. Life Sci.

1.1 Olfaction

Olfaction is a key means by which organisms perceive their surroundings. Chemical molecules in the environment relay important qualitative information about survival necessities, such as the locations of food and water sources. Potential dangers such as fire and predators are identified via chemical cues as well. Olfaction and other forms of chemodetection, for example pheromone detection, also influence many social behaviors, including mate selection and aggression. A diverse array of species share conserved olfaction principles, exemplifying the crucial role olfaction has served in organism survival through evolution (Ache and Young, 2005).

1.2 The mammalian olfactory epithelium

In humans and other mammals, olfaction occurs in the nose, specifically in a region towards the upper back portion of the nose called the olfactory epithelium (OE). The OE is comprised of three cell types that work together to enable the sense of smell. Actual chemodetection takes place in a population of specialized neurons known as olfactory sensory neurons (OSNs) (Figure 1.1). Supporting sustentacular cells surround the OSNs and are thought to detoxify noxious compounds, thus protecting OSNs by serving as both a chemical and physical barrier (Ding and Coon, 1988; Lazard et al., 1991; Nef et al., 1989). Equally important are the basal cells that lie at the base of the OE and function

as olfactory stem cells, replenishing damaged or old OSNs. Few neuronal cell types possess the capacity to regenerate, however OSNs are estimated to turn over every 70-90 days (Barber and Ronnett, 2000).

Morphologically, OSNs are bipolar cells that extend a single axon distally towards the olfactory bulb while a dendrite extends proximally, terminating near the edge of the OE at the dendritic knob (Figure 1.1). A large number of cilia project from each dendritic knob, extending into the external environment of the OE. It is here, on the cilia and readily accessible to inhaled environmental chemicals, that olfactory receptor (OR) proteins are localized. (Some reports also show evidence for OR expression on OSN axons; Barnea et al., 2004). A thin layer of mucus covers the OE and serves to trap inhaled odor molecules that then diffuse towards ORs or are bound by odorant binding proteins secreted into the mucus. Binding of an odorant molecule to the OR is the initial step in the cascade of olfactory perception.

1.3 Olfactory signaling in mammals

Mammalian ORs in the OE are activated by inhaled volatile odorants. A single odorant can serve as the ligand for multiple OR subtypes and individual ORs can have multiple ligands (Malnic et al., 1999). Additionally, structurally similar odorants can act as either agonists or antagonists (Oka et al., 2004). Through this combinatorial manner, ligand binding elicits a conformational change in the OR that activates appropriate downstream signaling cascades.

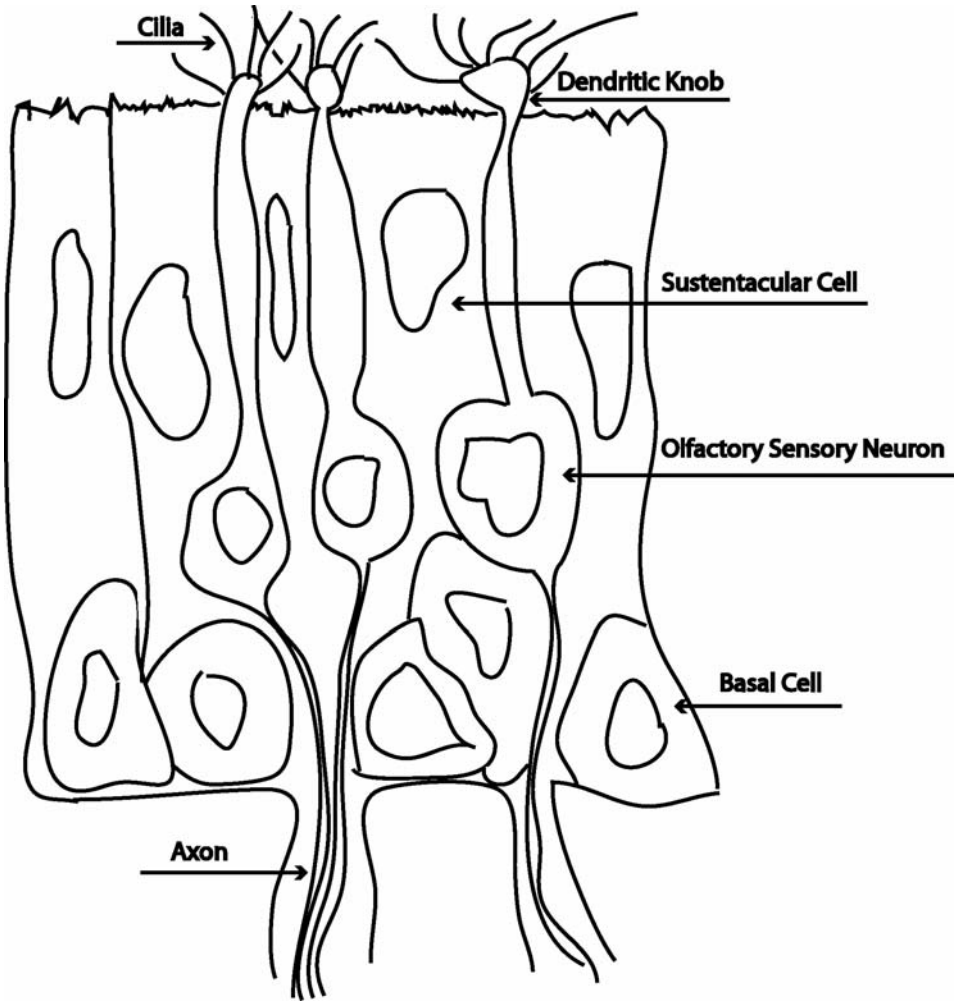


Figure 1.1. Anatomy of the mammalian olfactory epithelium. The olfactory epithelium is comprised of three distinct populations of cells. Olfactory sensory neurons (OSNs) extend a single dendrite that terminates in a knob at the interior surface of the nasal canal. Branching from each knob are multiple cilia that are the site of olfactory receptor expression. At the distal end, OSNs extend a single axon through the base of the epithelium towards the olfactory bulb. Axons of OSNs that express the same OR converge together. Sustentacular cells act as a sheath, surrounding and protecting the OSNs. Olfactory stem cells known as basal cells lie at the base of the epithelium and replace old or damaged OSNs.

In the most intensely studied OR signaling pathway (Figure 1.2), an activated OR couples to $G\alpha_{olf}$, a guanine nucleotide-binding protein (G-protein) enriched in the OE (Jones and Reed, 1989). $G\alpha_{olf}$ belongs to the family of G_s G proteins, which upon activation stimulate adenylyl cyclases via their α subunit resulting in the generation of cyclic AMP. Adenylyl cyclase III is the predominant form found in OSNs (Bakalyar and Reed, 1990). Increased levels of cyclic AMP activate cyclic-nucleotide-gated cation channels and the subsequent influx of Na^+ and Ca^{2+} causes depolarization of the cell (Dhallan et al., 1990). The increased concentration of intracellular Ca^{2+} also allows for opening of Ca^{2+}-gated Cl^- channels (Menini, 1999). High intracellular Cl^- concentrations in OSNs causes efflux of Cl^- upon channel opening and potentiation of the depolarization (Kurahashi and Yau, 1993).

Though $G\alpha_{olf}$ is the best-recognized coupling partner for ORs, quite a variety of other G proteins have been identified in vertebrate OE. These include $G\alpha_{s\ short}$, $G\alpha_{il}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, and $G\alpha_q$ (Schandar et al., 1998). There are also numerous reports citing the potential of ORs to signal through various pathways beyond cyclic AMP generation (Paysan and Breer, 2001). ORs have been demonstrated to signal through $G\alpha_s$ and $G\alpha_{15/16}$ (Kajiya et al., 2001) in addition to $G\alpha_{olf}$, and both longstanding and recent pieces of evidence suggest that ORs may couple to an unidentified G protein that activates phospholipase C, generating inositol 1,4,-5-trisphosphate and diacylglycerol (Huque and Bruch, 1986; Ko and Park, 2006; Schandar et al., 1998). Work presented in this thesis

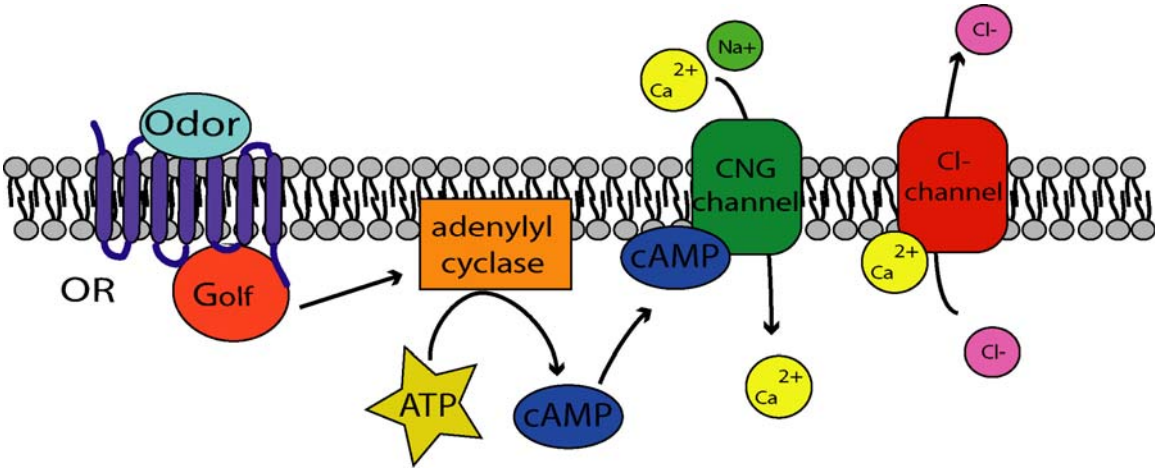


Figure 1.2. Canonical signaling pathway of native olfactory receptors. Inhaled environmental odorants bind and activate olfactory receptors (ORs) localized at the plasma membrane of olfactory sensory neuron (OSN) cilia. OSNs express high levels of G_{olf}, which is a G protein coupling partner for ORs. Stimulated G_{olf} activates adenylyl cyclase, resulting in cyclic AMP (cAMP) generation. cAMP binds and activates cyclic-nucleotide gated (CNG) channels allowing for influx of positive ions to initiate depolarization. Increases in intracellular Ca^{2+} lead to activation of Ca^{2+} regulated Cl^- channels, potentiation of the depolarization, and signal transduction through the OSN.

also demonstrates the capacity of an OR to couple to $G\alpha_{o,}$ leading to downstream activation of the mitogen-activated protein kinase pathway (Bush et al., 2007).

The depolarization elicited downstream of OR activation is propagated to the axon terminals of the OSNs, which synapse onto the dendrites of mitral and tufted cells in the olfactory bulb. Many thousands of such synapses form highly ordered structures called glomeruli. The axons of OSNs that express a particular OR target the same glomeruli, generally one located in each the medial and lateral hemispheres of the olfactory bulb (Mombaerts et al., 1996; Ressler et al., 1994). In this manner, signals originating from OSNs that express the same OR but may be scattered throughout the OE, converge at just two specific sites in the bulb. Thus specific odors activate defined patterns of glomeruli (Belluscio and Katz, 2001; Rubin and Katz, 1999). The positions of these glomeruli are remarkably conserved among animals of the same species, however it is not clear precisely how such defined axon convergence is accomplished. Evidence suggests that ORs themselves are a key determinant in the process (Bozza et al., 2002; Feinstein et al., 2004; Mombaerts et al., 1996; Ressler et al., 1994; Vassar et al., 1994). Odorant-evoked glomerular activity transduces through the olfactory bulb and output passes on to numerous higher brain regions including the olfactory cortex and the piriform cortex.

1.4 Identification of the olfactory receptor gene family

Early biochemical and electrophysiology studies implicated involvement of a G protein mediated pathway in olfaction. Isolated rat cilia exposed to odorants were shown to induce rapid activation of adenylyl cyclase and generation of cyclic AMP as well as inositol trisphosphate in response to some odorants (Boekhoff et al., 1990; Breer et al., 1990; Pace et al., 1985; Sklar et al., 1986). It was noted that adenylyl cyclase activation required guanosine triphosphate (GTP), suggesting involvement of receptor-coupled GTP-binding proteins. Operating under the assumption that G protein-coupled receptors (GPCRs) were indeed involved, degenerate primers corresponding to conserved GPCR sequences within the second and seventh transmembrane regions were generated. These primers were used in PCR reactions with template cDNA generated from rat OE RNA (Buck and Axel, 1991). The resultant PCR clones were found to contain features conserved among GPCRs and were also found to be enriched specifically in an olfactory neuron cDNA library. The identified clones also each shared motifs not general to the GPCR superfamily, indicative of a novel receptor family (Buck and Axel, 1991). In this manner, Buck and Axel successfully cloned the first 18 members of the rat OR family and opened the floodgates for current research in the field of olfaction.

1.4.1 Olfactory receptor genes and proteins

A significant fraction (1.4-4%) of all mammalian genes encode ORs (Mombaerts, 2004a), however approximately 25% of mice and 50% of human ORs have become pseudogenes through evolution. Notwithstanding, ORs still constitute the largest GPCR subfamily in mammals with nearly 1000 intact genes in rodents and greater than 300 in humans (Godfrey et al., 2004; Malnic et al., 2004). OR genes are typically around 1kb in size, usually intronless, and found on nearly every chromosome. OR genes are also found to be expressed in many different tissues, as is reviewed in section 1.4.2.

Indeed, the majority of olfaction research has focused on ORs expressed in the context of their chemosensory role in OE. It has been shown that only a small subset of OSNs express each OR gene. In rodents, individual ORs are expressed randomly throughout one of four zones covering the surface of the OE (Ressler et al., 1993). The significance of such spatial organization is not yet clear. Current dogma holds that each OSN expresses only one particular OR, however controversy exists over this point as the evidence is not conclusive (Mombaerts, 2004b). What is clearer is that ORs are expressed via allelic exclusion, such that only one allele is expressed in each neuron, even though two alleles are present for each gene (Chess et al., 1994). ORs appear to be expressed at unequal levels owing to both unequal numbers of expressing cells as well as unequal levels of transcript per expressing cell. In fact, the transcript levels of ORs in the OE can vary up to 300-fold (Young et al., 2003).

Mammalian ORs belong to the class A group of GPCRs. The majority of GPCRs are divided amongst three groups called class A, B, and C (also I, II, and III). Class A is the largest in number by far and receptors in this class are often described as “rhodopsin-like” owing to their structural similarity to the class A prototype, rhodopsin. Similarly, class B may be referred to as the “secretin-like” receptor family and class C as the metabotropic glutamate or pheromone receptor family. In addition to their class A GPCR status, ORs are further categorized amongst themselves into two classes, with 10% of ORs belonging to class I and the remainder to class II. Class I ORs are the so-called “fish-like” ORs, because they resemble ORs first identified in fish (Ngai et al., 1993) that were originally thought to be obsolete in mammals. However, it is now known that many mammalian class I OR genes are intact and encode functional receptors, some of which have been demonstrated to bind volatile odorants, as opposed to the water soluble chemicals that are generally associated with fish olfaction (Malnic et al., 1999). Further subfamilies of ORs have been established based upon functional studies indicating that ORs with $\geq$60% amino acid sequence identity tend to bind structurally related odorants (Kajiya et al., 2001; Malnic et al., 1999). The number of ORs belonging to each subfamily ranges from one to nine in humans and members of a subfamily tend to be encoded by genes at a single chromosomal locus. Human ORs are categorized into 172 subfamilies, while mice have 241 subfamilies (Godfrey et al., 2004; Malnic et al., 2004).

As evidenced by the large number of subfamily groups, ORs possess a great deal of sequence diversity, ranging in sequence identity from 34-99% (Malnic et al., 2004). The greatest sequence variability is found in the third, fourth, and fifth transmembrane domains, constituting regions of possible ligand binding (Fuchs et al., 2001). Though diverse, several common structural elements unite ORs. ORs are relatively short GPCRs, with most being only 300-350 amino acids long. They possess short N and C termini and lack an N-terminal signal sequence. Furthermore, most ORs have an unusually long second extracellular loop that contains two conserved cysteines. Several consensus motifs also characterize ORs, including LHTPMY in intracellular loop one, MAYDRYVAIC in transmembrane domain three, and PMLNPF in transmembrane domain seven (Buck and Axel, 1991). Class I and class II status of an OR is also determined by specific motifs (reviewed in Gaillard et al., 2004).

1.4.2 Olfactory receptor expression outside of the olfactory epithelium

Interestingly, a number of ORs exhibit ectopic expression. mRNAs of multiple ORs have been shown to be expressed in the developing rat heart (Ferrand et al., 1999). Evidence also exists for OR expression in the ganglia of the autonomic nervous system (Weber et al., 2002) and cerebral cortex pyramidal neurons (Otaki et al., 2004). Two ORs termed prostate specific GPCR (PSGR) and prostate overexpressed GPCR (POGR) show differential expression in human, rat, and mouse tissues. In humans, little PSGR and POGR expression is detected in the OE, while high levels are seen in the prostate. Conversely,

rodents show the greatest expression in the OE, with significant PSGR and POGR expression also observed in the rat liver and mouse colon (Yuan et al., 2001). In general, the functional significance of ectopically-expressed ORs is unclear. However, emerging evidence suggests that ORs may indeed serve a role in chemodetection outside of the nose. The human OR 17-4 and the mouse OR mOR23, which are strongly expressed in spermatids, have been demonstrated to influence sperm chemotaxis when stimulated with a synthetic agonist (Spehr et al., 2003; Spehr et al., 2006b). Further studies are needed to better understand the role of ORs expressed in non-olfactory tissues.

1.5 Olfactory receptor pharmacology

While the identification of ORs laid the foundation for decoding vertebrate olfaction, and netted discoverers Linda Buck and Richard Axel a Nobel Prize for their seminal work in this area (Buck and Axel, 1991), characterization of OR pharmacology and signaling mechanisms has been limited. Very few members of the vast OR family have identified ligands (reviewed in Mombaerts, 2004a), and as a result the overwhelming majority of these specialized receptors remain orphans. A key obstacle hindering OR characterization has been difficulty in efficiently expressing these receptors in heterologous cells. When expressed in common cell culture systems, the bulk of OR proteins are detected intracellularly, with very little localization to the plasma membrane (McClintock and Sammeta,

2003). Consequently, reliable results from traditional ligand screening and signaling assays have been difficult to obtain.

1.5.1 Regulation of olfactory receptors by kinases and arrestins

Studies in native cilia preparations have demonstrated that olfactory responses undergo rapid termination that is dependent on the actions of protein kinase A and protein kinase C (Boekhoff and Breer, 1992). Additionally, odorant application to cilia preparations has also been found to cause transient phosphorylation of cilia proteins (Boekhoff et al., 1992). These data support the postulation that activation of ORs, like many other GPCRs, may be highly regulated through desensitization and internalization mechanisms elicited by downstream second messenger-activated proteins.

In addition to regulation by second messenger-dependent kinases, olfactory desensitization is also regulated by receptor-specific kinases. Functional studies have demonstrated a key role for the G protein-coupled receptor kinase 3 (GRK3) in OR signal termination in purified olfactory cilia preparations (Dawson et al., 1993; Schleicher et al., 1993) and GRK3 knockout mice exhibit a loss of odorant-induced desensitization (Peppel et al., 1997; Schleicher et al., 1993). However, recent GeneChip expression profiling studies revealed only low expression of GRK3 in OSNs (Sammeta et al., 2007) and thus further investigation will be required to conclusively determine the identity of the kinase(s) mediating receptor-specific OR phosphorylation in native cells. In any case, OR desensitization is due, at least in part, to phosphorylation of sites within

the receptor's third intracellular loop (Mashukova et al., 2006). Finally, subsequent to phosphorylation-induced desensitization, ORs have been shown to be internalized via β-arrestin2 association and clathrin-mediated endocytosis (Dawson et al., 1993; Mashukova et al., 2006).

Given the evidence supporting robust desensitization of ORs in native tissue, it is natural to wonder whether the poor cell surface expression of heterologously-expressed ORs is due to constitutive activity of the receptors accompanied by persistent internalization. In accordance with this scenario, it was reported that a small portion of heterologously-expressed human OR 17-40 properly traffics to the plasma membrane where it then rapidly internalizes via clathrin-mediated endocytosis, even in the absence of agonist (Jacquier et al., 2006). Conversely, heterologously-expressed human OR 2AG1, a proportion of which is reported to properly localize to the plasma membrane, was found to remain stable at the cell surface until agonist simulation, whereupon it underwent clathrin-dependent endocytosis (Mashukova et al., 2006). Findings from other groups have demonstrated that heterologously-expressed, unstimulated ORs co-localize specifically with endoplasmic reticulum markers (Gimelbrant et al., 1999; Lu et al., 2003) rather than endosomal markers, suggesting that in most cases constitutive internalization is an unlikely explanation for the lack of OR protein detected at the plasma membrane of heterologous cells.

1.6 Trafficking difficulties of GPCRs as a whole

Like the ORs, many other GPCRs also exhibit poor plasma membrane localization upon expression in heterologous cells. This is especially true among families of sensory GPCRs. Members of the bitter taste receptor (Chandrashekar et al., 2000), V2R vomeronasal receptor (Loconto et al., 2003), and trace amine-associated receptor families (Borowsky et al., 2001) all fail to localize correctly in heterologous expression systems. Similarly, multiple non-sensory GPCRs also suffer intracellular retention when expressed heterologously (reviewed in Prinster et al., 2005). It is not clear why certain GPCRs localize properly at the plasma membrane of native cells but are retained intracellularly when expressed in heterologous cells. It has therefore been widely hypothesized that heterologous cell culture lines may lack one or more critical components present in native cells that are required for proper localization of certain receptors.

To overcome poor heterologous surface expression, several molecular tricks have been developed that can successfully enhance the trafficking of some GPCRs. For example, through addition of the membrane targeting sequence from the serotonin 5-HT3 receptor (Wetzel et al., 1999; Yasuoka et al., 2000) or addition of the N-terminal rhodopsin sequence, which may possess a forward targeting signal or simply provide additional glycosylation sites that are important for membrane localization (Kajiya et al., 2001; Katada et al., 2003; Krautwurst et al., 1998), a small number of ORs have been successfully studied in

heterologous systems. Other studies have foregone the benefits of experimentation in heterologous cells, and instead studied endogenous ORs identified via RT-PCR in native OSNs (Malnic et al., 1999) or utilized adenoviral approaches or gene targeting strategies to overexpress defined ORs in OSNs (Bozza et al., 2002; Ivic et al., 2002; Malnic et al., 1999; Touhara et al., 1999; Zhao et al., 1998). Such techniques have successfully matched OR-ligand pairs and mapped axon convergence of certain receptors. Despite these successes, however, overall there have been limited advances in ligand identification and receptor characterization, especially considering the enormity of the OR repertoire. Thus, over the past several years there has been tremendous interest in understanding how interactions with other proteins might control OR trafficking.

1.6.1 An olfactory receptor chaperone in *C. elegans*

The first evidence supporting a role for additional factors in the proper localization of ORs came from observations in *Caenorhabditis elegans*. Early studies on olfaction-deficient worms led to the isolation of the odorant response abnormal 4 (ODR-4) gene, whose protein product was later determined to be expressed specifically in *C. elegans* chemosensory neurons. ODR-4 is required for proper plasma membrane localization of the *C. elegans* olfactory receptor ODR-10 and is thought to aid in receptor folding, sorting, or transport (Dwyer et al., 1998). Subsequent experiments in Chinese hamster ovary cells demonstrated that co-expression with *C. elegans* ODR-4 alleviates the intracellular retention of the rat OR U131, but not that of the rat OR 5 (Gimelbrant

et al., 2001), suggesting that unique chaperones might exist for different ORs. A distantly-related human ortholog of ODR-4 (hODR-4) has been identified (Lehman et al., 2005), however it is not yet clear if the mammalian version of this protein plays a similar role in regulating OR trafficking as its *C. elegans* counterpart.

1.6.2 Heterodimerization as an influence on olfactory receptor trafficking

GPCRs physically associate with a great variety of cellular proteins, including other GPCRs. The functional significance of this phenomenon, termed GPCR dimerization/oligomerization, is an active area of current research. In the most compelling and well-characterized instances of GPCR dimerization, that of the $GABA_B$ and taste receptors, dimerization appears to be an obligatory requirement to generate a functional receptor at the plasma membrane. When expressed alone, the $GABA_BR1$ subunit suffers intracellular retention due to an endoplasmic reticulum retention motif in its C-terminus. However co-expression with the $GABA_BR2$ subunit appears to mask this retention motif, presumably through C-terminal coiled-coil domain interactions, and the $GABA_BR1/GABA_BR2$ heterodimer is found to be functional at the plasma membrane (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). For taste receptors, differential dimerization partners also impart distinct pharmacological properties. Co-expression of the taste receptor T1R3 with T1R1 yields a functional taste receptor that responds to umami (the taste of certain amino acids such as

glutamate and aspartate), while co-expression of T1R3 with T1R1 generates the sweet taste receptor (Nelson et al., 2002; Nelson et al., 2001).

In the class C family of GPCRs, the $GABA_B$ and taste receptors represent instances where heterodimerization has clear roles in the regulatory mechanisms of receptor expression and generation of novel pharmacology. Likewise, multiple class A rhodopsin-like GPCRs have also been demonstrated to physically interact, though the functional consequences are often more subtle. Modest alterations in ligand binding, internalization, and desensitization profiles have been observed for various heterodimers of the somatostatin, purinergic, and opioid receptor families. Class A heterodimerization has also been demonstrated to influence cross-talk between different receptor families, including both synergy and antagonism of signaling, and generation of new signaling through novel G-protein coupling (reviewed in Breit et al., 2004; Kroeger et al., 2003; Prinster et al., 2005). Additionally, emerging evidence has begun to indicate a role for dimerization in the maturation and membrane localization of class A GPCRs (reviewed in Bulenger et al., 2005) Specific examples can be found among receptors of the adrenergic receptor (AR) family. The α_{1D}-AR exhibits intracellular retention in a variety of cell lines (Chalothorn et al., 2002; Hirasawa et al., 1997), which can be alleviated through co-expression and heterodimerization with the closely related α_{1B}-AR (Hague et al., 2006; Hague et al., 2004c). Similarly, co-expression with the β_2-AR also results in robust plasma membrane localization and functional activity of the α_{1D}-AR (Uberti et al., 2005).

The theme of heterodimerization influencing GPCR trafficking will be examined in this dissertation in the context of ORs.

1.6.2.1 The role of heterodimerization in Drosophila olfactory receptor trafficking

Drosophila ORs are encoded by at least 61 genes that bear little sequence homology to their mammalian counterparts (Clyne et al., 1999; Gao and Chess, 1999). Regardless, however, of their apparent separate lines of evolution, *Drosophila* and mammalian ORs are both organized in a remarkably similar fashion. With the exception of a broadly expressed OR called OR83b, the remaining *Drosophila* OR genes are expressed only in a small subset of fly OSNs. Furthermore, OSNs expressing the same OR converge at a single glomerulus, and distinct patterns of glomerular activity appear to transduce *Drosophila* olfactory signals. Thus many of the same principles that characterize mammalian olfaction are conserved in the fruit fly.

Also bearing resemblance to mammalian ORs, *Drosophila* ORs too exhibit trafficking deficiencies when expressed in heterologous cells. However, recent advances in the field of *Drosophila* olfaction have indicated that insect ORs require receptor heterodimerization for proper localization and function. Evidence suggests that all typical ORs must heterodimerize with the divergent OR83b in order to correctly localize and function in olfactory sensory neurons of the fly (Larsson et al., 2004; Neuhaus et al., 2005). In heterologous cells, functional expression of typical *Drosophila* ORs is also significantly enhanced

upon expression with OR83b (Neuhaus et al., 2005). However, recent findings suggest that the transmembrane topology of *Drosophila* ORs may be quite distinct from their mammalian counterparts (Benton et al., 2006) and thus it is not clear how closely analogies can be drawn between heterodimerization of *Drosophila* ORs and mammalian GPCRs.

1.7 Neurotransmitter regulation of olfaction

A multitude of hormones and nucleotides have been demonstrated to influence olfactory perception. Antagonists of adrenergic and muscarinic acetocholine receptors block some odorant-evoked currents in patched OSNs (Firestein and Shepherd, 1992). Furthermore, adrenaline has been shown to enhance odorant contrast via effects on Na^+ and T-type Ca^{2+} currents in newt OSNs (Kawai et al., 1999), while dopamine has been found to decrease the odor sensitivity and activity of mouse OSNs (Hegg and Lucero, 2004). In addition, odor sensitivity appears to also be regulated by nucleotides. Extracellular purines are well-established co-transmitters and neuromodulators, particularly in the sensory systems. Adenosine is a key regulator of vision, while ATP plays a modulatory role in the inner ear. In the olfactory system, exogenous and endogenous ATP significantly reduces odor responsiveness, whereas puringeric receptor antagonists increase odor-induced signaling in mouse OE slice preparations (Hegg et al., 2003). The mechanisms by which the aforementioned hormones and nucleotides regulate olfactory perception are not fully resolved. Whether neurotransmitters regulate olfactory perception at its initial stages or at

a later point of signal transmission has not been examined. One possibility is that cross-talk occurs between neurotransmitter receptors and ORs, potentially due to receptor-receptor interactions.

1.8 Objectives of this dissertation

Based upon the aforementioned studies detailing GPCR translocation to the plasma membrane upon co-expression with specific heterodimerization partners (section 1.6.2.), this dissertation sought to explore whether association with specific interacting partners might similarly influence olfactory receptor trafficking. The initial objective of this dissertation was to screen non-olfactory receptor GPCRs for potential trafficking effects on particular ORs. GPCRs that positively affected the trafficking of examined ORs were further analyzed for expression in the OE, physical association with the ORs, and affect on OR functionality. The final objectives of this dissertation were to examine structural elements that might influence OR trafficking and determine whether ORs are capable of homodimerization.

CHAPTER 2:

Olfactory Receptor Interactions with Adrenergic Receptors[1]

[1]A portion of this chapter is published: Hague C, Uberti MA, Chen Z, Bush CF, Jones SV, Ressler KJ, Hall RA, and Minneman KP (2004) Olfactory receptor surface expression is driven by association with the β2-adrenergic receptor. Proc. Natl. Acad. Sci. U.S.A.**101**:13672-13676.

2.1 Introduction

Perception of smell begins with stimulation of olfactory receptors (ORs) on neurons within the olfactory epithelium (OE), leading to excitation and propagation of currents to the main olfactory bulb (Barber and Ronnett, 2000; Buck, 2000). ORs are class A GPCRs that have been demonstrated to signal through stimulation of $G\alpha_{olf}$, which leads to activation of type III adenylyl cyclase and opening of cAMP-gated cation channels (Mombaerts, 2004a). Since the completion of the human and mouse genome sequencing projects, approximately 350 receptors in humans (Malnic et al., 2004) and approximately 1,000 receptors in mice (Godfrey et al., 2004) have been identified, presumably to aid in the selective recognition of >100,000 different odors. However, the mechanism by which the olfactory system selectively recognizes specific odors remains unclear. It was initially hypothesized that each OSN expresses a single OR and that the axons of OSNs expressing the same OR then converge in the main olfactory bulb (Chess et al., 1994; McClintock and Sammeta, 2003). However, increasing evidence suggests that detection is substantially more complex than previously thought. For example, OSNs may not be restricted to expression of a single OR subtype (Li et al., 2004). In addition to ORs, OSNs can express many other receptors, which facilitate modulation of olfactory responses by hormones and neurotransmitters. For example, epinephrine stimulation of endogenous β-adrenergic receptors (ARs) has been proposed to modify the signaling of co-expressed ORs within OSNs (Kawai et al., 1999).

Furthermore, multiple OR subtypes can respond to the same ligand, a single OR can respond to multiple ligands (Bozza et al., 2002; Kajiya et al., 2001; Krautwurst et al., 1998), and structurally similar odorant ligands can act as either agonists or antagonists (Oka et al., 2004). Thus, as the complexity of the olfactory system becomes increasingly clear, the need to develop simple assays to allow mass screening of ligand–receptor interactions becomes increasingly important.

To date, the primary problem preventing the characterization of the OR family has been the inability to obtain significant surface expression of wild-type receptors in heterologous systems (McClintock and Sammeta, 2003). Upon heterologous transfection, essentially all ORs remain trapped within the endoplasmic reticulum, where they are unable to respond to agonist. Receptor mutations, such as C-terminal transmembrane truncation, N-terminal addition of rhodopsin sequences, N-terminal addition of epitope tags, or construction of OR/β_2-AR chimeras (Gantz et al., 1991; Gimelbrant et al., 1999; Ivic et al., 2002; Kajiya et al., 2001; Krautwurst et al., 1998; Levasseur et al., 2003; Wetzel et al., 1999) have been required to obtain OR surface expression. Although these techniques have proven useful for specific applications, the inability to examine wild-type ORs limits their applicability.

Like ORs, other class A GPCRs, such as α_{1D}-ARs (Chalothorn et al., 2002; Hague et al., 2004a; McCune et al., 2000), α_{2C}-ARs (von Zastrow et al., 1993), adenosine 2b (Sitaraman et al., 2002), and bitter-taste receptors (Chandrashekar et al., 2000), are known to be largely intracellular when

expressed heterologously. Previously, we showed that the α_{1B}-AR promotes the surface expression of intracellular α_{1D}-AR through direct physical association after co-transfection in human embryonic kidney (HEK) 293 cells (Hague et al., 2004a; Uberti et al., 2003). Mutation and truncation studies suggested that this did not involve signaling pathways or the soluble N- or C-terminal extensions, but only the hydrophobic core and/or associated loops. Because ORs consist almost exclusively of such a hydrophobic core and associated loops (Buck and Axel, 1991), we explored the possibility that receptor-receptor interactions might influence OR trafficking. Olfactory neurons are known to express ARs (Kawai et al., 1999), so we specifically examined whether ORs might physically associate with ARs to facilitate surface expression. We used the mouse 71 (M71) OR because it is one of the few ORs with a known ligand (Bozza et al., 2002). Using a variety of techniques, we found that co-expression with the β_2-AR results in a profound translocation of functional M71 to the cell surface in HEK-293 cells. We also found evidence for persistent physical association of the two receptors on the cell surface by co-immunoprecipitation and co-internalization studies in response to receptor-specific ligands and co-localization of M71 and β_2-AR mRNA in mouse OE.

2.2 Experimental procedures

2.2.1 Constructs

M71 in pcDNA3.1+ was amplified by PCR using specific primers containing XbaI and KpnI restriction sequences for insertion into pEGFP-N3.

Hemagglutinin (HA)-tagged β_1- and β_2-AR in pcDNA3.1+ were obtained from H. Kurose (Kyushu University, Hakozaki, Japan), HA-tagged β_3-AR from S. Collins (Duke University Medical Center), and HA-tagged α_2-AR from L. Limbird (Vanderbilt University, Nashville, TN). HA-tagged α_{1A}-AR α_{1B}-AR -, and α_{1D}-AR were created earlier (Uberti et al., 2003; Vicentic et al., 2002).

2.2.2 Cell culture and transfection

HEK-293 cells were propagated in DMEM with sodium pyruvate containing 10% heat-inactivated FBS, 100 µg/ml streptomycin, and 100 units/ml penicillin at 37°C in a humidified atmosphere with 5% CO_2. Confluent plates were subcultured at a ratio of 1:5 for transfection. HEK-293 cells were transfected with 3 µg of DNA of each construct for 12 h by using Lipofectamine 2000, and cells were used for experimentation 48–72 h after transfection.

2.2.3 Luminometer-based surface expression

HEK-293 cells transiently transfected with FLAG-M71-GFP with and without HA-tagged AR subtypes were split into poly D-lysine-coated 35-mm dishes and grown overnight at 37°C. Cells were rinsed three times with phosphate buffered saline (PBS), fixed with 2% paraformaldehyde in PBS for 30 min, and rinsed three times with PBS again. Next, cells were incubated in blocking buffer (2% nonfat milk in PBS, pH 7.4) for 30 min and were then incubated with horseradish peroxidase-conjugated M2-anti-FLAG antibody in blocking buffer for 1h at room temperature. Cells were washed three times with

blocking buffer, once with PBS, and then incubated with enhanced chemiluminescence reagent (Pierce) for 15 s. Luminescence was determined by using a TD20/20 luminometer (Turner Designs, Sunnyvale, CA). Mean values ± SEM were calculated as percent absorbance in arbitrary units and were statistically compared by using one-way ANOVA and post hoc comparison using Dunnett's test, with $P < 0.01$ being considered significant.

2.2.4 Confocal microscopy

Cells transiently transfected with HA- or GFP-tagged constructs were grown on sterile coverslips, were fixed for 30 min with 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and were rinsed three times with PBS containing 0.5% normal horse serum (PBS+). For anti-HA immunostaining, fixed coverslips were blocked for 1 h in blocking buffer (PBS containing 1% BSA, 5% normal horse serum) containing 0.01% Triton X-100 to permeabilize cells. Anti-HA antibody was added to coverslips overnight at 4°C at 1:500 dilution in blocking buffer, washed three times with PBS+ and incubated with Rhodamine red-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature at 1:500 dilution in blocking buffer. Coverslips were washed three times with PBS and mounted onto slides using Vectashield mounting medium. Cells were scanned with a Zeiss LSM 510 laser scanning confocal microscope as described (Hague et al., 2004a). For detecting GFP, fluorescein isothiocyanate fluorescence was excited by using an argon laser at a wavelength of 488 nm, and the absorbed wavelength was detected for 510–520 nm for GFP. For

detecting rhodamine red, rhodamine fluorescence was excited by using a helium–neon laser at a wavelength of 522 nm.

2.2.5 Immunoprecipitation and immunoblotting

HEK-293 cells expressing FLAG-M71-GFP ORs with and without HA-tagged ARs were harvested by scraping in ice-cold PBS and were washed by repeated centrifugation and homogenization. Cell lysates were solubilized, immunoprecipitated with anti-FLAG M2 affinity resin, and probed by using anti-FLAG M2 or anti-HA monoclonal antibodies as described (Pupo et al., 2003).

2.2.6 Cyclic AMP assays

The protocol used to measure cAMP formation in HEK-293 cells is a modification of a widely used prelabeling protocol (Guerrero and Minneman, 1999). HEK-293 cells were split into 24-well plates 24 h before experimentation. Because HEK-293 cells do not easily take up 3H-adenine, 3H-adenosine was used to prelabel cells. Cells were prelabeled with 1 ml of fresh media containing 1 µCi (1 Ci = 37 GBq) of 3H-adenosine for 2 h. Cells were then washed once with 1 ml of Krebs buffer (120 mM NaCl/5.5 mM KCl/2.5 mM $CaCl_2$/1.2 mM NaH_2PO_4/1.2 mM $MgCl_2$/20 mM $NaHCO_3$/11 mM glucose/0.029 mM Na_2EDTA), and 1 ml of Krebs buffer at 37°C, pH 7.4, containing 200 µM 3-isobutyl-1-methylxanthine was added. Stock concentrations of acetophenone (Fisher) were dissolved in Krebs buffer containing 10% ethanol, such that final ethanol concentrations in cells were 0.1%. Isoproterenol was dissolved in Krebs buffer.

Cells were incubated with drugs for 10 min, and reactions were stopped by addition of 77% trichloroacetic acid. A 50-µl aliquot of 10 mM cAMP was added as a carrier, and tubes were sonicated for 5 s and centrifuged for 5 min at 20,000 x g. Then 50-µl aliquots were removed to determine total radioactivity incorporated. 3H-cAMP formed was isolated by sequential Dowex (Guerrero and Minneman, 1999) and alumina chromatography. Eluants from alumina columns were collected, 5 ml of scintillation fluid was added, and 3H-cAMP was quantified by using a liquid scintillation counter. Data are expressed as fold stimulation compared with vehicle-treated control and statistically compared by using an unpaired two-tailed t test, with $P < 0.05$ considered significant.

2.2.7 *In situ* hybridization

In situ hybridization was performed as described (Ressler et al., 1993; Ressler et al., 1994). The M71 and β_2-AR clones were linearized and antisense riboprobes were generated with SP6 RNA polymerase. Young (p8) mice were killed with deep anesthesia, and noses were rapidly dissected at 4°C and were then fresh-frozen on dry ice. Cryostat sections (30 µm) were placed on SuperFrost Plus slides, postfixed proteinase digested, and blocked. Overnight hybridizations of sections with 35S-UTP labeled riboprobes were performed at 52°C. After a stringent wash protocol, slides were apposed to autoradiography film (Kodak Maximum Resolution) and were digitally scanned at 2,400 dpi by using ADOBE PHOTOSHOP.

2.3 Results

2.3.1 Co-expression with β_2-AR results in trafficking of M71 to the plasma membrane.

Earlier work (McClintock and Sammeta, 2003) has demonstrated that essentially all ORs are sequestered at intracellular sites when heterologously expressed. To examine this issue for the OR M71, we created a M71 construct containing N-terminal FLAG and C-terminal GFP epitopes to facilitate detection. By using a quantitative luminometer-based assay, we examined FLAG-M71-GFP cell-surface expression in unpermeabilized HEK-293 cells. As shown in Fig. 2.1, a very low amount of M71 surface expression was detected when this construct was expressed alone. We then screened all nine AR subtypes (α_1-, α_2-, and β-ARs) for their ability to enhance M71 trafficking to the surface. Remarkably, a 6- to 8-fold increase in M71 OR surface expression was observed upon co-transfection with the β_2-AR. However, none of the other eight AR subtypes increased M71 surface expression, suggesting that this interaction is highly specific. Interestingly, the specificity of this interaction was supported by the inability of the β_2-AR to promote cell-surface expression of FLAG-tagged rat I7 or human 17–40 ORs (data not shown).

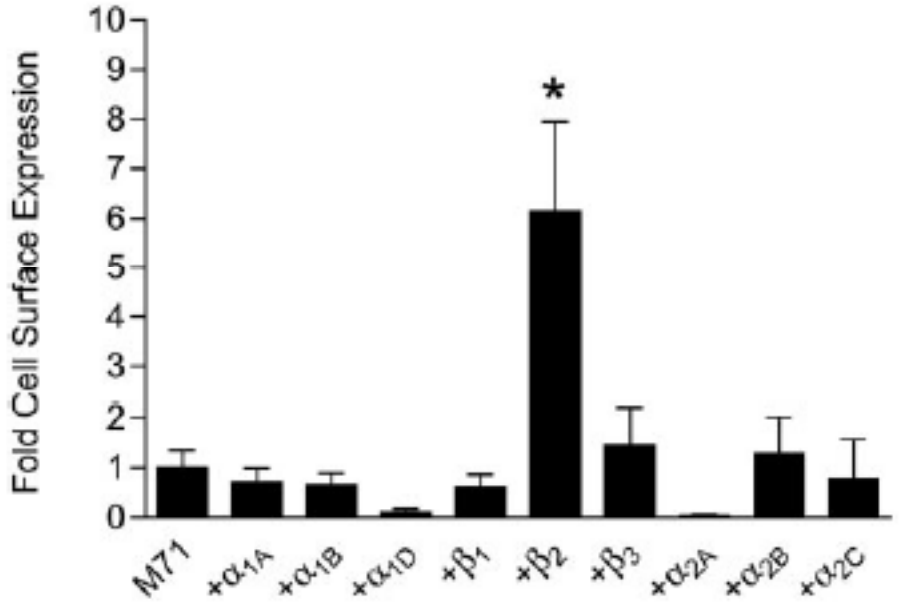


Figure 2.1. Specificity of M71/β_2-AR trafficking enhancement. HEK-293 cells were transiently co-transfected with FLAG-M71-GFP and each of the nine AR subtypes. Cell-surface expression was determined by using a luminometer-based assay. The values are represented as fold surface expression over M71 alone. Only β_2-AR was found to significantly promote the surface expression of M71. Data are expressed as mean ± SEM of three to eight experiments (*, $P < 0.01$ compared with M71 alone).

We also examined the intracellular localization of FLAG-M71-GFP in HEK-293 cells by using confocal microscopy. As shown for other ORs (McClintock and Sammeta, 2003), M71 was almost exclusively retained in intracellular compartments following heterologous expression (Fig. 2.2A Left). However, when co-expressed with the β_2-AR containing an N-terminal HA tag, M71 was quantitatively translocated to the plasma membrane (Fig. 2.2A Center). In contrast, co-expression with HA-α_{1B} -AR (Fig. 2.2A Right) or HA-β_1-AR (data not shown) resulted in no change in M71 localization. By using rhodamine staining to identify HA-β_2-AR localization, we found that HA-β_2-AR and M71 exhibited almost complete colocalization (Fig. 2.2B) and that M71 surface expression did not occur in an adjacent cell that did not express the β_2-AR. These data confirm and extend the above observations from the luminometer-based assay through use of an independent technique.

2.3.2 The β_2-AR physically associates with M71 to promote surface localization.

To determine whether translocation of M71 to the cell surface was due to a direct physical interaction with the β_2-AR in HEK-293 cells, FLAG-M71-GFP was co-expressed with either HA-β_2-AR or HA-α_{1B}-AR, solubilized, and immuno-precipitated with an anti-FLAG antibody. FLAG- and HA-tagged proteins were then detected by Western blotting. As shown in Fig. 2.3 Upper, FLAG-M71-GFP was detected at approximately 54 kDa in cells transfected with this construct. Membranes were stripped and reprobed by using anti-HA antibodies to detect

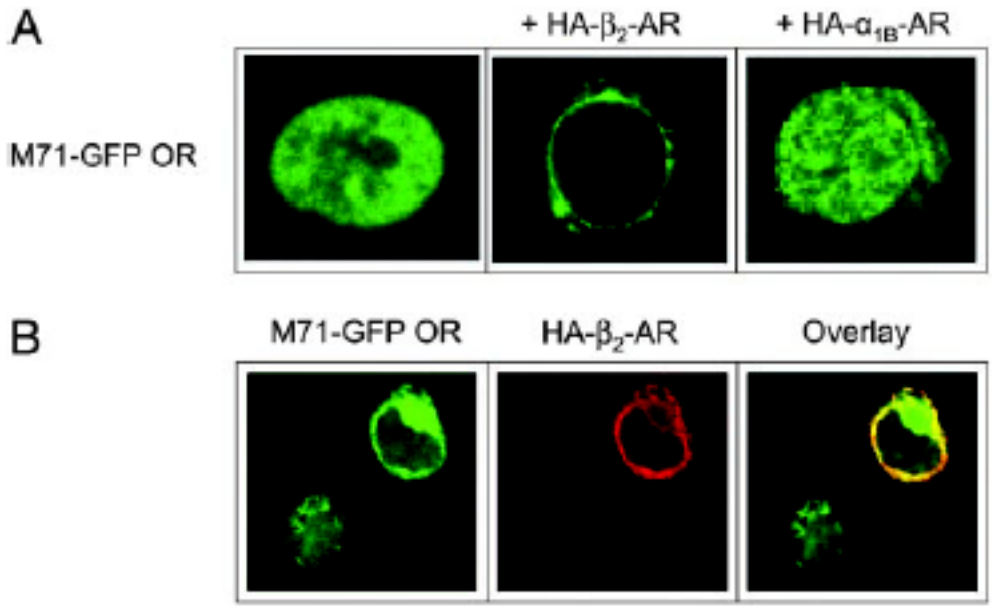


Figure 2.2. Confocal imaging of M71 in HEK-293 cells reveals translocation to the plasma membrane upon co-expression with β_2-AR. (A) FITC fluorescence imaging of FLAG-M71-GFP alone (Left), + HA-β_2-AR (Center), or + HA-α_{1B}-ARs (Right). (B) Confocal imaging of HEK-293 cells co-expressing FLAG-M71-GFP and HA-β_2-AR by using FITC (488 nM) (Left) for GFP, rhodamine (522 nM) for anti-HA (Center), or overlay of both images (Right).

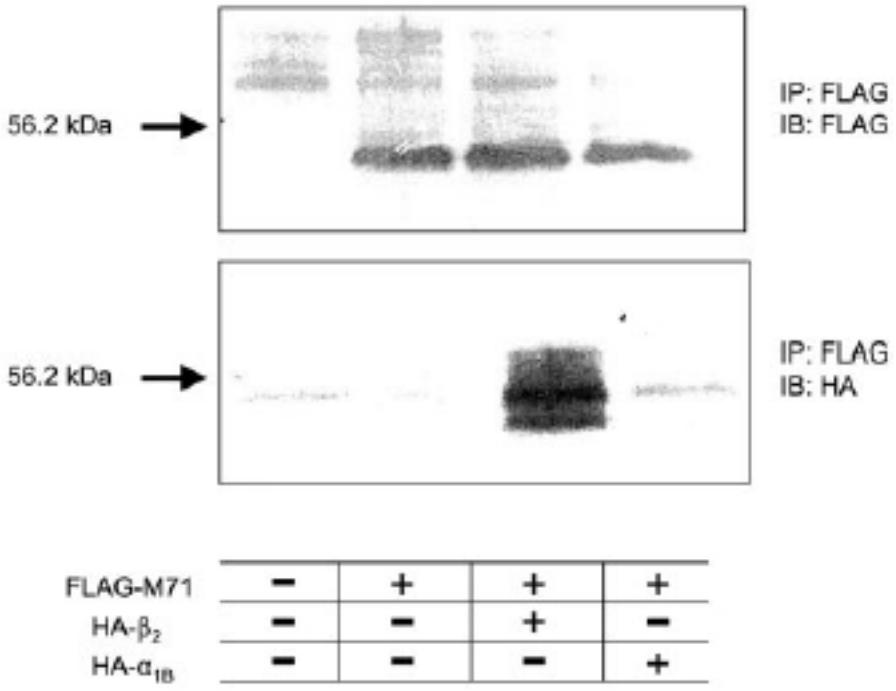


Figure 2.3. Physical association between M71 and β_2-AR. HEK-293 cells were cotransfected with FLAG-M71-GFP alone or with HA-β_2-AR or HA-α_{1B}-ARs. Cells were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-FLAG (Upper) or anti-HA (Lower) antibodies. A physical complex was found between M71 and β_2-AR but not between M71 and α_{1B}-ARs.

AR subtypes (Fig. 2.3 Lower). Dense immunostaining was observed at approximately 50 kDa in membranes co-transfected with FLAG-M71-GFP and the HA-β_2-AR, but not HA-α_{1B}-ARs, demonstrating selective co-immunoprecipitation. Therefore, these data suggest that the β_2-AR promotes M71 cell-surface localization through a direct physical interaction.

2.3.4 Wild-type M71 is functional upon co-expression with the β_2-AR.

We next determined whether M71 would initiate functional responses on trafficking to the cell surface by the β_2-AR. Unlike previous studies that used chimeric or modified ORs to artificially induce surface expression, we used a wild-type M71 construct. cAMP accumulation was measured in HEK-293 cells that were untransfected, transiently transfected with wild-type M71, or transiently co-transfected with HA-β_2-AR and wild-type M71. Fig. 2.4 shows that untransfected cells did not respond to either the M71 agonist acetophenone or the β_2-AR agonist isoproterenol. Cells expressing M71 alone were also unresponsive to both agonists. However, cells expressing both M71 and the β_2-AR showed robust (3–7-fold) stimulation of cAMP formation by either acetophenone or isoproterenol (Fig. 2.4), demonstrating that surface localization of M71 by co-expression with the β_2-AR results in functional responses to M71 stimulation.

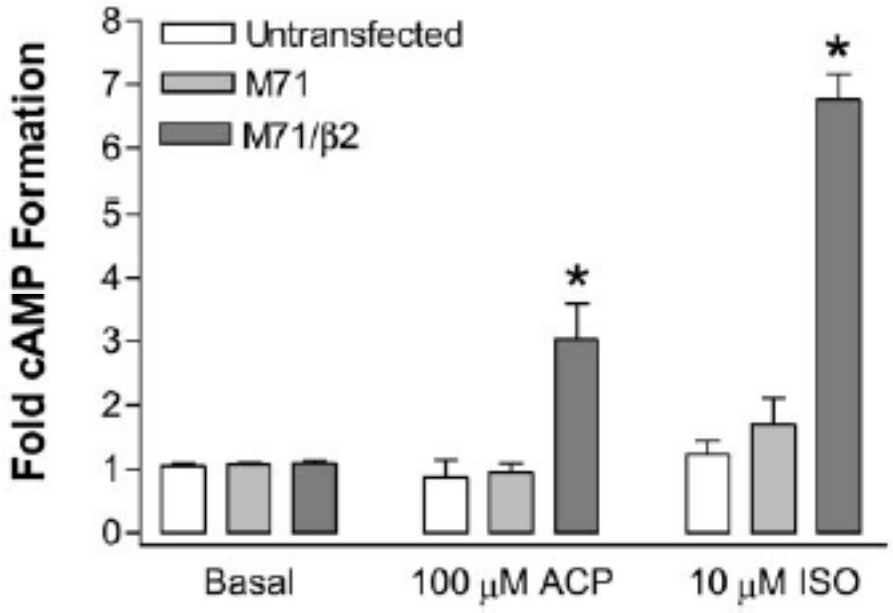
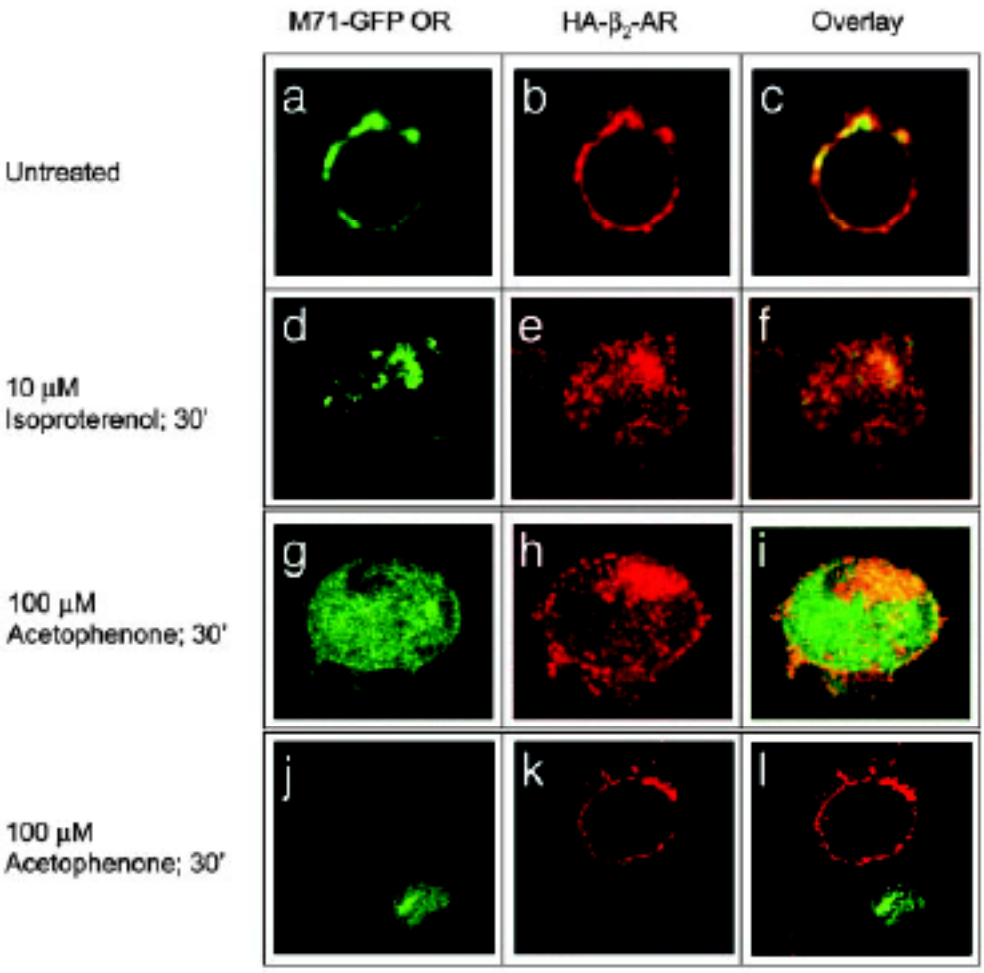


Figure 2.4. Co-expression with β_2-AR results in wild-type M71 coupling to cAMP responses. HEK-293 cells were transiently transfected with M71 alone or in combination with HA-β_2-AR. Cells were prelabeled with 3H-adenosine for 2 h and stimulated for 10 min with 10 μM isoproterenol (ISO) or 100 μM acetophenone (ACP). Data are mean ± SEM of six to eight experiments (*, $P < 0.01$ compared with basal).

2.3.5 Selective agonist stimulation results in co-internalization of M71-GFP and HA-β_2-AR.

After agonist stimulation, β_2-AR is rapidly desensitized through phosphorylation by G protein-coupled receptor kinases (GRKs) and the subsequent binding of β arrestins, leading to their internalization into clathrin-coated vesicles (Kohout and Lefkowitz, 2003). To determine whether the β_2-AR and M71 remain physically associated throughout this process, we determined whether chronic exposure to selective agonists would cause co-internalization of the two receptors. As shown in Fig. 2.5, stimulation of HEK-293 cells co-expressing FLAG-M71-GFP and HA-β_2-AR with 10 μM isoproterenol for 30 min (Fig. 2.5 d–f) resulted in significant internalization of both β_2-AR and M71. Similarly, stimulation with 100 μM acetophenone for 30 min resulted in robust internalization of both receptors (g–i). However, acetophenone stimulation did not promote internalization of β_2-AR in cells that did not express M71 (j–l). These studies suggest that β_2-AR and M71 persistently associate on the cell surface, as well as during the endocytic process that follows agonist stimulation.

Figure 2.5. Co-internalization of M71 and β_2-AR. HEK-293 cells were co-transfected with FLAG-M71-GFP and HA-β_2-AR and grown on sterile coverslips (a–c). Cells were stimulated with either 10 μM isoproterenol (d–f) or 100 μM acetophenone (g–l) for 30 min, fixed, immunostained, and visualized with confocal microscopy by using FITC (488 nm) to observe GFP fluorescence (Left) or rhodamine (522 nm) to observe anti-HA fluorescence (Center). (Right) An overlay of GFP and rhodamine fluorescence is shown. Stimulation with acetophenone induced robust internalization of not only M71 but also β_2-AR when the two receptors were co-expressed (g–i). In contrast, acetophenone had no effect on β_2-AR subcellular localization when M71 was not present, as shown in the top cell in j–l. Similarly, isoproterenol induced co-internalization of the two receptors when they were expressed together (d–f).


M71-GFP OR
HA-β2-AR
Overlay
Untreated
10 μM
Isoproterenol; 30'
100 μM
Acetophenone; 30'
100 μM
Acetophenone; 30'
a
b
c
d
e
f
g
h
i
j
k
l

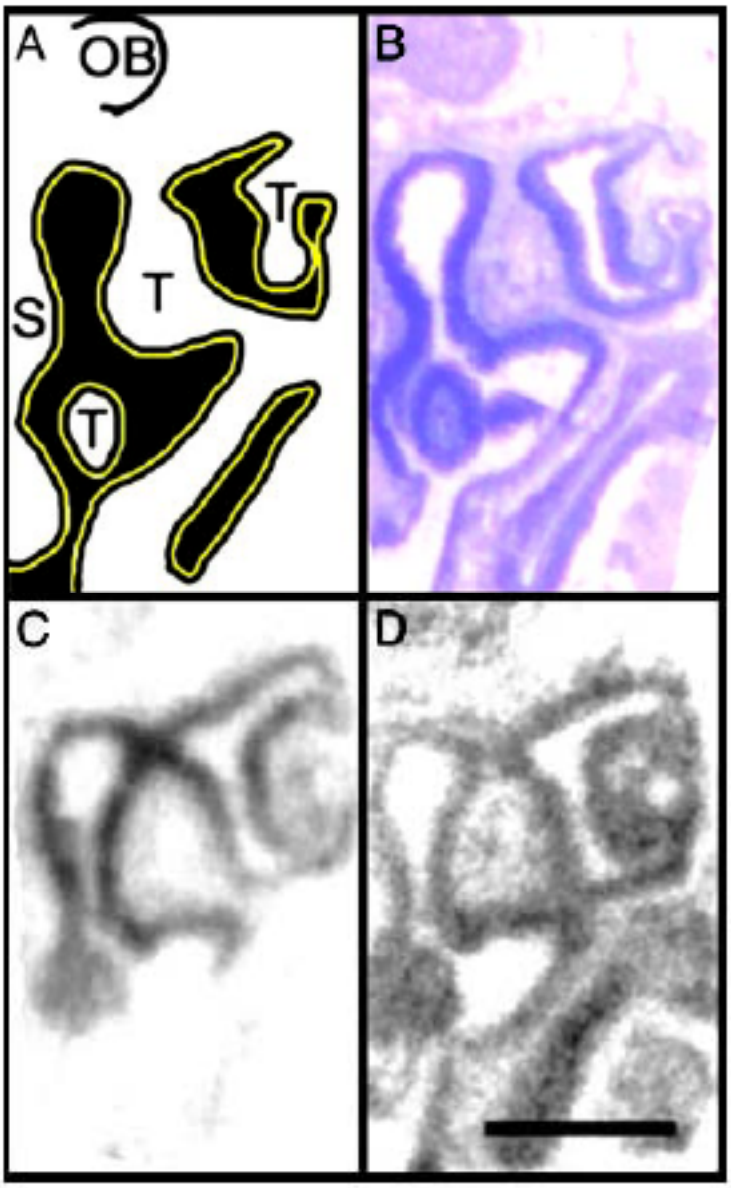
2.3.6 M71 and β_2-AR co-localize in mouse olfactory epithelium.

To determine whether M71 and the β_2-AR are co-expressed in OSNs, *in situ* hybridization was performed on freshly isolated mouse nasal cavity sections by using specific riboprobes (Fig. 2.6). M71 and β_2-AR mRNAs were selectively expressed with a high degree of colocalization in the dorso-medial receptor zone. Interestingly, β_2-AR were more widely expressed than M71, consistent with previous functional data demonstrating the widespread existence of β_2-AR in OSNs (Kawai et al., 1999).

2.4 Discussion

The inability to obtain heterologous OR expression has directly hindered the characterization of this very large and important family of class A GPCRs for over a decade. In this study, we demonstrate that a mouse OR can be translocated to the cell surface of heterologous cells in a functional manner through persistent physical association with the β_2-AR. Thus, it seems that ORs can be added to the growing list of intracellular GPCRs that require specific GPCR partners for chaperoning to the cell surface. This phenomenon was first reported for class C $GABA_B$ receptors, which require assembly of two distinct seven-transmembrane proteins to form a single functional receptor (Marshall et al., 1999). A second and more complex example is the T1R1 family of taste receptors (Nelson et al., 2001). This family of class C GPCRs contains three subtypes (T1R1, T1R2, and T1R3) that require obligate assembly of two distinct

Figure 2.6. Co-expression of M71 and β_2-AR mRNAs in olfactory epithelium. (A) A schematic diagram of half of a mouse nasal cavity, demonstrating the septum (S) in the middle and the olfactory turbinates (T), which extend from the walls into the air space into which odorants flow (black). The entire cavity is covered with OE containing OSNs (yellow stripe). The anterior portion of the olfactory bulb (OB) lies above the cribiform plate superior to the nasal cavity. (B) Cresyl violet staining demonstrates mature olfactory epithelium as dense purple staining lining the cavity. (C) In situ hybridization demonstrates M71 ORs (dark signal) are selectively expressed in the dorso-medial receptor zone. (D) In situ hybridization demonstrates β_2-AR is also expressed in mature OSNs. Notably, β_2-AR is colocalized with M71 in addition to the other ventrolateral expression zones. (Bar, 1 mm.)


A
OB
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subunits to form a single functional receptor. Interestingly, one complex (T1R1/T1R3) forms an amino acid umami receptor (Nelson et al., 2002), whereas another (T1R2/T1R3) forms a sweet-taste receptor with a completely distinct pharmacology (Nelson et al., 2001). Similar complexes occur with other members of the small class C family of GPCRs, including the metabotropic glutamate receptors (Pin and Acher, 2002). However, until recently, this phenomenon has been restricted to the class C GPCR subfamily. Increasing evidence now supports the concept of physical interactions between the much larger class A family of GPCRs (Bouvier, 2001). For example, it has been shown that co-expression with α_{1B}-AR promotes surface expression of normally intracellular α_{1D}-AR through direct physical association (Hague et al., 2004a; Uberti et al., 2003). Interestingly, it has been suggested that β_2-AR must first form multimeric complexes in the endoplasmic reticulum for surface expression (Salahpour et al., 2004). This result raises the possibility that many, if not all, GPCRs must form multiprotein complexes to facilitate surface expression. For some receptors, such as the β_2-AR, homomeric associations may be sufficient to allow for trafficking to the plasma membrane, whereas for other receptors, interactions with other specific receptor types may be required for surface expression.

The data presented in this chapter offers proof-of-concept evidence that ORs such as M71 can associate with non-OR GPCRs, such as β_2-AR, when over-expressed in heterologous cells. A subsequent question of interest is whether these receptor-receptor interactions can also occur in native OSNs when

the receptors are expressed at their endogenous levels. An important role for OR–AR interactions *in vivo* has been suggested by previous studies in which OR stimulation was found to be attenuated by β-AR antagonists (Firestein and Shepherd, 1992) and by stimulation of β-ARs in olfactory neurons (Kawai et al., 1999). Although we have shown that β_2-AR and M71 are co-expressed in OE, further studies are required to determine whether β-AR regulation of olfactory responses *in vivo* depends on the direct physical association between the β_2-AR and the ORs that we have described here.

In conclusion, our data demonstrate that M71 is expressed and functional at the cell surface of HEK-293 cells through persistent physical association with the β_2-AR, thereby providing a molecular mechanism by which ORs may be functionally expressed in olfactory neurons. Because stimulation of M71 and β_2-AR results in receptor co-internalization, these studies also shed light on potential mechanisms underlying the desensitization of olfactory responses as well as potential mechanisms underlying adrenergic regulation of olfaction. Finally, co-expression of ORs with other GPCRs may serve as a general mechanism for obtaining OR surface expression and responsiveness in heterologous cells, allowing for more detailed analysis of this enormous and poorly understood GPCR family.

CHAPTER 3:

Olfactory Receptor Interactions with Purinergic Receptors [1]

[1] A portion of this chapter is published: Bush CF, Jones SV, Lyle AN, Minneman KP, Ressler KJ and Hall RA (2007) Specificity of olfactory receptor interactions with other G Protein-Coupled Receptors. J. Biol. Chem. 282:19042-19051.

3.1 Introduction

A major obstacle hindering the study of OR pharmacology and signaling has been difficulty expressing functional ORs in heterologous cells, primarily owing to their poor trafficking to the plasma membrane (McClintock et al., 1997). Thus, the molecular determinants underlying the impaired cell surface localization of ORs in heterologous cells is a topic of intense research interest. A significant enhancement in the plasma membrane localization of the rat OR 5 is seen upon truncation of the receptor's sixth and seventh transmembrane domains and the C-terminus, which suggests that the C-terminal regions of ORs may potentially contain ER retention signals that impair plasma membrane trafficking (Gimelbrant et al., 1999). In order to block or overcome such ER retention signals, ORs may require association with accessory proteins to improve their trafficking. Such an accessory protein may be absent in heterologous cells, leading to non-functional ORs trapped inside the cell. As reviewed in Chapter 1 (1.6.1 and 1.6.2.1), evidence from the chemosensory systems of several species demonstrates the necessity for accessory proteins to properly localize ORs at the plasma membrane. Briefly, mutation of the *Caenorhabditis elegans* protein ODR-4, which has been proposed to aid in receptor folding, sorting, or transport, inhibits OR insertion into the plasma membrane (Dwyer et al., 1998) and *Drosophila* olfaction has been found to depend upon heterodimerization between conventional ORs and an atypical OR named OR83b, which is required for correct localization and functionality of fly

ORs (Benton et al., 2006; Larsson et al., 2004). In mammals, proteins belonging to the RTP family help translocate some ORs to the cell surface and enhance responses to odorants in HEK-293T cells (Saito et al., 2004).

The work shown in Chapter 2 of this dissertation demonstrates that association with the β_2-adrenergic receptor (AR) results in enhanced surface expression and functionality of the OR M71 in heterologous cells (Hague et al., 2004b). A natural question of interest following this finding was whether GPCRs other than the β_2-AR are capable of assembling with M71 to promote its surface expression or otherwise alter its functionality. GPCRs have been demonstrated to associate with multiple heterodimer partners and can exhibit altered pharmacological properties depending on their interacting partner. As noted in Chapter 1 (1.6.2), taste receptor responsiveness to amino acids or sweet stimuli is dependent on the specific association of the T1R3 receptor with either T1R1 or T1R2 respectively (Nelson et al., 2002). An example is also seen among the opioid receptor family, where the μ-opioid receptor heterodimerizes with both the κ and δ-opioid receptors. Subtype selective agonists show altered affinities and altered rank order of affinities for μ-κ versus μ-δ heterodimers versus the opioid receptors expressed alone (George et al., 2000; Jordan and Devi, 1999). Beyond altering receptor pharmacology, dimerization partners are also thought to influence signaling mechanisms. Agonist activation of the μ, κ or δ-opioid receptors expressed alone results in pertussis-toxin sensitive inhibition of forskolin-stimulated adenylyl cyclase activity. Conversely, agonist application to cells co-expressing the μ and δ-opioid receptor subtypes results in inhibition of

forskolin-stimulated adenylyl cyclase activity that is not pertussis-toxin sensitive, suggesting the μ-δ dimer may couple to a G protein distinct from μ or δ expressed alone (George et al., 2000).

In the present Chapter, we screened 42 distinct non-olfactory GPCRs for their ability to enhance the plasma membrane trafficking of M71. The screen included dopamine receptors including the dopamine D2 receptor that is suggested to be the most highly expressed non-OR GPCR in OSNs by a recent micro-array study (Sammenta et al., 2007). Other receptors screened were from families where at least one receptor subtype is reportedly expressed in the OE and/or olfactory bulb and included the histamine H1-3 receptor subtypes (Jahn et al, 1995), 2 melanocortin receptor subtypes (Alvaro et al., 1996), the five muscarinic achetocholine receptors (Gomeza et al., 1999), the opioid receptors (Buzas and Cox, 1997), several purinergic receptors (Hegg et al., 2003; Kaelin-Lang et al., 1999), the serotonin 5-HT_{1A} receptor (Hardy et al., 2005), other ORs, and multiple metabotropic glutamate receptor subtypes (Ulas et al., 2000). Finally, we screened several of the trace amine associated receptors that also exhibit trafficking deficits in heterolgous cells and were recently shown to be a new class of chemosensory receptor that is highly expressed in the olfactory epithelium (Liberles and Buck, 2006).

3.2 Experimental Procedures

3.2.1 Receptor constructs

The FLAG-M71-GFP construct, WT-M71 construct, and α_{1A}-, α_{1B}-, and α_{1D}-AR constructs were generated as previously described (Hague et al., 2004b; Uberti et al., 2003; Vicentic et al., 2002). The rat I7 construct was amplified from rat genomic DNA via PCR using Pfu turbo (Stratagene) with a forward primer corresponding to nucleotides 1-25 and a reverse primer corresponding to nucleotides 958-981 (GenBank M64386). The hOR17-40 construct was amplified similarly from human genomic DNA with a forward primer corresponding to nucleotides 1-25 and a reverse primer corresponding to nucleotides 921-945 (GenBank X80391). The mOR171-4 construct was amplified from mouse genomic DNA with a forward primer corresponding to nucleotides 3-20 and a reverse primer corresponding to nucleotides 915-933 (GenBank AY073236). PCR products were inserted into pEGFP-N3 modified to contain a FLAG-tag via an XbaI restriction enzyme site in the forward primer and either a KpnI (rat I7, mOR171-4) or BamHI (hOR17-40) restriction enzyme site in the reverse primer. FLAG-M71-GFP was subcloned into the pBK vector to generate a FLAG-tagged M71 construct without the C-terminal GFP. α_{2A}-, α_{2B}-, and α_{2C}-AR constructs were kindly provided by Lee Limbird (Vanderbilt University Medical Center). β_1- and β_2-AR and chimera constructs were kindly provided by Hitoshi Kurose (Kyushu University, Hakozaki, Japan). The β_3-AR construct was kindly provided by Sheila Collins (CIIT Centers for Health Research). The

dopamine D2 receptor construct was kindly provided by David Sibley (National Institutes of Health). Histamine H1–3 receptor constructs were kindly provided by Tim Lovenberg (The R. W. Johnson Pharmaceutical Research Institute). Muscarinic M1–5 acetylcholine receptor constructs were kindly provided by Allan Levey (Emory University School of Medicine). Opioid receptor constructs, μ and δ, were kindly provided by Ping-Yee Law (University of Minnesota Medical School). The P2Y1 receptor (P2Y1R) construct was kindly provided by Ken Harden (University of North Carolina, Chapel Hill). The dopamine D1 and D5 receptor constructs, melanocortin 3 and 4 receptor constructs, P2Y2 receptor (P2Y2R) construct, adenosine A1, A2A (A2AR), A2B, and A3 receptor constructs, and trace amine associated receptor (TAAR) 1, 3-5 constructs were purchased from the UMR cDNA Resource Center. The serotonin 5HT1A receptor construct was kindly provided by John Raymond (Medical University of South Carolina). Metabotropic glutamate receptor constructs, 4b, 7a, and 8 were kindly provided by Jeff Conn (Emory University School of Medicine). TAAR-2 receptor construct was kindly provided by Dr. Kenneth Jones (Synaptic).

3.2.2 Cell culture and transfection

All tissue culture media and related reagents were purchased from Invitrogen. HEK-293 cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) at 37°C with 5% CO2. 80-95% confluent cells in 10-cm tissue culture dishes were transfected with 1-3 μg of cDNA mixed with 15 μl

Lipofectamine 2000 in 5 ml of serum-free medium. Following overnight incubation, complete medium was added and cells were trypsinized and re-plated.

For confocal microscopy experiments, a high transfection efficiency was achieved through electroporation using the Nucleofector® and following the manufacturer's protocol (Amaxa). Briefly, HEK-293 cells were trypsinized, collected by centrifugation, and resuspended in Nucleofector solution along with 0.7 μg of cDNA per construct. This suspension was then subjected to electroporation in the Nucleofector®, followed by addition of complete medium and plating of cells directly onto tissue culture treated glass slides (BD Biosciences). Cells were grown for 24 hours.

3.2.4 Western blotting

Samples were resolved by SDS-PAGE on 4-20% Tris-Glycine gels, followed by transfer of protein to nitrocellulose membranes (Bio-Rad). The membranes were incubated in blocking buffer (2% non-fat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM HEPES, pH 7.4) for 30 minutes and then incubated with primary antibody for either 1 hour at room temperature or overnight at 4°C. Next, the membranes were washed three times in blocking buffer and incubated with either a horseradish peroxidase-conjugated (HRP) secondary antibody or a fluorescent-conjugated secondary antibody for 30 minutes at room temperature, followed by three blocking buffer washes. Proteins bound by HRP-conjugated secondaries were visualized via enzyme-linked chemiluminescence using ECL

reagent (Pierce). Proteins bound by fluorescent-conjugated secondary antibody were detected using the Odyssey imaging system (Li-Cor).

3.2.5 Surface luminometer assay

HEK-293 cells transiently transfected with ORs alone or co-transfected with ORs plus other GPCR subtypes were split into poly D-lysine-coated 35-mm dishes and grown overnight at 37°C. Cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde, and washed with PBS again. Cells were then incubated in blocking buffer (2% nonfat milk in PBS, pH 7.4) for 30 minutes, followed by incubation with HRP-conjugated M2-anti-FLAG antibody (1:600, Sigma) in blocking buffer for 1 hour at room temperature. Cells were washed twice with blocking buffer, twice with PBS, and then incubated with SuperSignal Pico ECL reagent (Pierce) for 15 seconds. Luminescence of the entire 35-mm dish was determined using a TD-20/20 luminometer (Turner Designs). Mean values ± SEM were calculated as percent absorbance in arbitrary units and were normalized to total protein in experiments where different cell densities were a factor.

3.2.6 Immunohistochemistry on nasal epithelium slices

Adult female M71-IRES-taulacZ (M71-lacZ) (Vassalli et al., 2002), P2Y1R-knockout (KO) (Fabre et al., 1999), and P2Y2R-KO (Cressman et al., 1999) transgenic mice were perfused with ice-cold paraformaldehyde and the olfactory epithelium was dissected. Following 1 hour post-fixation, tissue was

decalcified at 4°C in 250 mm EDTA for one week. After freezing in optimal cutting temperature compound (Tissue-Tek OCT), tissue was sectioned at 25 μM using a Leica cryostat and sections were adhered to Superfrost Plus slides (VWR). Sections were blocked for three hours in blocking buffer (10% normal donkey serum (NDS), 0.1% Triton-X-100 in PBS, pH 7.4) followed by overnight incubation at room temperature with anti-β-galactosidase (1:300, Promega) plus either anti-P2Y1R, P2Y2R, (both 1:25, Zymed) or A2AR (1:25, Chemicon) primary antibodies in PBS plus 2.5% NDS. After three 10 minute washes in wash buffer (PBS plus 0.1% Triton-X-100), sections were incubated with anti-mouse Alexa-Fluor 488-conjugated and anti-rabbit Alexa-Fluor 546-conjugated secondary antibodies in PBS plus 2.5% NDS for 1 hour. Sections were washed three times for 10 minutes each in wash buffer and then DAPI stained, followed by two brief water rinses. Slides were mounted in Vectashield (Vector Labs) and analyzed on a Zeiss LSM 510 laser scanning confocal microscope.

3.2.7 Confocal microscopy analysis of transfected cells

Nucleofected cells grown on glass slides were rinsed with PBS, fixed in 4% paraformaldehyde, and washed for 5 minutes three times with PBS. Fixed cells were permeabilized and blocked by incubating in blocking buffer (1x PBS, 2% bovine serum albumin, 0.04% saponin, pH 7.4) for 1 hour. Next, cells were incubated with mouse anti-FLAG antibody (1:1000, Sigma) plus either rat anti-hemagglutinin (HA) antibody (1:1000, Roche), or rabbit anti-P2Y2R antibody (1:300, Zymed) for 1 hour at room temperature. Following three 5 minute

washes with blocking buffer, cells were incubated for 30 minutes with anti-mouse Alexa-Fluor 488-conjugated secondary antibody plus either anti-rat Alexa-Fluor 546-conjugated or anti-rabbit Alexa-Fluor 546-conjugated secondary antibody (1:250, Molecular Probes). Cells were washed in blocking buffer three times for 5 minutes, DAPI stained, rinsed twice with water, dehydrated through ethanol, and mounted with Vectashield. A Zeiss LSM 510 laser scanning confocal microscope was used to examine cells.

3.2.8 Co-immunoprecipitation

Transfected cells were harvested in 500 μl ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 1.0% Triton X-100, 5mM EDTA) and rotated end-over-end at 4°C for 30 minutes to solubilize. Unsolubilized membranes were pelleted through centrifugation. 100 μl of the supernatant was reserved to verify construct expression and 20 μl 6x sample buffer was added. The remaining supernatant was incubated with 60 μl of anti-FLAG antibody-conjugated agarose beads rotating at 4°C. Following at least 4 hours of incubation, the beads were pelleted and washed 5 times with 1 ml of lysis buffer. Next, 150 μl of 2x sample buffer was added to elute the proteins. 20 μl of lysate and immunoprecipitated samples were loaded onto gels and analyzed by Western blotting as described above.

3.2.9 ERK activation assays

Transfected HEK-293 cells grown in 35-mm dishes were starved in serum-free minimum essential medium overnight. For pertussis toxin (PTX) pre-

treatment, 10 ng/ml PTX was added to media 24 hours before the experiment. To stimulate cells, 100 μM acetophenone (Fluka, stock solution prepared in ethanol and diluted to working concentration in PBS) was added directly to the starvation medium for 2 minutes at 37°C. At the end of the stimulation, the media was removed and 80 μl of sample buffer was added. Samples were sonicated, heated to 85°C for 5 min, and centrifuged briefly at 17,000 x g. The proteins were resolved by SDS-PAGE, as described above, and extracellular regulated kinase 1/2 (ERK 1/2) was visualized using monoclonal anti-phospho p42/44 and rabbit anti-p42/44 antibodies (1:1000, Cell Signaling) to blot for phosphorylated and total mitogen-activated ERK 1/2, respectively. Fluorescent-conjugated anti-mouse and anti-rabbit secondary signals (1:10,000, Rockland) were detected using the Odyssey imaging system, and band densities were quantified using Odyssey imaging software (Li-Cor).

3.3 Results

3.3.1 Plasma membrane localization of the OR M71 is enhanced upon co-expression with the purinergic receptors P2Y1R, P2Y2R, and A2AR.

As shown in Chapter 2 of this dissertation, we have previously reported that association of the OR M71 with the β_2-AR alleviates intracellular retention and yields functional M71 localized at the plasma membrane (Hague et al., 2004b). To determine the specificity of such GPCR-OR interactions and identify whether other GPCRs are similarly capable of enhancing M71 plasma membrane

localization, we conducted a screen co-expressing M71 with a multitude of other GPCRs. M71 tagged at the N-terminus with FLAG and at the C-terminus with GFP (FLAG-M71-GFP) was expressed alone and in combination with each of the other GPCRs by transient transfection in HEK-293 cells. Plasma membrane levels of M71 were quantified by detection with an anti-FLAG HRP-conjugated antibody in unpermeabilized cells via a luminometer assay. When expressed alone, only a small amount of M71 was detected at the plasma membrane. Co-expression with the vast majority of receptors examined had no significant effect on M71 surface expression. Strikingly, however, three purinergic receptor subtypes, P2Y1R, P2Y2R and A2AR, significantly increased M71 plasma membrane expression by 4-8 fold, comparable to the previously-reported effect of co-expression with β_2-AR (Figure 3.1).

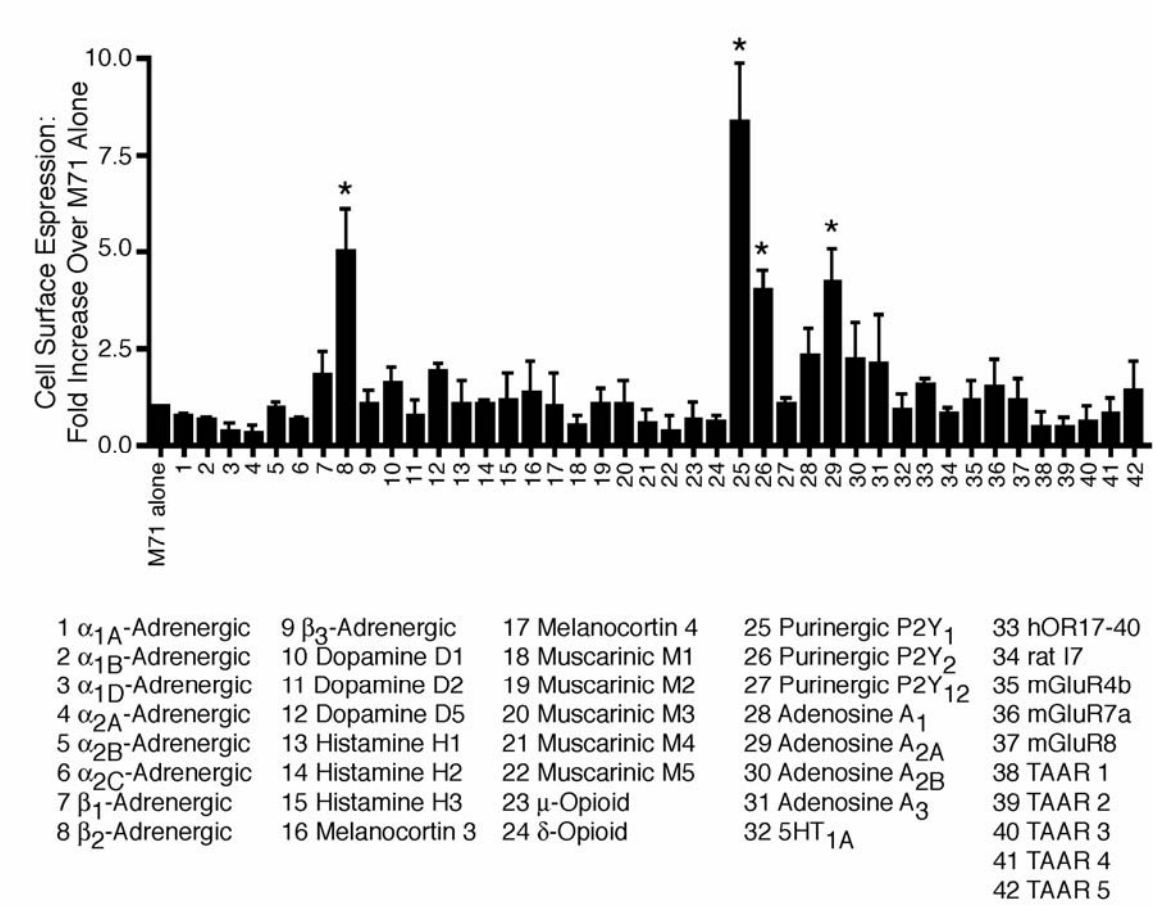
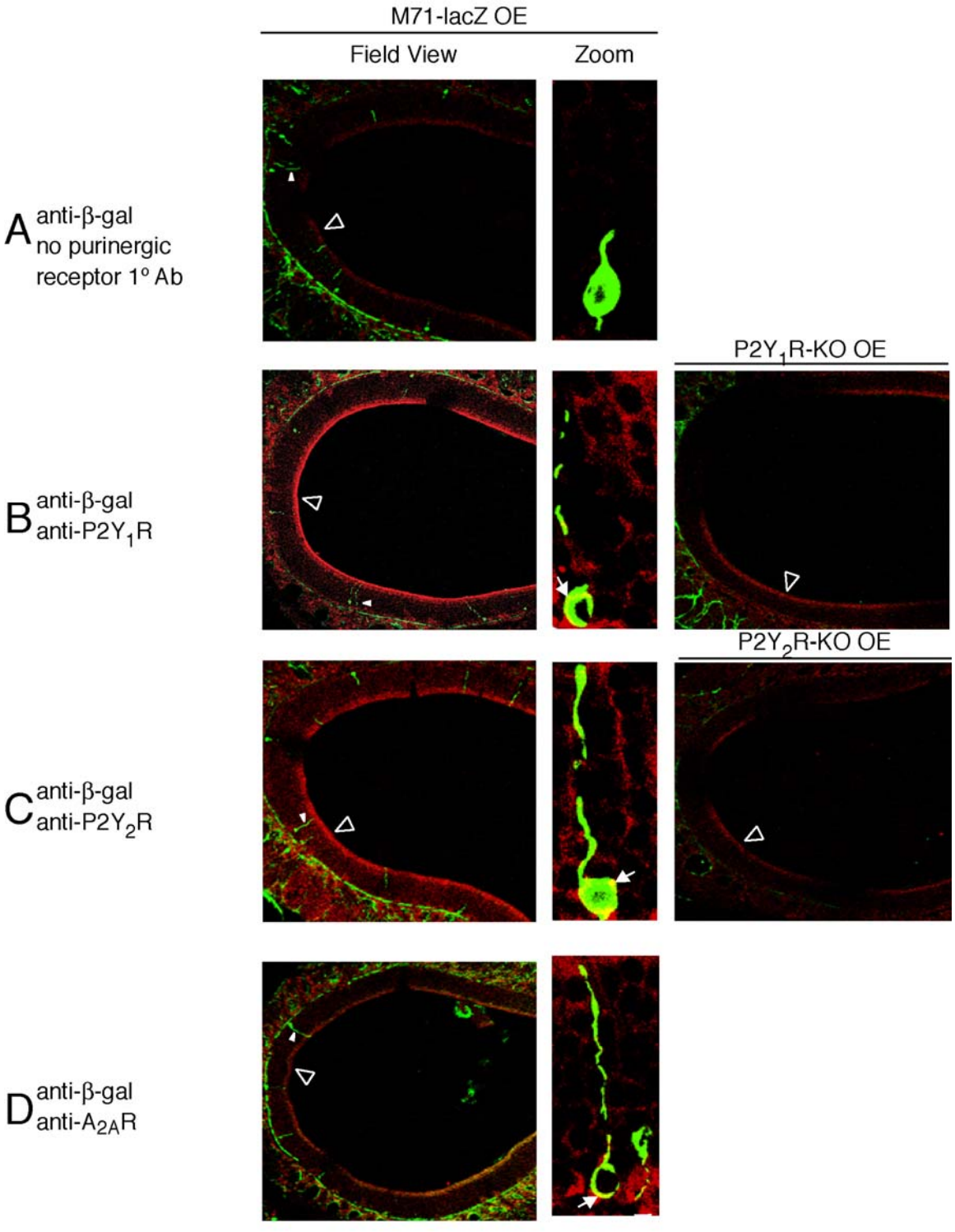


Figure 3.1. Enhanced M71 plasma membrane localization upon co-expression with β_2-AR, P2Y1R, P2Y2R, and A2AR. FLAG-M71-GFP was expressed alone or co-expressed with 42 other GPCRs in HEK-293 cells. Plasma membrane expression of M71 in unpermeabilized cells was detected via a luminomteter assay following incubation with an anti-FLAG HRP-conjugated antibody. Each bar represents data from at least 3 independent experiments and shows the fold increase in cell surface expression compared to M71 expressed alone. One-way ANOVA, followed by Dunnett's post-hoc test was used to determine statistical significance (* indicates $p < 0.001$).

3.3.2 P2Y1R, P2Y2R, and A2AR show overlapping expression with M71-positive olfactory sensory neurons.

We performed immunohistochemistry on cryostat sections of olfactory epithelial tissue to elucidate whether P2Y1R, P2Y2R, and A2AR are expressed in M71-positive OSNs. In order to circumvent the lack of an M71-specific antibody, we utilized M71-lacZ transgenic mice for our studies. These mice express the β–galactosidase gene under control of the M71 promoter such that all cells expressing M71 also express β-galactosidase (Vassalli et al., 2002). Thus, by labeling sections with an anti-β-galactosidase primary antibody, we identified M71-positive OSNs distributed in the dorso-medial zone of the nasal epithelium, as previously described (Mombaerts et al., 1996; Vassalli et al., 2002). Using antibodies specific for P2Y1R, P2Y2R, and A2AR, we found each of the purinergic receptors to be expressed in olfactory epithelial tissue (Figure 3.2). The expression of all three receptors appeared to be ubiquitous throughout the epithelial layer and not restricted to any one population of cells. Both the P2Y1R and P2Y2R showed particularly intense expression on the luminal edge of the epithelium, where OSN cilia extend and ORs are expressed (Figure 3.2: field view). High magnification images showed direct overlap (yellow) of the purinergic receptors expression with M71-positive OSNs (Figure 3.2: zoom) and all M71-positive OSNs observed exhibited co-staining with the purinergic receptors. Tissue labeled without purinergic receptor primary antibody exhibited a low level of auto-fluorescence. Control experiments in sections from P2Y1R-KO and P2Y2R-KO mice showed similar auto-fluorescence levels to those

Figure 3.2. Expression of P2Y1R, P2Y2R, and A2AR in M71-positive olfactory sensory neurons. Coronal sections (25 μm) of olfactory epithelium (OE) from M71-lacZ mice were immunostained with anti-β-galactosidase primary antibody followed by Alexa-Fluor 488-conjugated secondary antibody to detect M71-expressing OSNs (green). The purinergic receptors were detected by incubation with specific anti-P2Y1R (B), anti-P2Y2R (C), and anti-A2AR (D) primary antibodies followed by Alexa-Fluor 546-conjugated secondary antibodies (red). To determine background tissue fluorescence, M71-lacZ sections were incubated without purinergic receptor primary antibody. As a further control, anti-P2Y1R and anti-P2Y2R antibodies were incubated with OE sections from P2Y1R-KO and P2Y2R-KO mice (B, C, far right). White arrowheads indicate M71-positive OSNs, open arrowheads indicate the luminal edge of the OE, and white arrows indicate purinergic receptor staining that overlaps with M71-positive OSNs.


M71-lacZ OE
Field View
Zoom
A anti-β-gal
no purinergic
receptor 1° Ab
P2Y1R-KO OE
B anti-β-gal
anti-P2Y1R
P2Y2R-KO OE
C anti-β-gal
anti-P2Y2R
D anti-β-gal
anti-A2AR

without primary antibody, suggesting the labeling observed with the purinergic receptor antibodies was specific.

3.3.3 M71 physically associates with P2Y1R, P2Y2R, and A2AR.

The observed enhancement of the plasma membrane localization of M71 upon co-expression with P2Y1R, P2Y2R, and A2AR receptors, together with confirmation that these purinergic receptors are expressed with M71 in native tissue, suggested that M71 might physically interact with each of these GPCRs. Thus, co-immunoprecipitation studies were performed to determine if M71 can associate in physical complexes with P2Y1R, P2Y2R, and A2AR. FLAG-M71-GFP was expressed together with each of the purinergic receptors and cell lysates were subjected to immunoprecipitation with anti-FLAG antibody-conjugated agarose beads. Equal levels of expression were observed for M71 transfected alone or co-transfected with the purinergic receptors and levels of M71 immunoprecipitated were also similar with each of the co-transfected purinergic receptor (data not shown). FLAG-M71-GFP expression was detected as a unique band slightly higher than the 37 kDa protein marker in lysate and immunoprecipitated samples (Figure 3.3A). Immunoprecipitation of M71 from cells co-expressing HA-P2Y1R yielded a dense immunoreactive band upon blotting with anti-HA antibody (Figure 3.3B). In addition, both P2Y2R and HA-A2AR were also robustly co-immunoprecipitated with M71 (Figure 3.3C-D). Conversely, a GPCR that does not enhance the cell surface expression of M71, the δ-opioid receptor, was not found to co-immunoprecipitate with M71 (Figure

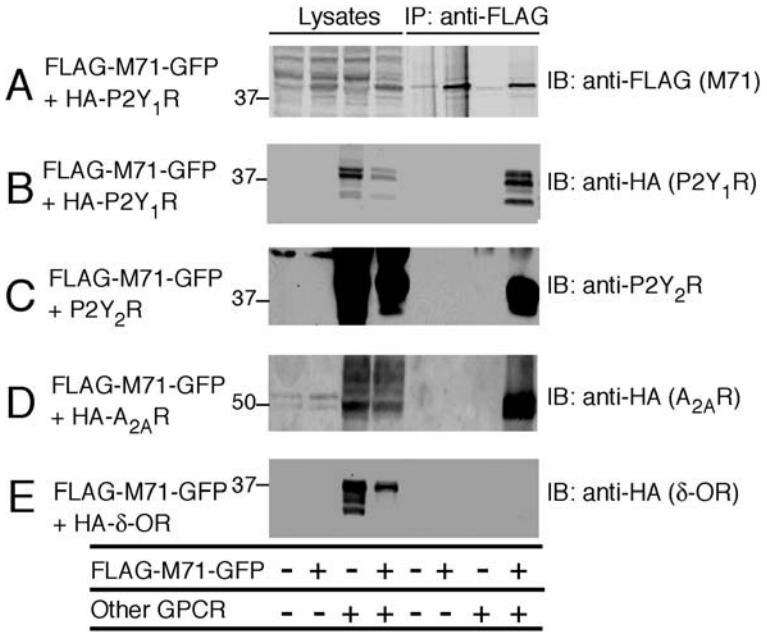


Figure 3.3. Physical association of M71 with P2Y1R, P2Y2R, and A2AR. HEK-293 cells were transfected with FLAG-M71-GFP alone or in combination with P2Y1R. After harvesting and solubilization, cell lysates were incubated with anti-FLAG antibody-conjugated agarose beads and immunoprecipitated. Samples were resolved via SDS-PAGE and anti-FLAG antibody was used to detect M71 (A) and anti-P2Y1R antibody was used to detect P2Y1R (B). In additional experiments, FLAG-M71-GFP was co-expressed with P2Y2R and blots were probed with a specific anti-P2Y2R antibody (C) or FLAG-M71-GFP was co-expressed with HA-A2AR and blots were probed with anti-HA antibody (D). In each case, strong immunoreactivity at the appropriate size of the co-expressed receptor was detected in the co-transfected lane, indicative of co-immunoprecipitation between M71 and each of the purinergic receptors. Each of these experiments was performed at least three times, with similar results.

3.3E). These data demonstrate the ability of M71 to form stable complexes with specific purinergic receptors in a cellular context.

To further verify the cellular localization of M71, we studied transfected HEK-293 cells via confocal microscopy. P2Y1R, P2Y2R, and A2AR effectively trafficked to the plasma membrane when expressed alone in HEK cells (data not shown). FLAG-M71, however, exhibited a diffuse staining throughout the entirety of the cytoplasm when expressed alone (Figure 4A). Conversely, upon co-transfection with HA-P2Y1R, P2Y2R, or HA-A2AR, a significant amount of M71 localized to the plasma membrane where it co-localized well with the various purinergic receptors (Figure 3.4B-D). These data suggest that the purinergic receptors P2Y1R, P2Y2R, and A2AR are able to interact in a physical complex with M71 that facilitates localization of the OR to the plasma membrane. Furthermore, the co-localization of M71 and the purinergic receptors at the cell surface indicated by confocal microscopy suggests a persistent association that may potentially have functional consequences.

3.3.4 Agonist stimulation of M71 co-expressed with β_2-AR, P2Y1R, and P2Y2R, but not A2AR results in activation of the MAPK pathway.

OSNs expressing M71 have been shown to respond to the aromatic ketone acetophenone (ACP) (Bozza et al., 2002). We previously found that ACP stimulation of wild-type M71 (WT-M71) expressed in HEK-293 cells did not result in detectable receptor signaling, consistent with the lack of receptor expressed at the plasma membrane, but stimulation of WT-M71 co-expressed with β_2-AR did

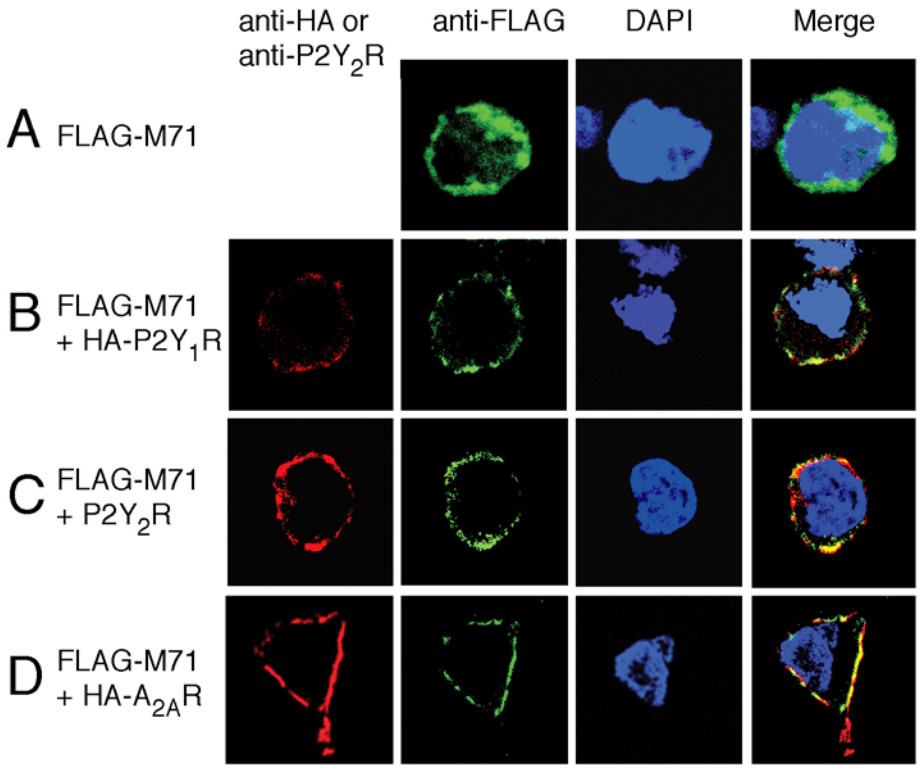


Figure 3.4. Co-localization of M71 with P2Y1R, P2Y2R, and A2AR at the plasma membrane. FLAG-M71 was transfected in HEK-293 cells either alone, or in combination with HA-P2Y1R, P2Y2R, or HA-A2AR. Anti-FLAG primary antibody followed by Alexa-Fluor 488-conjugated secondary (green) was used to detect FLAG-M71. P2Y2R was detected by anti-P2Y2R antibody, while HA-P2Y1R and HA-A2AR were detected by anti-HA antibody. All three purinergic receptors were visualized using Alexa-Fluor 546-conjugated secondary antibody (red). DAPI staining of the nuclei is shown in blue. FLAG-M71 expressed alone was localized diffusely throughout cells (A). Co-transfection of FLAG-M71 with HA-P2Y1R, P2Y2R, and HA-A2AR resulted in translocation of M71 to the plasma membrane, where it was co-localized with the various purinergic receptors (B-D).

result in significant cAMP generation (Hague et al., 2004b). These studies demonstrated that when in complex with β_2-AR, heterologously-expressed WT-M71 can signal via cAMP generation, as has been reported for many examples of odorant-induced signaling in native OSNs (Gaillard et al., 2004; Mombaerts, 2004a). Based on these previous findings, we examined cAMP generation in response to ACP stimulation of WT-M71 co-expressed with P2Y1R, P2Y2R, or A2AR. These experiments, however, revealed no evidence of ACP-induced cAMP generation, even with co-transfection of the specialized OSN G-protein, $G\alpha_{olf}$ (data not shown).

In addition to cAMP formation, other signaling pathways that are known to be activated in response to OR stimulation in native OSNs include formation of inositol (1,4,5) bis-phosphate (IP3) and activation of the extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway (Ko and Park, 2006; Miwa and Storm, 2005). ACP stimulation of WT-M71 co-expressed with the various purinergic receptors did not result in detectable accumulation of IP3 (data not shown). However, we did observe small increases in the phosphorylation of ERK 1/2 in response to ACP when WT-M71 was co-expressed with the various purinergic receptors or β_2-AR (Figure 5). Although these ACP-induced increases in phospho-ERK 1/2 were not statistically significant, we pursued further studies of this type to see if the effects could somehow be enhanced.

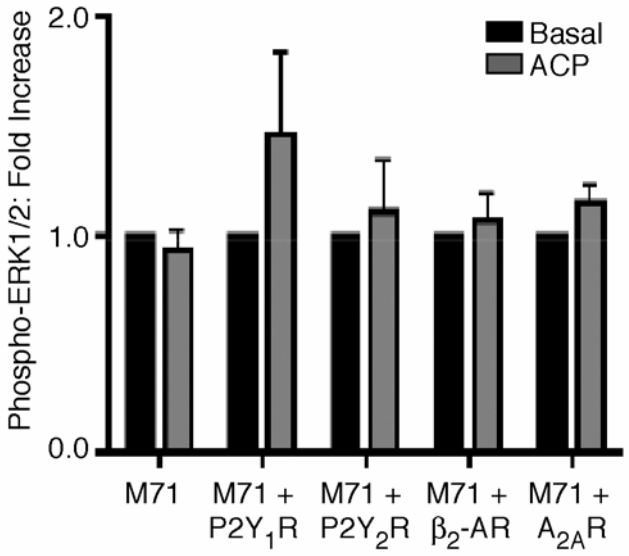
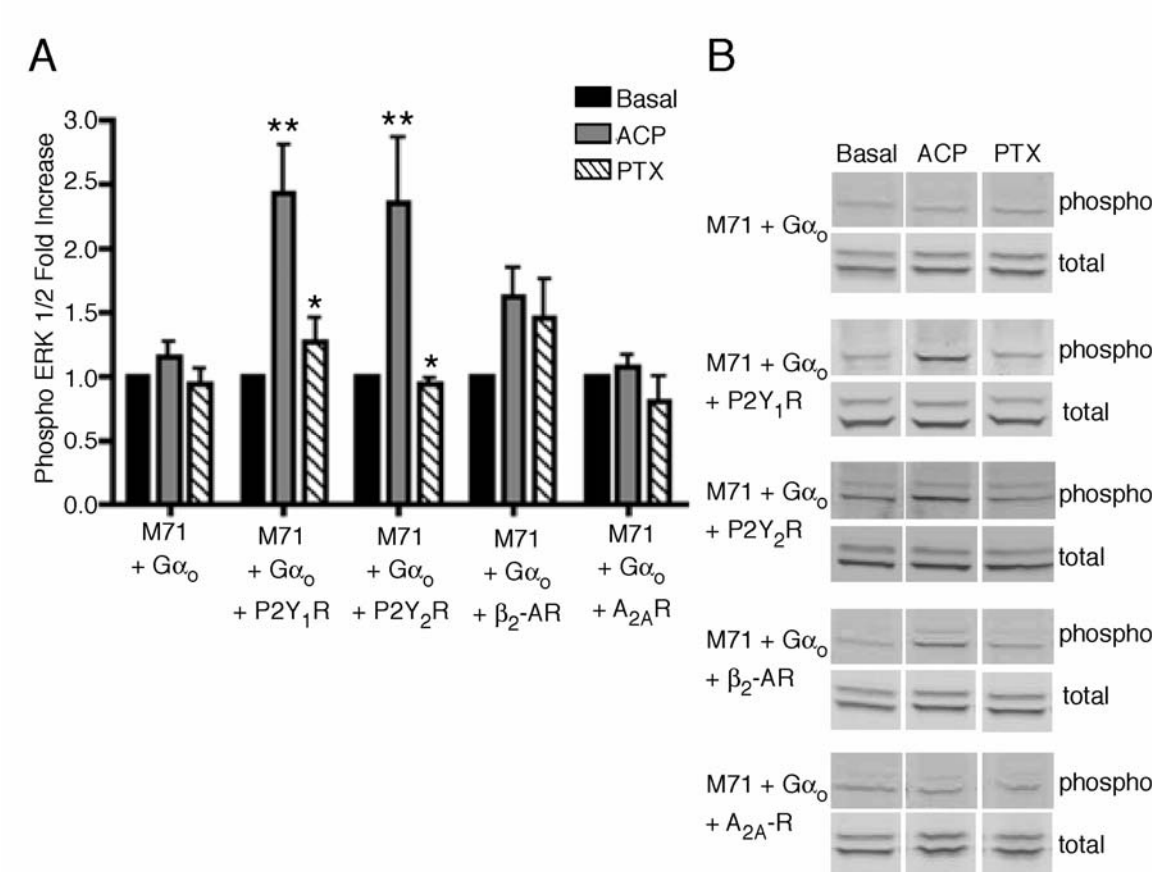


Figure 3.5. Acetophenone stimulation of M71 co-expressed with P2Y1R, P2Y2R, β_2-AR, or A2AR does not cause significant ERK 1/2 phosphorylation. WT-M71 was co-transfected with P2Y1R, P2Y2R, β_2-AR, or A2AR in HEK-293 cells and stimulated for 2 minutes with 100 μm ACP. No significant increase in phosphorylation of ERK 1/2 was found compared to basal levels. Bars and error bars represent means ± SEM from 3-6 independent experiments. In each experiment, the quantification of phospho-ERK 1/2 immunoreactive bands was normalized to the immunoreactive bands for total ERK 1/2.

There is no consensus as to which G protein(s) mediate OR signaling through the IP3 and MAPK pathways, and it is likely that many if not most ORs are capable of promiscuous G protein coupling (Kajiya et al., 2001; Krautwurst et al., 1998). While subsets of OSNs exhibit differential G protein expression, it has been reported that all OSNs express $G\alpha_o$ (Wekesa, 1999). Interestingly, both P2Y1R and P2Y2R, as well as β_2-AR, are well known to couple to pertussis toxin-sensitive G $\alpha_{i/o}$, while the A2AR receptor has not been reported to couple to G $\alpha_{i/o}$ (Chen and Chen, 1998; Filippov et al., 1998; Vasquez, 2002).

Given the abundance of $G\alpha_o$ in the olfactory epithelium, we re-examined the capacity of M71 to mediate ACP-induced changes in ERK 1/2 phosphorylation by performing MAPK activation assays in the presence of co-transfected $G\alpha_o$. Under these conditions, we observed ACP stimulation of cells co-expressing WT-M71, $G\alpha_o$, and either P2Y1R or P2Y2R resulted in significant increases in ERK 1/2 phosphorylation. ACP stimulation of WT-M71 co-expressed with $G\alpha_o$ and β_2-AR exhibited more modest increases in phospho-ERK 1/2, while ACP stimulation of WT-M71 co-expressed with $G\alpha_o$ and A2AR had no effect on ERK 1/2 phosphorylation levels. Pre-treatment of cells with PTX, which inactivates $G\alpha_o$, resulted in a marked decrease in ACP-induced ERK 1/2 phosphorylation in cells co-expressing WT-M71 with P2Y1R or P2Y2R. (Figure 3.6A-B). Studies with a specific anti-$G\alpha_o$ antibody revealed that the levels of $G\alpha_o$ expression achieved in these experiments following transfection of HEK-293 cells were roughly comparable to the expression levels of $G\alpha_o$ in native OE tissue (data not shown). Together, these data demonstrate that M71 co-

Figure 3.6. M71 co-expressed with $G\alpha_o$ in addition to P2Y1R, P2Y2R, and β_2-AR exhibits increased phospho-ERK 1/2 signaling when stimulated with acetophenone. (A) HEK-293 cells were transfected with WT-M71 plus $G\alpha_o$ or WT-M71 plus $G\alpha_o$ and P2Y1R, P2Y2R, β_2-AR, or A2AR. Unstimulated cells were harvested alongside cells exposed to 2-minute stimulation with ACP. Some cells were pre-treated for 24 hours with pertussis toxin (PTX). ACP stimulation of M71 co-expressed with P2Y1R and P2Y2R caused significant increases in ERK 1/2 phosphorylation (n = 12-16, **p < 0.01), which was markedly reduced by PTX pre-treatment (n = 3-4, *p < 0.05). A more modest enhancement of ERK 1/2 phosphorylation occurred in ACP-stimulated cells expressing M71 together with $G\alpha_o$ and β_2-AR while no increases in phospho-ERK 1/2 resulted from stimulation of M71 plus $G\alpha_o$ and A2AR (n = 5). The graph represents pooled data analyzed by 2-way ANOVA and Bonferroni post-hoc tests. Bars and error bars represent means $\pm$ SEM. Representative data for each experimental condition are shown in Panal B.


A
Basal
ACP
PTX
Phospho ERK 1/2 Fold Increase
3.0
2.5
2.0
1.5
1.0
0.5
0.0
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**
*
*
M71 + Gαo
M71 + Gαo + P2Y1R
M71 + Gαo + P2Y2R
M71 + Gαo + β2-AR
M71 + Gαo + A2AR
B
Basal
ACP
PTX
M71 + Gαo
phospho
total
M71 + Gαo + P2Y1R
phospho
total
M71 + Gαo + P2Y2R
phospho
total
M71 + Gαo + β2-AR
phospho
total
M71 + Gαo + A2A-R
phospho
total

expressed with P2Y1R or P2Y2R is functional at the cell surface and capable of coupling to $G\alpha_o$ in an agonist-regulated fashion.

3.3.5 OR interactions with other receptors shows specificity.

We next assessed whether co-expression with β_2-AR, P2Y1R, P2Y2R, and A2AR might generally result in enhanced plasma membrane localization for many ORs, or if these effects might be specific to particular OR classes. In previous confocal studies, we noted that the β_2-AR did not appear to enhance the surface localization of two ORs that are distantly related to M71: hOR17-40 and rat I7 (Hague et al., 2004b). Similarly, in the current analysis, co-expression with β_2-AR, P2Y1R, P2Y2R, or A2AR did not significantly alter hOR17-40 or rat I7 plasma membrane expression as assessed in luminometer assays (Figure 3.7). We also examined the effects of co-expression with the purinergic receptors and β_2-AR on the surface expression of an OR more closely-related to M71, mOR171-4, which is a M71 subfamily member that shares ~67% amino acid identity with M71 (Godfrey et al., 2004). In luminometer assays of FLAG-mOR171-4-GFP transfected HEK-293 cells, co-expression with β_2-AR and A2AR significantly elevated levels of the OR at the plasma membrane, while co-expression with P2Y1R and P2Y2R also modestly enhanced mOR171-4 cell surface expression (Figure 3.7). Additionally, co-immunoprecipitation studies demonstrated the ability of mOR171-4 to associate with β_2-AR, P2Y1R, P2Y2R, and A2AR in a cellular context (Figure 3.8). These data suggest that β_2-AR, P2Y1R, P2Y2R, and A2AR are not general OR chaperones, but that instead

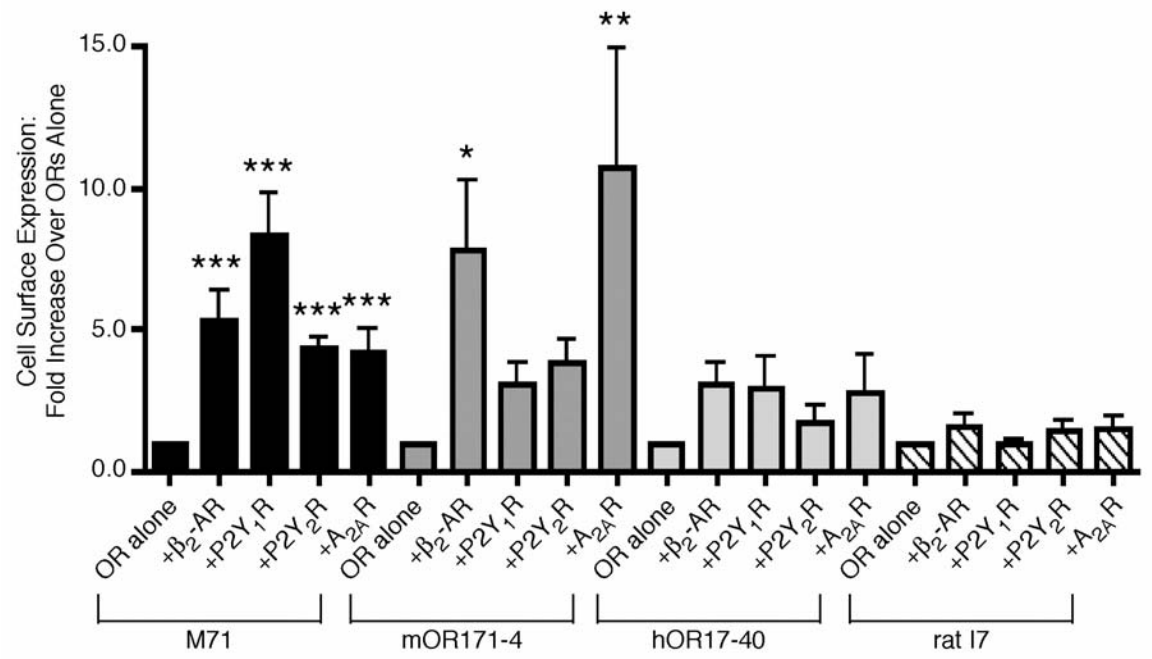


Figure 3.7. Specificity of OR surface expression enhancement by co-expression with P2Y1R, P2Y2R, β_2-AR, and A2AR. P2Y1R, P2Y2R, β_2-AR, and A2AR were co-expressed with three ORs other than M71; FLAG-mOR171-4-GFP, which shares 67% amino acid identity with M71, FLAG-hOR17-40-GFP (46% identity with M71) and FLAG-rat-I7-GFP (45% identity with M71). The bars show means ± SEM for fold increases in cell surface expression following co-expression compared to each OR expressed alone. Each data set was analyzed individually by one-way ANOVA and Dunnett's post hoc test (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

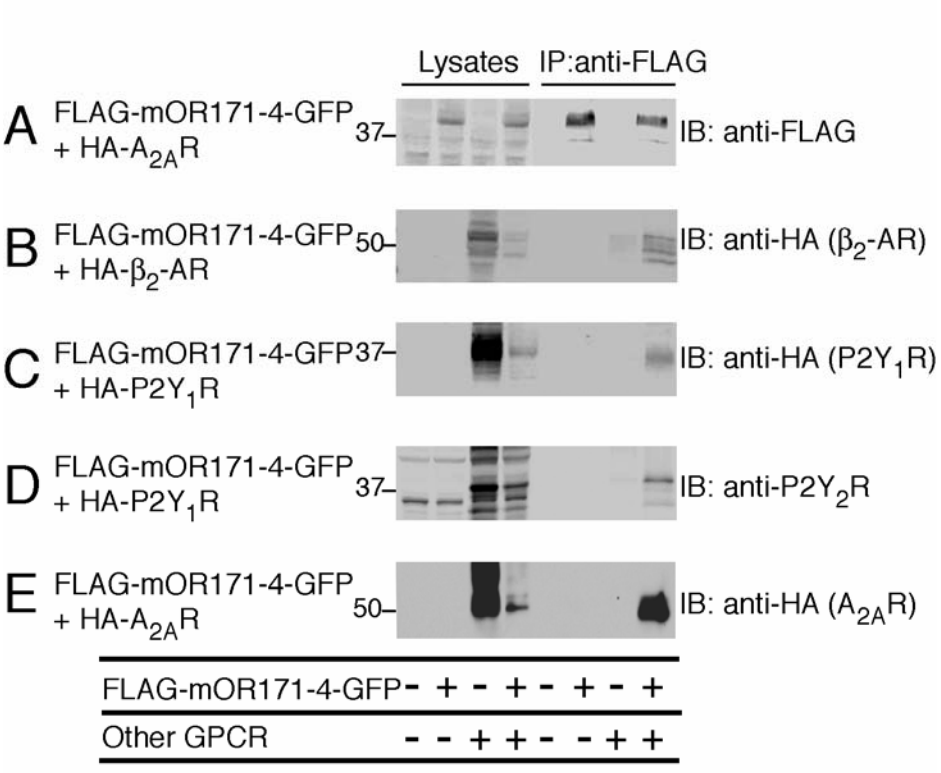


Figure 3.8. Co-immunoprecipitation of P2Y1R, P2Y2R, β_2-AR, and A2AR with mOR171-4. HEK-293 cells were transfected with FLAG-mOR171-4-GFP alone or FLAG-mOR171-4-GFP plus HA-A2AR. Cells were harvested, solubilized, and cell lysates were incubated with anti-FLAG antibody-conjugated agarose beads. Following SDS-PAGE, anti-FLAG antibody was used to detect FLAG-mOR171-4-GFP. Additional experiments were performed co-expressing FLAG-mOR171-4-GFP with HA-β_2-AR (B), HA-P2Y1R (C), P2Y2R (D), and HA-A2AR (E). Western blotting using either anti-HA antibody (B, C, E) or anti-P2Y2R antibody (D) revealed robust co-immunoprecipitation of each receptor with mOR171-4.

these GPCRs interact specifically with particular classes of ORs, with these interactions facilitating OR plasma membrane localization.

3.3.6 The second transmembrane domain of β_2-AR is necessary for β_2-AR-facilitated M71 plasma membrane localization.

To identify structural elements that allow specific GPCRs to enhance the cell surface localization of certain ORs, we utilized chimeras that have the transmembrane domains (TMDs) of β_2-AR sequentially replaced with the TMDs of β_1-AR (Kikkawa et al., 1998). Although the β_1-AR and β_2-AR are closely related, only the β_2-AR significantly increases levels of M71 at the plasma membrane. Chimera 1, in which the N-terminus and TMD1 of β_2-AR are replaced by those of β_1-AR, and chimera 3, in which TMD7 is replaced by that of β1-AR, both exhibited robust enhancement of M71 surface localization, similar to wild-type β_2-AR. Conversely, chimera 2, which contains the TMD2 of β_1-AR, was completely unable to enhance M71 levels at the plasma membrane. In addition, chimera 4, in which both TMD2 and TMD7 of β_2-AR are replaced by those of β_1-AR, was also incapable of localizing M71 to the cell surface (Figure 3.9). These data indicate that TMD2 is necessary for β_2-AR-mediated enhancement of M71 plasma membrane expression.

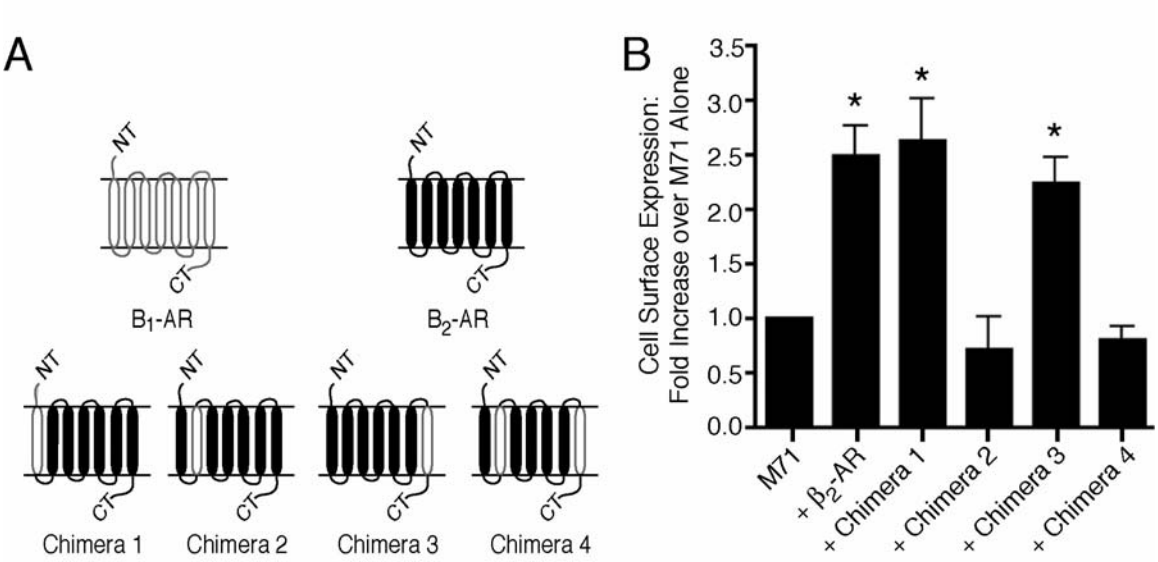


Figure 3.9. β_1/β_2-AR chimera effects on M71 plasma membrane localization. (A) HEK-293 cells were transfected with M71 plus wild-type β_2-AR or chimeras in which various TMDs of the β_2-AR were replaced with those of the β_1-AR. The chimera junctions occurred at the following amino acid positions in the human β_1-AR and β_2-AR sequences: chimera 1, β_1 1-84/β_2 60-413; chimera 2, β_2 1-71/β_1 97-131/β_2 107-413; chimera 3, β_2 1-295/β_1 347-381/β_2 331-413; chimera 4, β_2 1-71/β_1 97-131β_2 107-295/ $\beta_1$347-381/ β_2 331-413. (B) Plasma membrane levels of M71 were quantitated through surface luminometer assays and data from 3 independent experiments was analyzed by one-way ANOVA, using Dunnett's post-hoc analysis (*$p < 0.01$).

3.4 Discussion

The data shown here demonstrate that plasma membrane levels of the OR M71 in HEK-293 cells are significantly enhanced by co-expression with three subtypes of purinergic receptors, P2Y1R, P2Y2R, and A2AR. We further found that M71 co-immunoprecipitates as well as co-localizes with each of the purinergic receptors in HEK-293 cells, and that P2Y1R, P2Y2R, and A2AR are each present in M71-expressing OSNs *in vivo*. These data suggest that certain non-OR GPCRs can associate with and facilitate the surface expression of M71. These receptor-receptor interactions appear to be highly specific, since the vast majority of the 42 GPCRs that we examined had no significant effect on the localization of M71. Several other examples have been described whereby a GPCR that is retained intracellularly when expressed alone in heterologous cells can be liberated to the plasma membrane upon co-expression and association with another GPCR (Hague et al., 2006; Prinster et al., 2006; Uberti et al., 2003). The most well-studied example of this occurrence is the intracellular retention of $GABA_BR1$, which is alleviated by co-expression with $GABA_BR2$ to form a functional heterodimer at the plasma membrane (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). Co-expression of $GABA_BR1$ with 35 other GPCRs, however, does not affect $GABA_BR1$ surface trafficking, exemplifying the specificity of this interaction (Balasubramanian, 2004).

Interactions between receptors can potentially serve as the basis for receptor-receptor cross-talk. With respect to OR interactions with non-OR

GPCRs, it is interesting to note that OR signaling and olfaction in general are known to be modulated by various hormones and neurotransmitters. For example, adrenaline strongly enhances odorant contrast in newt olfactory receptor cells (Kawai et al., 1999), and dopamine has been demonstrated to suppress odorant-induced Ca^{2+} signaling in mouse OSNs and depress overall OSN excitability (Hegg and Lucero, 2004). Most relevant to this study, purinergic nucleotides have been found to reduce odor responsiveness in cultured mouse OSNs (Hegg et al., 2003). In addition, the expression of the purinergic receptor subtypes P2Y1R and P2Y2R has previously been characterized in olfactory epithelium (Gayle and Burnstock, 2005; Hegg et al., 2003) consistent with our findings in the current study. Thus, the present data, taken together with previous findings, suggest that purinergic receptors *in vivo* may associate with certain ORs, such as M71, to promote OR surface expression and regulate OR functionality. This model for the regulation of mammalian ORs by receptor heterodimerization bears similarity to recent findings in the field of *Drosophila* olfaction, where typical ORs have been found to require heterodimerization with an atypical OR, OR83b, in order to achieve proper localization and activity (Benton et al., 2006; Larsson et al., 2004).

The association of the OR M71 with P2Y1R, P2Y2R, and A2AR, whether by direct physical dimerization or via interactions in a multi-protein complex, offers a novel mechanism by which nucleotides may modulate olfaction. Direct associations between ORs and other GPCRs might also potentially alter receptor conformation in a way that results in new pharmacological properties, as has

been established for heterodimers between taste receptors (Nelson et al., 2001; Zhao et al., 2003). In the case of ORs, differential interacting partners could create altered affinities for odorants or contribute to the ability of ORs to be activated by multiple odorants (Buck, 2004).

In addition to potential effects on receptor pharmacology, OR associations with other GPCRs may also influence OR signaling pathways. In our studies, we observed weak activation of the MAPK pathway in response to agonist stimulation of M71 co-expressed with P2Y1R, P2Y2R, and β_2-AR. Strikingly, however, agonist stimulation of M71 co-expressed with exogenous $G\alpha_o$ in addition to P2Y1R, P2Y2R, or β_2-AR resulted in much more significant ACP-induced phosphorylation of ERK 1/2. Notably, P2Y1R, P2Y2R, and β_2-AR have all been demonstrated to signal via $G\alpha_{i/o}$ (Chen and Chen, 1998; Filippov et al., 1998; Vasquez, 2002). A2AR, however, is not known to couple to $G\alpha_{i/o}$, and thus M71 interacting with A2AR may signal through an alternate pathway that does not result in phosphorylation of ERK 1/2. OR signaling through $G\alpha_o$ has not been previously reported, but a number of studies do suggest an ability of ORs to couple to G proteins besides $G\alpha_{olf}$, for example $G\alpha_s$ and $G\alpha_{15/16}$ (Kajiya et al., 2001). In addition, $G\alpha_o$ has been strongly implicated in olfactory signaling. Goa-1, the *C. elegans* orthologue of mammalian $G\alpha_o$, has been shown to modulate olfactory habituation (Matsuki et al., 2006) and $G\alpha_o$ knockout mice exhibit dramatically impaired olfaction (Luo et al., 2002; Matsuki et al., 2006). We propose that association with other GPCRs, such as P2Y1R, P2Y2R, and β_2-AR, imparts to M71 the ability to initiate signaling through coupling to $G\alpha_o$. Further

studies may clarify how the downstream effects of OR signaling through $G\alpha_o$ differ from those that occur by OR signaling through $G\alpha_{olf}$.

Using receptor chimeras, we found that replacing the second TMD of the β_2-AR with TMD2 of β_1-AR abolishes β_2-AR-mediated enhancement of M71 at the plasma membrane. Protein alignments, however, did not reveal any obvious motif similarities in TMD2 among β_2-AR, P2Y1R, P2Y2R, and A2AR, which were not found in other GPCRs, suggesting the structural elements that mediate interaction with M71 may vary from receptor to receptor. Indeed, the TMDs implicated in GPCR dimerization appear to be highly receptor-dependent. TMD6 of β_2-AR has been shown to constitute a necessary interface for receptor homodimerization, whereas this domain was determined to be of limited importance for dopamine D1 receptor dimerization (George et al., 1998; Hebert et al., 1996). Oligomerization of the yeast α-factor receptor was reported to be mediated by the N-terminus, TMD1 and TMD2, and two independent groups identified TMD4 as the interface of dopamine D2 receptor homodimers (Guo et al., 2003; Lee et al., 2003; Overton and Blumer, 2002). CCR5 receptor dimerization appears to depend on residues in TMD1 and TMD4 and oligomerization of the A2AR has been demonstrated to involve the fifth TMD (Hernanz-Falcon et al., 2004; Thevenin et al., 2005). Most recently, oligomerization of the cholecystokinin receptor was shown to be most influenced by TMD7 (Harikumar et al., 2006). In summary, the necessity of TMD2 for β_2-AR mediated enhancement of M71 surface localization adds to the growing

consensus that the mechanisms of GPCR dimerization are based on unique structural complexities distinct to particular interacting partners.

Our results indicate that not all ORs share the propensity to associate with non-OR GPCRs such as β_2-AR, P2Y1R, P2Y2R, and A2AR. We found that an OR with 67% identity to M71 does associate with β_2-AR and the purinergic receptors, whereas two ORs with 46% or less identity to M71 do not. ORs with greater than 60% identity are thought to be activated by similar types of odorants and are therefore classified into the same subfamily (Godfrey et al., 2004). We speculate that non-OR GPCRs such as β_2-AR, P2Y1R, P2Y2R, and A2AR may interact with specific subfamilies of ORs, but not all ORs, to facilitate cell surface expression and modulate responsiveness to odorants. Furthermore, such OR interactions with other receptors may act in concert with OR associations with accessory proteins (Saito et al., 2004) to control OR trafficking. Considering the enormous size of the OR family, a number of distinct mechanisms are likely to contribute to the regulation of OR plasma membrane localization and functionality.

Chapter 4

Structural Determinants Governing Olfactory Receptor Trafficking

4.1 Introduction

4.1.1 Elements governing olfactory receptor trafficking

As demonstrated in Chapters 2 and 3, OR interactions with other receptors can promote OR surface expression in heterologous cells. One plausible mechanism to explain these data is that ORs may possess ER retention signals that are blocked by associations with other receptors. Given this possibility, it is a point of interest to define the structural determinants that lead to OR trafficking deficits, as such information may help clarify how these deficits are overcome by interactions with other receptors. Moreover, the aforementioned examples of OR heterodimerization with other GPCRs justify an examination of whether or not ORs are also capable of homodimerization, as has been reported for a number of distinct GPCRs (Bouvier, 2001; Dean et al., 2001; Javitch, 2004).

4.1.1.1 Endoplasmic retention motifs on intracellular regions of transmembrane proteins

Proteins of varying topology possess endoplasmic reticulum (ER) retention motifs that affect their intracellular trafficking. A variety of lysine-based ER retention motifs have been well characterized including KDEL in some luminal proteins and K(X)KXX (lysine in the -3 and -4/-5 position) in some plasma membrane proteins (Michelsen et al., 2005). Another, lesser-studied class of ER retention motifs that has been identified is arginine-based. ER retention of the

previously described $GABA_BR1$ receptor is thought to occur through the RSRR motif in its C-terminus (Margeta-Mitrovic et al., 2000). Various other plasma membrane localized proteins such as the NR1 subunit (KRRR) of the N-methyl $_D$-aspartate receptor and the Kir6.2 pore-forming subunit (LRKR) of the K_{ATP} channel also possess arginine-based ER retention motifs (Michelsen et al., 2005).

Arginine-based ER retention motifs have been identified in many different cytosolic domains of membrane proteins, most commonly in the C-terminal regions. For the RXR motif, the key determinant of the motif's activity is sufficient exposure for potential binding proteins in the ER (Michelsen et al., 2005). Conversely, the precise mechanism by which ER retention motifs are inactivated is not all together clear, though steric masking, as is proposed to occur with the $GABA_B$ receptors (Margeta-Mitrovic et al., 2000), is an obvious possibility.

To date, no ER retention motifs have been identified in ORs. It has been demonstrated, however, that truncation of the rat OR 5 prior to its 6^{th} transmembrane domain enhances receptor trafficking to the plasma membrane (Gimelbrant et al., 1999). Moreover, class II ORs contain several conserved basic residues within their C-termini, directly following the N-P-x-x-Y motif that is conserved in many GPCRs at the cytoplasmic interface of the plasma membrane. The role of these conserved basic residues is currently unknown and we wondered if they might potentially constitute part of an ER retention signal. We therefore examined, via mutagenesis, whether one or more of three

basic C-terminal residues might be important in the heterologous cell surface expression of the rat I7 OR.

4.1.1.2 N-terminal retention motifs

As mentioned above (4.1.1.1), it has previously been demonstrated that an OR truncated prior to its 6th transmembrane domain properly localizes at the plasma membrane of heterologous cells. It has been speculated that such truncation might eliminate intramolecular interactions between the OR N and C-termini, which may contribute to OR intracellular retention (Gimelbrant et al., 1999). Removal of the N-termini has been successfully used to facilitate cell surface localization of other intracellularly-retained GPCRs. For example, N-terminal truncation was shown to alleviate α_{1D}-AR ER retention (Hague et al., 2004a). Similarly, removing the majority of the large N-termini from the orphan GPCRs GPR37 and GPR56 also facilitates their plasma membrane localization (personal communication, J. Dunham and K. Paavola, Emory University). In an effort to determine the trafficking importance of OR N-termini, we removed the majority of the N-terminus of the rat I7 OR and monitored the cell surface expression of this mutant.

4.1.1.3 Post-synaptic density-95, Discs large, Zona occludens-1 (PDZ) binding motifs

(PDZ) binding motifs are short C-terminal peptide sequences recognized by PDZ domain-containing proteins, many of which are scaffolds that have been

demonstrated to be important interacting partners for a number of GPCRs (Balasubramanian et al., 2007; Chen et al., 2006; Paquet et al., 2006). PDZ domain-containing scaffolds cluster functionally-related proteins in close proximity to each other to enhance signaling efficiency. Furthermore, association with PDZ domain-containing proteins can regulate the internalization and recycling of stimulated GPCRs.

PDZ domain-containing proteins are classified into three groups based on the PDZ motifs they recognize, which are typically determined by the amino acids at the 0 and –2 positions in the motif, though residues at the –1 and –3 positions can also affect binding. Class I PDZ domains recognize a –S/T-X-ϕ sequence, class II PDZ domains recognize a -ϕ/Ψ-X-ϕ sequence, and class III PDZ domains recognize a –D/E-X-V sequence (ϕ, hydrophobic residue: Ψ, aromatic residue: X, any residue) (Vaccaro and Dente, 2002). The human OR 2AG1 has an ideal C-terminal class 1 PDZ binding motif (-S-T-L), which is unusual amongst ORs. Interestingly, 2AG1 is one of the few ORs that has been reported to successfully localize at the plasma membrane of heterologous cells without modification of the protein (Mashukova et al., 2006). We wondered whether the 2AG1 PDZ binding motif might impart the receptor with the ability to traffic efficiently to the cell surface in heterolgous cells, and we therefore examined the trafficking consequence of adding the 2AG1 C-terminal PDZ motif onto a non-PDZ motif containing OR.

4.1.1.4 Chemical chaperones

The misfolding and mislocalization of various proteins has been shown to improve upon treatment with compounds known as “chemical chaperones.” One of these compounds in particular, 4-phenylbutyrate (4-PB), has been demonstrated to alleviate ER retention of the misfolded ΔF508 cystic fibrosis transmembrane conductance regulator (CFTR), thus restoring chloride channel activity in cystic fibrosis patients (Rubenstein and Lyons, 2001; Zeitlin et al., 2002). 4-PB has additionally been found to exhibit chaperone activity on protein aggregation *in vitro*. Furthermore, 4-PB treatment restores surface expression of the normally ER-aggregated orphan GPCR, GPR37 (Pael-R) (Kubota et al., 2006). The affect of 4-PB appears to occur through a reduction of ER-stress and cell death without induction of certain ER chaperone proteins. It is speculated that 4-PB interacts with misfolded proteins directly to prevent aggregation between hydrophobic regions, however the exact mechanisms by which chemical chaperones such as 4-PB work are not fully understood (Kubota et al., 2006). We examined whether treatment with 4-PB could alleviate the intracellular retention of heterologously-expressed ORs.

4.1.1.5 The human ortholog of C. elegans odorant response abnormal 4 (ODR-4)

As previously discussed in Chapter 1 of this dissertation (1.6.1), the ODR-4 gene of *C. elegans* encodes a protein that is specifically localized to chemosensory neurons and is necessary for *C. elegans* OR plasma membrane

expression. ODR-4 is thought to aid in the folding, sorting, or transport of ORs (Dwyer et al., 1998). The human ortholog of ODR-4 (hODR-4) has more recently been identified and its potential to influence the trafficking of ORs or other proteins is unknown (Lehman et al., 2005). We examined whether the hODR-4 is able to enhance mammalian OR trafficking similar to its *C. elegans* counterpart.

4.1.1.6 N-terminal signal sequences and glycosylation sites

The addition of N-terminal signal leader sequences has successfully been employed to enhance the plasma membrane expression of various GPCRs. For ORs specifically, addition of the signal leader sequences from the serotonin receptor type 3, rhodopsin and vasopressin 1 receptors have all helped facilitate cell surface localization of some ORs in heterologous cells (Kajiya et al., 2001; Katada et al., 2003; Katada et al., 2004; Krautwurst et al., 1998; Wetzel et al., 1999; Yasuoka et al., 2000). The aforementioned signal leader sequences also contain sites for potential N-glycosylation. Evidence suggests that N-terminal glycosylation is critical for the proper surface localization of mOR-EG (Katada et al., 2004) and indeed, the majority of ORs contain a highly conserved potential N-glycosylation site at their N-terminus. Though the mechanism(s) by which signal leader sequences enhance GPCR trafficking are not all together clear, they still present a useful way to enhance functional expression of ORs at the cell surface of heterologous cells. We modified some of the ORs under study in our

laboratory to contain an N-terminal rhodopsin tag (Rho-tag) and examined their surface localization and functionality.

4.1.2 Olfactory receptor homodimerization

It is now well-accepted that GPCRs do not function exclusively as isolated monomeric entities. Instead, work over much of the last decade indicates that GPCRs oligomerize to generate higher order functioning units. GPCR oligomerization includes heterodimerization (associations between two different types of receptors) as well as homodimerization (associations between two receptors of the same type), and indeed it seems that many GPCRs are capable of both. As discussed previously in this dissertation (Chapter 1.6.2), the functional consequences of GPCR heterodimerization are numerous, varied, and not all together understood. The functional consequences of GPCR homodimerization, however, are even more poorly understood owing to the technical difficulties of differentiating between monomeric and dimeric receptor properties. One speculation for the physiological significance of dimerization as a whole is that it may be a general requirement for GPCRs to be successfully exported from the ER. Currently, however, there is limited evidence available to clarify the relationship between GPCR dimerization and trafficking. Heterodimerization is clearly necessary for ER-export of the $GABA_B$ receptor (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998) and, as previously mentioned, evidence suggests heterodimerization is also important for the cell surface localization of members of the adrenergic receptor family (Hague et al.,

2006; Hague et al., 2004c). Additionally, studies on the β_2-AR suggest that homodimerization is a prerequisite for its plasma membrane targeting (Hebert et al., 1998; Hebert et al., 1996; Salahpour et al., 2004). Given our findings that the cell surface expression of certain ORs is enhanced by specific heterodimerization events (Chapters 2 and 3), it is of further interest to determine whether ORs are also capable of homodimerzation, as this point is potentially central to understanding their trafficking prerogatives. We thus examined the homodimerization capacity of several different ORs.

4.2 Experimental Procedures

4.2.1 Constructs

The FLAG-mOR171-4-GFP, FLAG-rat I7-GFP, and FLAG-hOR17-40-GFP constructs were generated as previously described (Bush et al., 2007; Hague et al., 2004b). The rat I7-GFP and FLAG-rat I7 constructs were generated through subcloning into pcDNA3.1- and a FLAG-tagged version of the pBK CMV vector respectively. POGR and PSGR clones were purchased from ATCC and were amplified via PCR with forward primers corresponding to nucleotides 4-26 (PSGR) and 4-27 (POGR) and reverse primers corresponding to nucleotides 942-963 (PSGR) and 932-954 (POGR). Subsequently, POGR and PSGR were subcloned into both pCMV-Tag 2B (Stratagene) and pcDNA3.1+ (Invitrogen, modified to contain a FLAG-tag and the first 20 amino acids of bovine rhodopsin) using 5' EcoRI and 3' XhoI sites, thus producing N-terminally FLAG-tagged and

FLAG-rhodopsin-tagged constructs, respectively. FLAG-rat I7-GFP mutants were generated using a site-directed mutagenesis kit (Stratagene). The FLAG-rat I7-GFP construct lacking the N-terminal 21 amino acids was generated through PCR with forward and reverse primers corresponding to nucleotides 64-81 and 958-981, respectively. The resultant product was inserted into pEGFP-N3 (Clontech) via a 5' XbaI site and 3' KpnI site. The FLAG-rat I7-PDZ construct was created via 2 PCR reactions. In the first PCR, the forward primer corresponded to the FLAG-tag sequence (which precedes rat I7 in peGFP-N3) and the reverse primer corresponded to the last 24 nucleotides of the rat I7 sequence (minus the stop codon) followed by the last 18 nucleotides of OR2AG1. This PCR product was then used as the template for the second reaction with the same forward primer encoding a 5' EcoRI site and a reverse primer encoding a stop codon and a 3'XhoI site. This final product was inserted into pcDNA3.1+. The hODR-4, mOR454 and mOR828 constructs were kindly provided by Claire Komives (San Jose State University).

4.2.2 Cell culture and transfection

All tissue culture media and related reagents were purchased from Invitrogen. CHO cells were maintained in F12 medium plus 10% fetal bovine serum and 1% penicillin/streptomycin and HEK-293 cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) at 37°C with 5% CO2. 80-95% confluent cells in 10-cm tissue culture dishes were transfected with 1-3 μg of cDNA mixed

with 15 μl Lipofectamine 2000 in 5 ml of serum-free medium. Following overnight incubation, complete medium was added and cells were trypsinized and re-plated.

4.2.3 Western blotting

Samples were resolved by SDS-PAGE on 4-20% Tris-Glycine gels, followed by transfer of protein to nitrocellulose membranes (Bio-Rad). The membranes were incubated in blocking buffer (2% non-fat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM HEPES, pH 7.4) for 30 minutes and then incubated with primary antibody for either 1 hour at room temperature or overnight at 4°C. Next, the membranes were washed three times in blocking buffer and incubated with a fluorescent-conjugated secondary antibody for 30 minutes at room temperature, followed by three blocking buffer washes. Proteins bound by fluorescent-conjugated secondary antibody were detected using the Odyssey imaging system (Li-Cor).

4.2.3 Surface luminometer assay

Transiently transfected HEK-293 or CHO cells were split into poly D-lysine-coated 35-mm dishes and grown overnight at 37°C. Cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde, and washed with PBS again. Cells were then incubated in blocking buffer (2% nonfat milk in PBS, pH 7.4) for 30 minutes, followed by incubation with HRP-conjugated M2-anti-FLAG antibody (1:600, Sigma) in blocking buffer for 1 hour at room

temperature. In experiments using HA-tagged ORs, cells were incubated in mouse anti-HA primary antibody for 1 hour, washed three times with blocking buffer, then incubated with sheep anti-mouse HRP-conjugated secondary antibody. Cells were washed twice with blocking buffer, twice with PBS, and then incubated with SuperSignal Pico ECL reagent (Pierce) for 15 seconds. Luminescence of the entire 35-mm dish was determined using a TD-20/20 luminometer (Turner Designs). Mean values ± SEM were calculated as percent absorbance in arbitrary units.

4.2.4 Cyclic AMP Assays

The protocol used to measure cAMP formation in HEK-293 cells is a modification of a widely used prelabeling protocol (Guerrero and Minneman, 1999). Transfected HEK-293 cells were split into poly-D-lysine coated 24-well plates 24 h before experimentation. Because HEK-293 cells do not easily take up 3H-adenine, 3H-adenosine was used to pre-label cells. Cells were prelabeled with .25 ml of fresh media containing 1 µCi (1 Ci = 37 GBq) of 3H-adenosine for 2 h. Labeling media was aspirated and cells were incubated in .25ml Na-Elliot buffer (137 mM NaCl/5mM KCl/1.2 mM MgCl2/.44 mM KH2PO4/4.2mM NaHCO3/20mM HEPES/10 mM glucose) containing 200 µM 3-isobutyl-1-methylxanthine, pH 7.4, at 37°C for 10 minutes. Stock concentrations of various potential ligands were dissolved either in straight Na-Elliot buffer, or in Na-Elliot buffer containing 10% ethanol, such that final ethanol concentrations in cells were 0.1%. Cells were incubated with potential ligands for the time points

specified in the results section, and reactions were stopped by addition of 100ul 77% trichloroacetic acid. A 50µl aliquot of 10 mM cyclic AMP (cAMP) was added as a carrier. 3H-cAMP formed was isolated by sequential Dowex (Guerrero and Minneman, 1999) and alumina chromatography. Eluants from alumina columns were collected, 10 ml of 30% scintillation fluid was added, and 3H-cAMP was quantified by using a liquid scintillation counter.

4.2.5 Co-immunoprecipitation

Transfected cells were harvested in 500 µl of freshly made ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 1.0% Triton X-100, 5mM EDTA) and rotated end-over-end at 4°C for 30 minutes to solubilize. Unsolubilized membranes were pelleted through centrifugation. 100 µl of the supernatant was reserved to verify construct expression and 20 µl 6x sample buffer was added. The remaining supernatant was incubated with 60 µl of anti-FLAG antibody-conjugated agarose beads rotating at 4°C. Following at least 2 hours of incubation, the beads were pelleted and washed 6 times for 5 minutes each with 1 ml of high salt lysis buffer (10 mM HEPES, 150 mM NaCl, 1.0% Triton X-100, 5mM EDTA) rotating end-over-end at 4°C. Next, 100 µl of 2x sample buffer was added to elute the proteins. 20 µl of lysate and immunoprecipitated samples were loaded onto 4-20% gels and analyzed by Western blotting as described above. 12% gels were used to obtain better separation between the FLAG-rat I7-GFP and rat I7-GFP constructs in Figure 4.9.

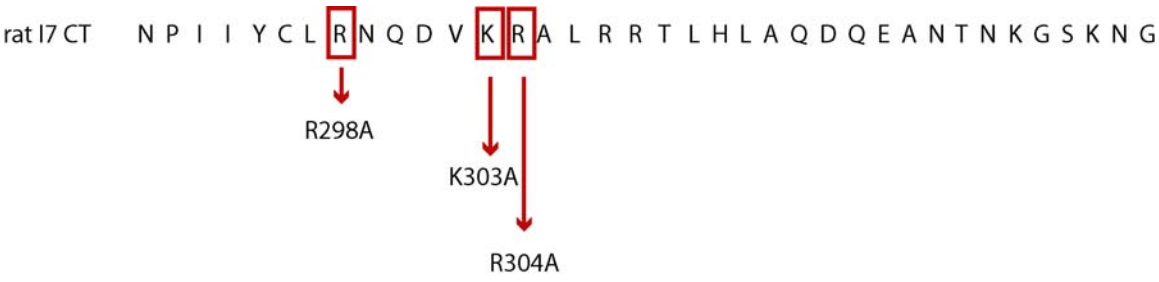
4.3 Results

4.3.1 Mutation of basic residues in the carboxyl-terminus does not enhance the surface expression of the rat I7 olfactory receptor.

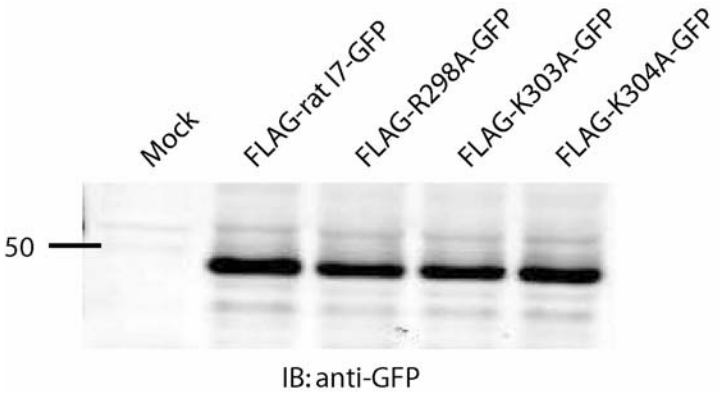
Class II ORs contain several conserved basic residues in their C-termini. Based on reports that arginine and lysine-based ER-retention motifs often occur in receptor C-termini (Michelsen et al., 2005), along with findings that elements in the C-terminus of ORs may be important in trafficking (Gimelbrant et al., 1999; Katada et al., 2004), we examined the importance of the three basic residues immediately following the 7th transmembrane domain in the rat I7 OR (Figure 4.1 A). Via site-directed mutagenesis, we generated lysine to alanine and arginine to alanine mutants to evaluate whether these residues were individually critical for receptor retention. The constructs possessed an N-terminal FLAG-tag and a C-terminal GFP-tag for detection purposes. We transiently expressed rat I7 and mutants ΔR298A, ΔK303A, and ΔR304A in HEK-293 cells and measured total protein expression and cell surface expression via Western blotting and a luminometer assay, respectively. As shown in Figure 4.1 B-C, the mutants' total protein expression levels were similar to those of wild-type rat I7 and they did not show an enhancement of the very low plasma membrane expression exhibited by the wild-type receptor.

Figure 4.1. Unchanged levels of total protein and surface-expressed protein for the wild-type rat I7 and I7 ΔK298A, ΔR303A, and ΔR304A mutants. (A) Class II ORs like the rat I7 contain multiple conserved basic residues in their C-termini (CT). We mutated three of the basic residues of the rat I7 OR to alanine via site-directed mutagenesis: ΔR298A, ΔK303A, and ΔR304A. (B) Mutants and wild-type receptor had a N-terminal FLAG-tag and C-terminal GFP tag. Total protein expression of the mutants was compared to wild-type receptor by Western blotting with an anti-GFP antibody. (C) Cell surface expression of the mutants and non-mutated rat I7 was observed via a luminometer assay after incubation with an anti-FLAG HRP-conjugated antibody. Cell surface expression of the FLAG-β_1-adrenergic receptor (β_1-AR) is shown as a positive control. Bars represent data from 3 independent experiments.

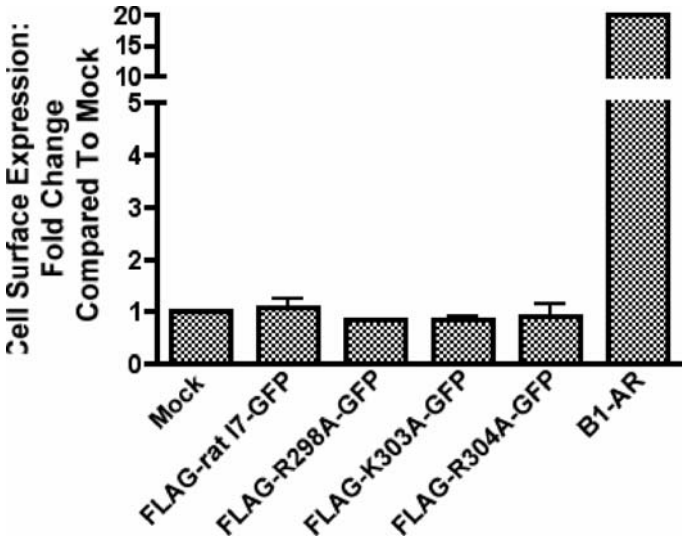
A


rat I7 CT N P I I Y C L R N Q D V K R A L R R T L H L A Q D Q E A N T N K G S K N G
R298A
K303A
R304A

B


Mock
FLAG-rat I7-GFP
FLAG-R298A-GFP
FLAG-K303A-GFP
FLAG-K304A-GFP
50
IB: anti-GFP

C

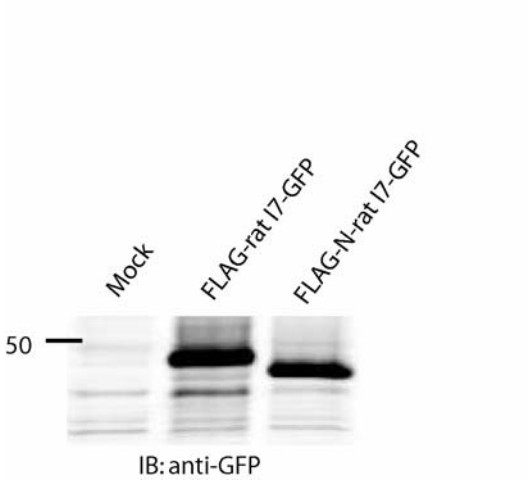

Cell Surface Expression:
Fold Change
Compared To Mock
20
15
10
5
4
3
2
1
0
Mock
FLAG-rat I7-GFP
FLAG-R298A-GFP
FLAG-K303A-GFP
FLAG-R304A-GFP
B1-AR

4.3.2 N-terminal truncation of the rat I7 olfactory receptor does not enhance its plasma membrane localization.

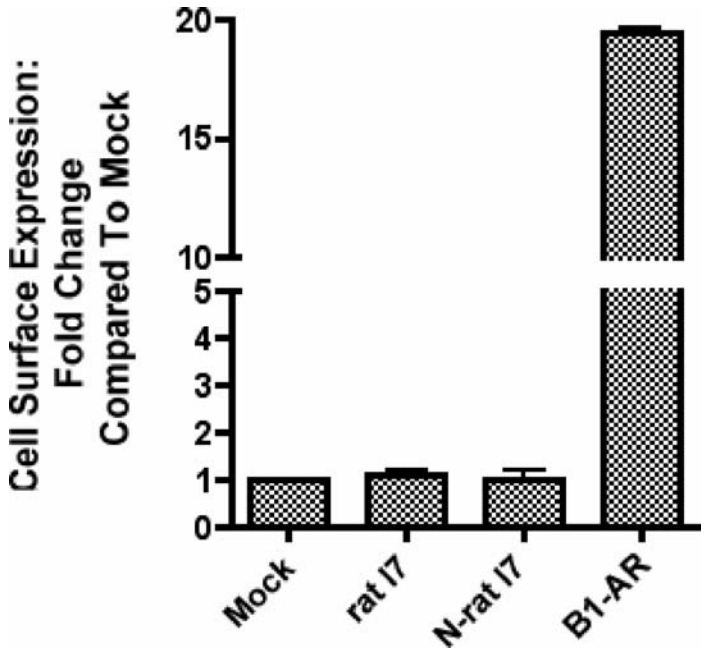
Truncation of the N-terminus can influence the cell surface trafficking of some GPCRs (Hague et al., 2004a). Additionally, it has been postulated that ORs are retained intracellularly due to intramolecular interactions between their N and C-termini (Gimelbrant et al., 1999). Thus, we truncated 21 of the 26 amino acids preceding the first transmembrane domain of the rat I7 receptor and compared the plasma membrane expression of this truncated receptor and the full-length receptor. We found no difference between the total protein or cell surface expression levels of the truncated and full-length rat I7 ORs, with both detectable at only very low levels at the plasma membrane (Figure 4.2).

Figure 4.2. N-terminal truncation of the rat I7 olfactory receptor does not alter its surface expression in heterologous cells. The N-terminal 21 amino acids of the rat I7 OR were removed through subcloning (N-rat I7). Total protein and cell surface protein levels of this construct were compared to a full-length rat I7 construct. Both contained N-terminal FLAG epitope tags and C-terminal GFP epitope tags. (A) The FLAG-N-rat-I7-GFP expressed at a protein level similar to full-length FLAG-rat I7-GFP. (B) The FLAG-N-rat I7-GFP and full-length FLAG-rat I7-GFP both showed similar lack of detectable plasma membrane expression. Cell surface expression of the β_1-AR is shown as a positive control. These data are from 3 independent experiments. Bars and error bars represent means $\pm$ SEM.

A


Mock
FLAG-rat I7-GFP
FLAG-N-rat I7-GFP
50
IB: anti-GFP

B


Cell Surface Expression:
Fold Change
Compared To Mock
20
15
10
5
4
3
2
1
0
Mock
rat I7
N-rat I7
B1-AR

4.3.3 Addition of the PDZ motif from the olfactory receptor 2AG1 to the C-terminus of the rat I7 olfactory receptor does not alter surface expression.

As demonstrated repeatedly throughout this dissertation, the rat I7 and most other olfactory receptors do not traffic efficiently to the plasma membrane upon expression in heterologous cells. Interestingly, the human OR 2AG1 has been reported to be successfully expressed at the cell surface when transfected in HEK-293 cells (Mashukova et al., 2006). In examining the sequence of 2AG1 for elements that might impart this unusual trafficking capability, we noticed that the carboxyl-terminus of 2AG1 contains a consensus class 1 PDZ binding motif. This is a unique feature, as the majority of ORs do not possess PDZ motifs. Interestingly, several of the receptors demonstrated to promote OR surface expression in Chapters 2 and 3, including the β_2-AR and P2Y1R (Hall et al., 1998), also possess class 1 PDZ-interaction motifs that play key roles in regulating receptor signaling and trafficking. It is currently unclear whether this observation is purely coincidence or has physiological relevance to OR interactions with these proteins. To test whether the 2AG1 PDZ motif, which would presumably allow for interaction with unidentified PDZ domain-containing proteins, could influence the surface trafficking of an OR other than 2AG1, we created a chimeric OR. We fused the final 7 amino acids, including the PDZ motif, of the 2AG1 receptor onto the C-terminus of the rat I7 OR and evaluated the chimera's cell surface localization. As seen in Figure 4.3, addition of the 2AG1 PDZ motif to the rat I7 did not alter the poor plasma membrane localization of rat I7.

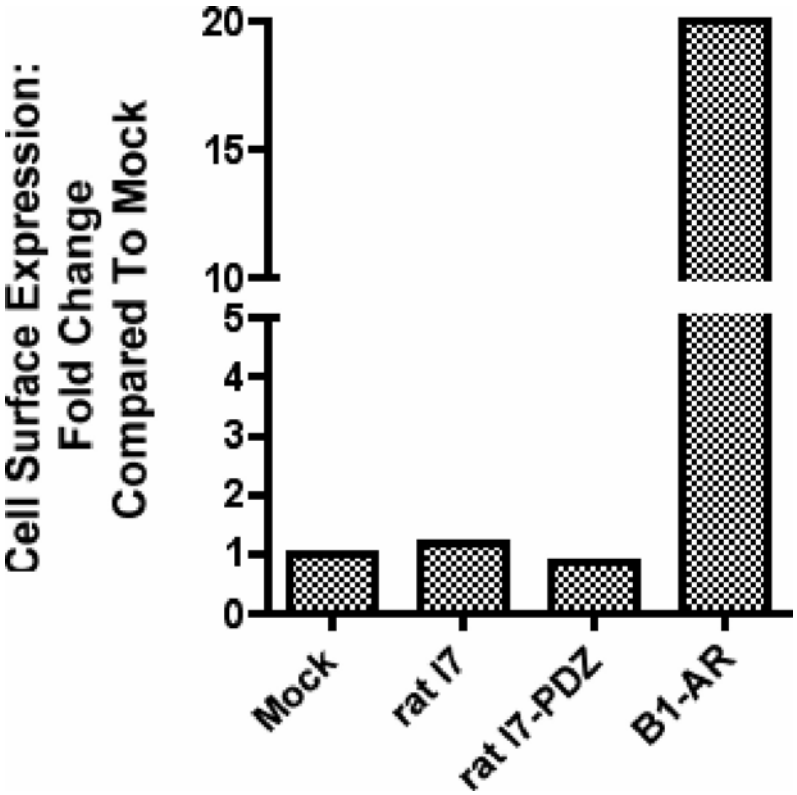


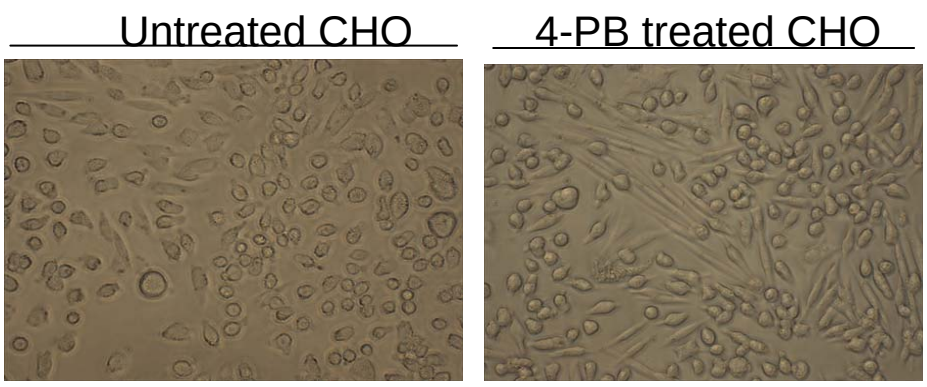
Figure 4.3. Addition of a PDZ-binding motif to the C-terminus of the rat I7 olfactory receptor does not enhance its cell surface localization. The C-terminal 7 amino acids of the human 2AG1 OR, including its class 1 PDZ motif, were fused onto the C-terminus of the rat I7 OR (rat I7-PDZ). Following transient expression in HEK-293 cells, plasma membrane levels of the chimera were detected via luminometer assay. Cell surface expression of the β_1-AR is shown as a positive control. Bars represent data from 2 independent experiments.

4.3.4 4-Phenylbutyrate treatment effects on heterologously-expressed olfactory receptors.

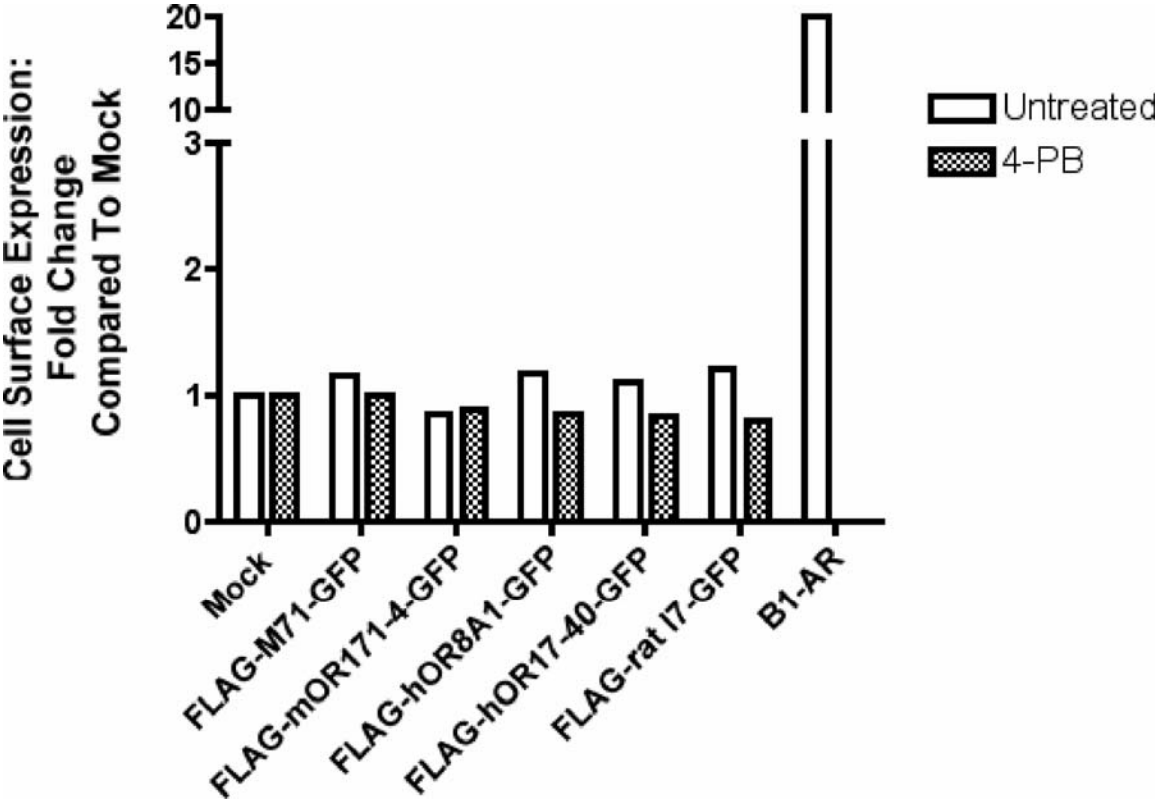
Treatment with 4-phenylbutyrate (4-PB) has been demonstrated to alleviate the ER retention of multiple misfolded proteins, including the ΔF508 mutant of the cystic fibrosis transmembrane conductance regulator (CFTR) (Rubenstein and Lyons, 2001; Zeitlin et al., 2002) and the orphan receptor GPR37 (Kubota et al., 2006). To investigate whether 4-PB treatment similarly alleviates OR intracellular retention, we transiently expressed the ORs M71, mOR171-4, hOR8A1, hOR17-40, and rat I7 in CHO cells. We opted to use CHO cells because they showed the characteristic 4-PB-induced change in cell morphology while HEK-293 cells did not. Cells were either left untreated or were treated for 48 hours with 3mM 4-PB. As shown in figure 4.4, no significant differences in OR cell surface detection were observed between untreated and 4-PB treated cells.

Figure 4.4. 4-phenylbutyrate treatment does not enhance the plasma membrane localization of several intracellularly retained olfactory receptors. The N-terminally FLAG-tagged ORs M71, mOR171-4, hOR8A1, hOR17-40, and rat I7 were transiently transfected in CHO cells and their levels of plasma membrane expression were evaluated after treatment with 4-PB. (A) Following 48 hours of 4-PB treatment, CHO cells exhibited an elongated morphology that is associated with 4-PB exposure. (B) Treated versus untreated cells showed no difference in the levels of cell surface detectable ORs as assessed by luminometer assay. The cell surface expression of the β_1-AR is shown as a positive control. Bars represent data from a single experiment.

A


Untreated CHO
4-PB treated CHO

B


Cell Surface Expression:
Fold Change
Compared To Mock
20
15
10
3
2
1
0
Untreated
4-PB
Mock
FLAG-M71-GFP
FLAG-mOR171-4-GFP
FLAG-hOR8A1-GFP
FLAG-hOR17-40-GFP
FLAG-rat I7-GFP
B1-AR

4.3.5 hODR-4 co-expression does not effect the cell surface localization of multiple olfactory receptors.

The *C. elegans* ODR-4 gene encodes a protein that is specifically localized to chemosensory neurons, is thought to aid in the folding, sorting, or transport of ORs, and is necessary for proper *C. elegans* OR plasma membrane expression (Dwyer et al., 1998). To determine whether the human ortholog of ODR-4 (h-ODR-4) (Lehman et al., 2005) is similarly able to effect mammalian ORs, mOR454, mOR828, rat I7, M71 and a prostate-expressed OR, prostate-specific GPCR (PSGR) were expressed alone or co-expressed with the hODR-4 construct and the cell surface expression of these ORs was monitored via the luminometer assay. As seen in Figure 4.5, none of the ORs' cell surface expression levels were significantly altered by co-expression with hODR-4. hODR-4 co-expression also did not alter the poor cell surface expression of several examined trace amine associated receptors, a new class of chemosensory GPCRs found to be highly expressed in the OE (data not shown.) Still, hODR-4 may be involved in the trafficking of a specific subset of ORs not yet examined, or, alternatively, hODR-4 may be involved in the trafficking of GPCRs found outside of the OE, given that it exhibits a much broader tissue distribution pattern than its ortholog in *C. elegans* (Lehman et al., 2005).

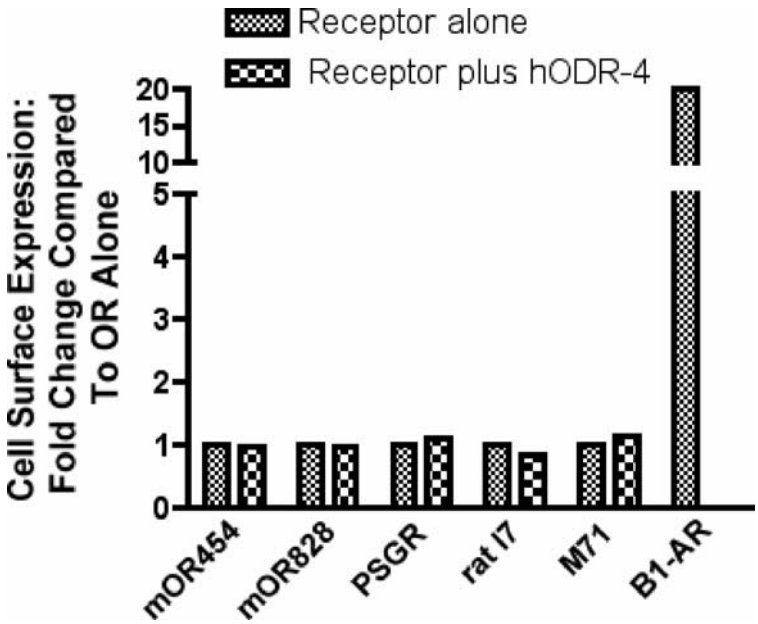


Figure 4.5. Co-expression with the odorant response abnormal 4 human ortholog does not influence the plasma membrane expression of a variety of olfactory receptors. The ORs HA-mOR454, HA-mOR828, FLAG-PSGR, FLAG-rat I7, and FLAG-M71 were expressed alone or co-expressed with human odorant response abnormal 4 (hODR-4) and plasma membrane levels were detected via a luminometer assay following incubation with the appropriate antibody(s) (either anti-FLAG HRP-conjugated antibody or monoclonal anti-HA 12CA5 primary antibody followed by sheep anti-mouse HRP-conjugated secondary antibody.) Co-expression with hODR-4 did not alter the plasma membrane associated levels of any of the examined olfactory receptors. The cell surface expression of the β_1-AR is shown as a positive control. Bars represent data from a single experiment.

4.3.6 Signal leader sequence addition enhances the cell surface localization of two prostate-expressed olfactory receptors.

N-terminal addition of the first 20 amino acids of rhodopsin has been reported to enhance the cell surface localization of a number of ORs, as well as other GPCRs (Chandrashekar et al., 2000; Kajiya et al., 2001; Katada et al., 2004; Krautwurst et al., 1998). In an attempt to improve the cell surface expression and functionality of the prostate-overexpressed GPCR (POGR) and prostate-specific GPCR (PSGR), which are ORs found in both the prostate and olfactory epithelium, we generated chimeric constructs that contained an N-terminal FLAG-Rho-tag. In luminometer assay, FLAG-Rho-POGR and FLAG-Rho-PSGR both showed significant elevations in the plasma membrane localization compared to FLAG-POGR and FLAG-PSGR constructs that did not contain a Rho-tag (Figure 4.6).

Subsequent to enhancing the surface expression of the ORs POGR and PSGR, we also wished to determine whether the Rho-tagged versions of these receptors were functional, as Rho-tagged ORs have previously been shown to respond to agonist stimulation (Chandrashekar et al., 2000; Kajiya et al., 2001; Katada et al., 2004; Krautwurst et al., 1998). At the onset of these experiments, little was known concerning POGR and PSGR beyond their high homology to each other, unique expression in human prostate tissue and over-expression in prostate cancer (Weigle et al., 2004; Xia et al., 2001; Xu et al., 2000). Using this limited information, we identified and screened several potential ligands using

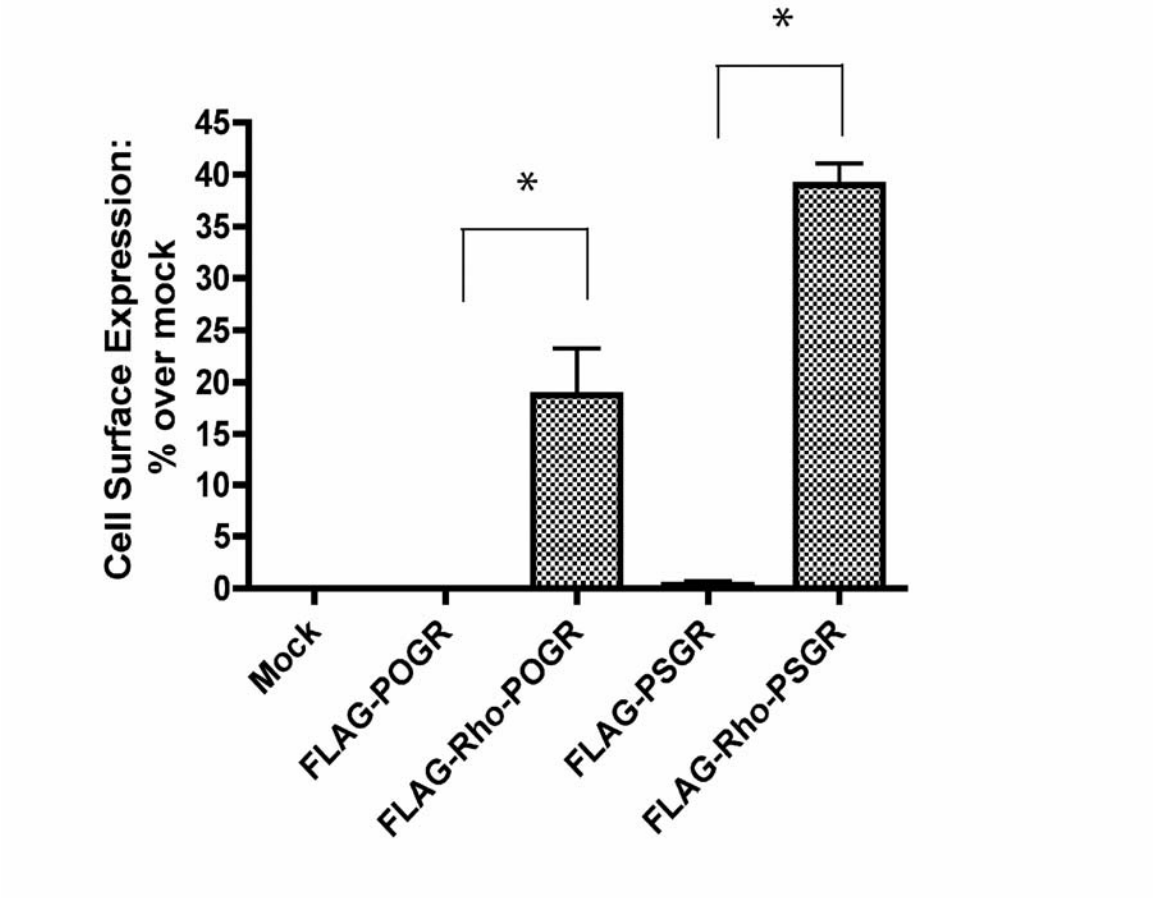


Figure 4.6. Addition of an N-terminal Rhodopsin-tag enhances surface localization of POGR and PSGR. POGR and PSGR were subcloned into a vector containing an N-terminal FLAG-tag followed by the first 20 amino acids of rhodopsin (Rho), thus generating FLAG-Rho-POGR and FLAG-Rho-PSGR. The constructs were transiently transfected into HEK-293 cells and OR surface presence was monitored via a luminometer assay. Bars and error bars represent means ± SEM. Data from 3 independent experiments was analyzed by one-way ANOVA, using Dunnett's post-hoc analysis (*$p < 0.01$).

FLAG-Rho-POGR and FLAG-Rho-PSGR transiently transfected HEK-cells in a cyclic AMP assay. Because POGR and PSGR are class 1 or “fish-like” ORs (Conzelmann et al., 2000), our screen included molecules that are detected by fish olfaction, such as bile acids, amino acids, and gonadal steroids (Hara, 1994). We also examined molecules suggested to have a GPCR-related role in prostate cancer, such as β_2-microglobulin (Huang et al., 2006). Table 4.1 outlines the screened compounds, briefly states their relevance, and indicates references. Despite these efforts, none of the examined compounds elicited cAMP accumulation upon treatment of FLAG-Rho-POGR or FLAG-Rho-PSGR transfected cells. Concurrent with our studies, a report was published showing that 3 and 4-methylvaleric acids (3 and 4-MVa) could stimulate Rho-tagged POGR mediated cAMP generation and were therefore putative ligands for POGR (Fujita et al., 2007). We were able to replicate these findings (Figure 4.7) and proceeded to examine compounds structurally related to 3 and 4-MVa for potential agonist activity at POGR and PSGR (Table 4.2, Figure 4.8). We found weaker, though significant activity by the parent compound, valeric acid (also demonstrated by Fujita et al., 2007), while valproic acid, γ-aminobutyric acid (GABA), and γ-hydroxybutyric acid (GHB) had no effect on POGR or PSGR mediated cAMP generation (Figure 4.8).

Table 4.1. Potential ligands screened for activation of POGR and PSGR

Potential Ligand	Relevance	References
β_2-microglobulin	-β_2-microglobulin activation of mystery GPCR in prostate cancer -$G_{\beta\gamma}$ signaling inhibition retards prostate tumor xenograft growth -MHC components are involved in individual odortypes	(Bard et al., 2000; Bookout et al., 2003; Huang et al., 2006; Spehr et al., 2006a)
Taurocholic Acid, Deoxycholate, Glycocholate	-Fish ORs are strongly activated by bile salts -A GPCR activated by bile acids was recently identified	(Choi et al., 2003; Kawamata et al., 2003; Lo et al., 1994; Rolen and Caprio, 2007)
L-Methionine, L-Alanine, L-Histadine, L-Leucine, L-Lysine, L-Asparagine, Glycine, L-Serine, Aspartic Acid, Glutamic Acid, L-Valine, L-Isoleucine, L-Adenine, L-Phenylalanine, L-Proline	-Fish ORs are strongly activated by amino acids	(Lindsay and Vogt, 2004; Lo et al., 1993)
Testosterone, Androstenol, β-estradiol	-Fish ORs are activated by gonadal steroids -Certain sex steroids are potent odorants in mammals (such as androstenol)	(Culig and Bartsch, 2006; Hara, 1994; Laska et al., 2005)
Acetophenone, Benzaldehyde, Citral, Amyl Acetate	- Odorants work in a combinatorial manner and can activate multiple ORs	(Malnic et al., 1999)

β_2-microglobulin, along with mixtures of a variety of bile acids, amino acids, steroids, and odorants were screened in cAMP assays for potential activation of Rho-tagged POGR or PSGR. Compounds were identified based on relevant evidence from the literature. None of these compounds showed significant stimulation of either POGR or PSGR-mediated cAMP generation.

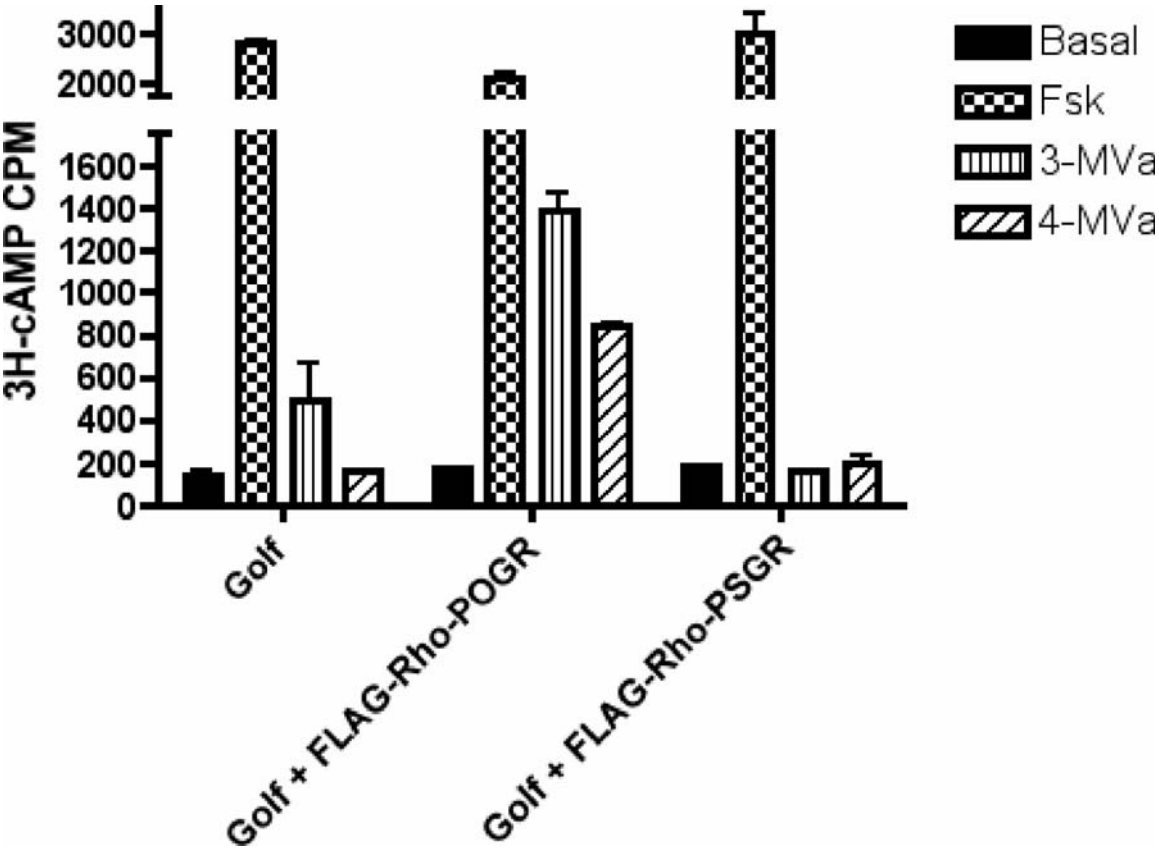
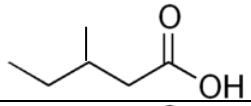
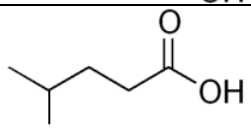
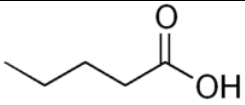
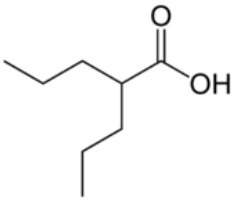
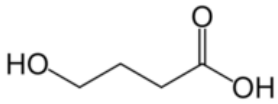
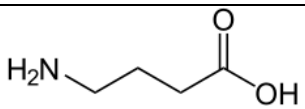


Figure 4.7. 3-methylvaleric acid and 4-methylvaleric acid stimulate cyclic AMP generation at POGR, but not PSGR. FLAG-Rho PSGR and POGR were transiently transfected into HEK-293 cells along with $G\alpha_{olf}$. Forskolin (Fsk) was used to validate cAMP production in the cells. Generation of cAMP was measured in response to stimulation with 3 and 4-methylvaleric (3 and 4-MVa) acids. POGR showed significant stimulation by both 3 and 4-MVa while PSGR did not. Raw data are shown as 3H-cAMP counts per minute (CPM) from a representative experiment.

Table 4.2. Endogenous and/or therapeutically-utilized compounds with structurally similarity to 3 and 4-methylvaleric acids

Compound	Structure
3-methylvaleric acid	
4-methylvaleric acid	
Valeric acid	
Valproic acid	
γ-hydroxybutyric acid (GHB)	
γ-aminobutyric acid (GABA)	

Physiologically relevant compounds structurally similar to 3 and 4-methylvaleric acid were identified. Valeric acid is the parent compound of 3 and 4-methyl-valeric acids and is naturally occurring in the valerian plant and is used over-the-counter as a sedative. Valproic acid differs from valeric acid by only a 3-carbon side chain and is used therapeutically for multiple CNS conditions. GHB is found endogenously at low levels and has a variety of biological activities. GABA is the endogenous ligand for both metabotropic and ionotropic GABA receptors. The mechanisms of action of valeric acid, valproic acid, and GHB are not clear.

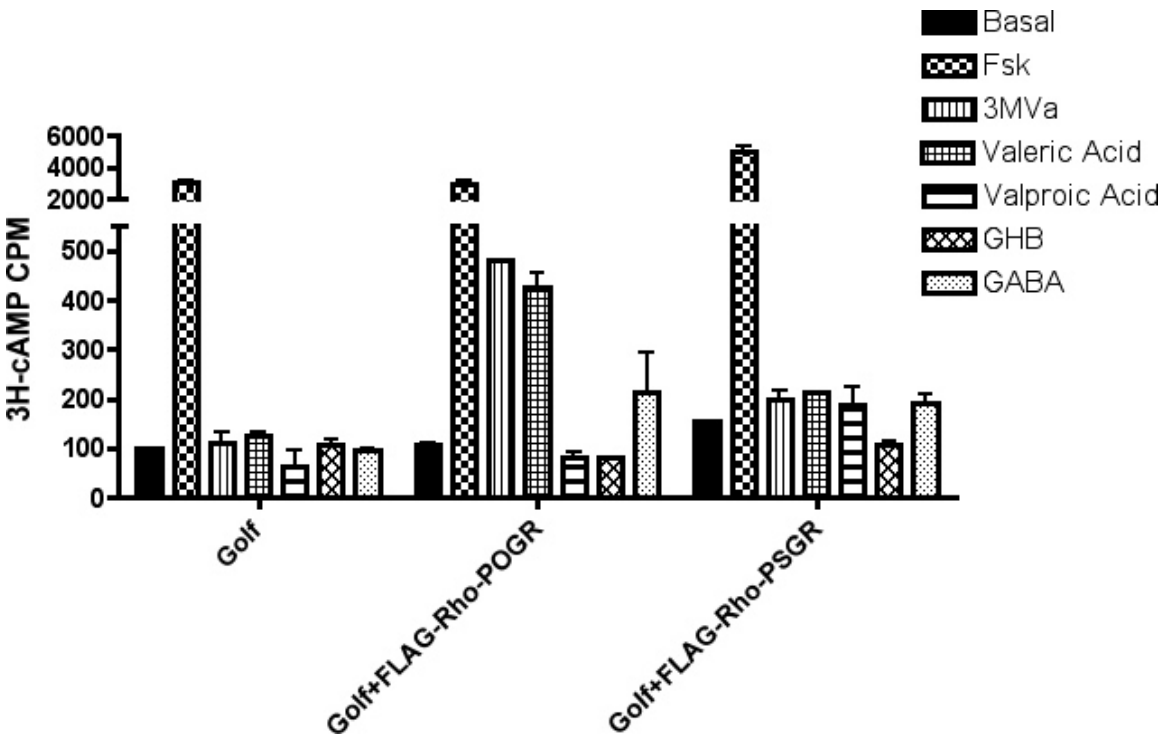


Figure 4.8. Valproic acid, GHB, and GABA are 3-methylvaleric acid structurally related compounds that do not activate POGR or PSGR mediated cAMP generation. HEK-293 cells transiently transfected with FLAG-Rho-POGR and PSGR plus $G\alpha_{olf}$ were treated with 3-methylvaleric acid or a variety of structurally similar compounds and cAMP generation was measured. POGR showed significant stimulation by both 3-methylvaleric and valeric acids, but no other compounds examined. PSGR did not appear to be activated by any of the tested compounds. Raw data are shown as 3H-cAMP counts per minute (CPM) from a representative experiment.

4.3.7 Homo and heterodimerization of olfactory receptors.

It is widely believed that most OSNs express only one OR (Serizawa, et al., 2003), and therefore heterodimerization between ORs may be unlikely for most OR subtypes. However POGR and PSGR are both known to be highly expressed in the human prostate. Given the data presented in earlier chapters concerning OR interactions with other receptors, we wondered if two ORs, such as POGR and PSGR might be capable of associating when co-expressed in heterologous cells. Furthermore, we were curious whether POGR and PSGR might be capable of homodimerization as well. To explore these possibilities we performed co-immunoprecipitation of differentially tagged versions of POGR and PSGR. As shown in Figure 4.9, N-terminally tagged FLAG-Rho POGR and PSGR constructs were co-expressed with C-terminally tagged GFP POGR and PSGR constructs in HEK-293 cells. Following immunoprecipitation with anti-FLAG antibody conjugated agarose, samples were resolved via SDS-PAGE, and blots were probed with anti-GFP antibodies. Bands corresponding to POGR-GFP and PSGR-GFP were detected in the co-expressed lanes, but not in lanes where these constructs were expressed alone. Furthermore, co-immunoprecipitation from cells co-expressing POGR and PSGR constructs indicate that these two closely-related receptors are able to heterodimerize with each other (Figure 4.9). These findings represent the first evidence indicative of OR homodimerization and also the first evidence indicative of heterodimerization between ORs.

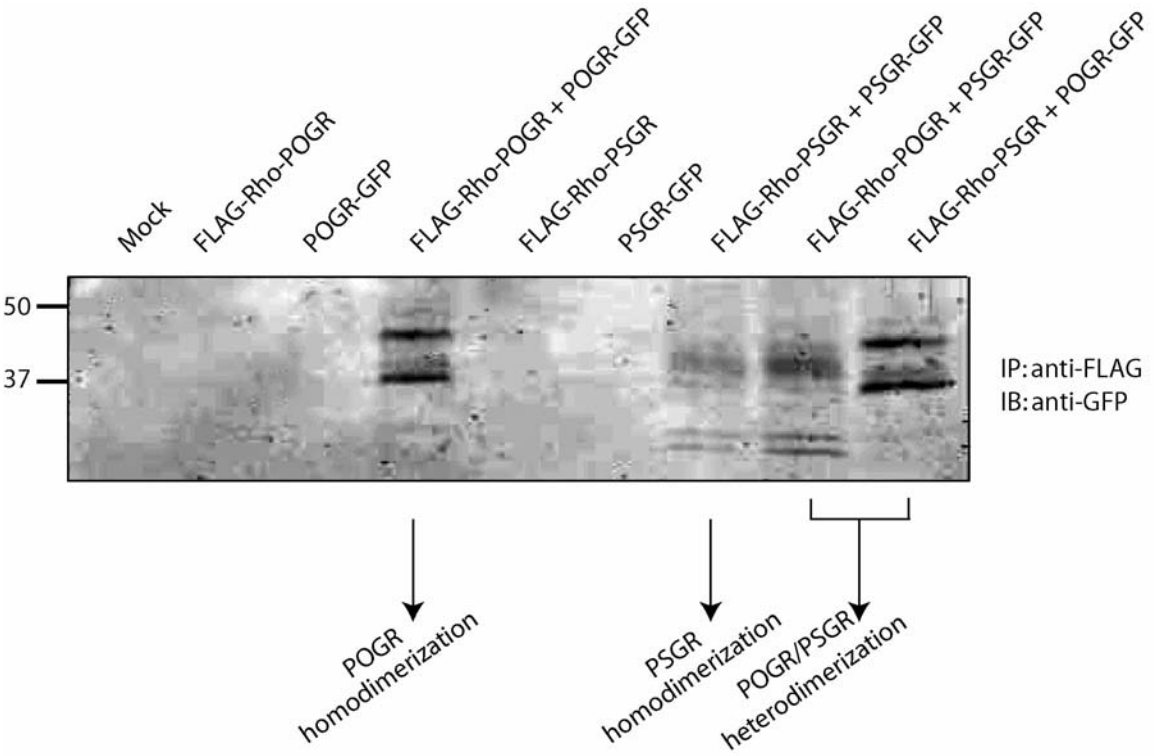


Figure 4.9. Co-immunoprecipitation using differentially tagged PSGR and POGR constructs reveals OR homodimerization and heterodimerization. N-terminally tagged FLAG-Rho-POGR and FLAG-Rho-PSGR constructs were expressed alone and co-expressed with C-terminally tagged POGR-GFP and PSGR-GFP to examine OR homodimerization. FLAG-Rho-POGR was also co-expressed with PSGR-GFP and FLAG-Rho-PSGR was co-expressed with POGR-GFP to examine potential OR heterodimerization. Anti-FLAG immunoprecipitates were resolved via SDS-PAGE. Immunoblotting with anti-GFP antibody revealed strongly immunoreactive bands in GFP-construct co-expressed lanes indicative of co-immunoprecipitation between FLAG-Rho and GFP-tagged constructs.

In order to explore whether other ORs beyond POGR and PSGR might also be capable of homodimerization, co-immunoprecipitation studies using differentially tagged constructs were performed to evaluate the homodimerization capacity of the rat I7 OR. N-terminally FLAG-tagged rat I7 (FLAG-rat I7) was expressed alone and co-expressed with C-terminally GFP-tagged rat I7 (rat I7-GFP) in HEK-293 cells. Cell lysates were incubated with anti-FLAG antibody conjugated agarose beads to immunoprecipitate FLAG-rat I7. Subsequent to sample resolution via SDS-PAGE, blots were probed with an anti-GFP antibody and revealed a clear band corresponding to rat I7-GFP in the co-transfected lane (Figure 4.10). No rat I7-GFP was immunoprecipitated when this construct was expressed alone.

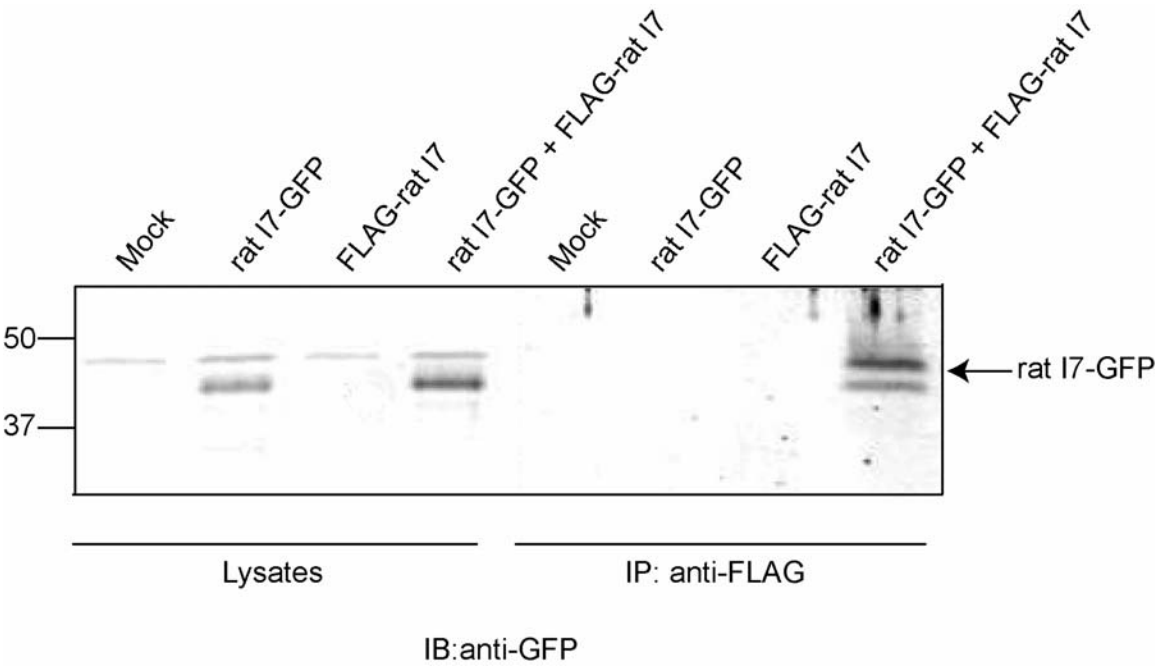


Figure 4.10 Homodimerization of the rat I7 olfactory receptor using differentially tagged receptor constructs. Two rat I7 constructs, one with an N-terminal FLAG-tag and the other with a C-terminal GFP tag, were expressed alone or co-expressed in HEK-293 cells. Rat I7-GFP was clearly detectable as an approximately 45 kD band following Western blotting of cell lysates with an anti-GFP antibody. Following cell lysate incubation with anti-FLAG agarose, a clear band corresponding to the rat I7-GFP construct was detectable in the co-expressed lane, indicating co-immunoprecipitation between FLAG-rat I7 and rat I7-GFP.

4.4 Discussion

Numerous and varied approaches have been employed to alleviate the intracellular retention suffered by some GPCRs when expressed in heterologous cells. We examined approaches previously demonstrated to be successful for other GPCRs, to see if they could specifically help ER-retained ORs. Mutation of ER retention motifs can target plasma membrane proteins to their appropriate location. Though no ER retention motif had yet been discovered in ORs, we wondered whether highly conserved basic residues in their C-terminus might constitute such a motif. We found that C-terminal mutants of the rat I7 OR (ΔK298A, ΔR303A, and ΔR304A) showed no apparent enhancement of cell surface localization. It remains possible that these residues are indeed important for ER retention, however, they might not be critical on an individual basis. Constructs with multiple mutations may behave differently than those with individual mutations. Alternatively, these residues might not be involved in receptor targeting at all and instead are conserved amongst ORs for another reason.

Truncation of the N-terminus is beneficial in targeting several ER-retained GPCRs, such as the α_{1D}-AR to the plasma membrane (Hague et al., 2004a). However, N-terminal truncation did not elicit a similar affect for the rat I7 OR in our studies despite suggestions that intramolecular interactions between the N and C-termini play a role in OR ER retention (Gimelbrant et al., 1999). The GPCRs (α_{1D}-AR, GPR56, GPR37) for which this approach has been successful

have all had significantly longer N-termini than ORs, which are characterized by their extremely short N and C-termini. We left the 5 amino acids preceding the first transmembrane region of the rat I7 intact due to stability concerns. It is possible that complete truncation, including these 5 amino acids, may have yielded an alteration in surface targeting. Alternatively, unlike the aforementioned GPCRs, the N-terminus of ORs may not be involved in the ER retention of these receptors.

While N-terminal truncation had no effect on OR membrane translocation in our hands, we did find that N-terminal addition of the rhodopsin signal leader sequence could increase the surface targeting of some ORs. Rho-tagged versions of both POGR and PSGR showed significant enhancement of cell surface expression versus non-Rho-tagged versions of these receptors. Addition of the Rho-tag allowed us to successfully study the pharmacology of POGR and PSGR in heterologous cells. We screened numerous molecules as potential ligands for these GPCR orphans (Table 4.1, Figure 4.8), and replicated findings that 3 and 4-methylvaleric acids stimulate POGR-mediated cAMP generation (Fujita et al., 2007). While this finding provides a means to further study the receptor, it does not shed light on the functional significance of ORs in the prostate, their potential involvement in prostate cancer, or their capacity to hetero- and homodimerize.

The functional consequences of the OR dimerization events described in this dissertation are largely unknown. Heterodimerization with the β_2-AR, P2Y1, P2Y2, and A2A receptors (Chapters 2 and 3) is not a universal means by which

the surface trafficking of all ORs is regulated. Yet receptor-receptor interactions may still constitute a critical step in OR surface expression. For the first time, evidence presented in this dissertation demonstrates heterodimerization between ORs as well as OR homodimerization and raises the possibility that such dimerization events might be a general occurrence among ORs. This is an intriguing proposition, as receptor-receptor interactions and their resultant consequences on receptor physiology have been described for a multitude of other GPCRs (Prinster et al., 2005), but have yet to be considered in the biological function of ORs.

Accumulating evidence suggests a role for dimerization events in the membrane targeting of GPCRs (reviewed in Minneman, 2007). Existence of a relationship between *hetero*dimerization and trafficking is clearly presented by evidence from the $GABA_B$ (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998) and adrenergic receptors (Hague et al., 2006; Hague et al., 2004c), and is further substantiated by evidence presented in Chapters 2 and 3 here. The relationship between GPCR *homo*dimerization and trafficking, however, is more poorly understood. Receptor subtypes beyond GPCRs, such as the insulin (Bass et al., 1998) and transforming growth factor β (Gilboa et al., 1998) receptors, require homodimerization as a prerequisite for ER export. Along these lines, dimerization has also been suggested to be a requirement for proper GPCR maturation (Bulenger et al., 2005). Homodimerization specifically has been reported to play a role in the maturation and the plasma membrane expression of the β_2-AR. A β_2-AR chimera containing the C-terminal ER-

retention motif of the $GABA_BR1$ subunit fails to traffic to the cell surface, and moreover inhibits the trafficking of wild-type β_2-AR in co-expression studies. Additionally, a β_2-AR mutant incapable of dimerizing also fails to traffic to the cell surface (Salahpour et al., 2004). Experiments using a mutant α_{1B}-AR and mutant frizzled 4 receptor have shown similar results; ER-retained mutants of both of these receptors act as dominant negatives and inhibit the surface targeting of wild-type α_{1B}-AR or frizzled 4 respectively upon co-expression (Kaykas et al., 2004; Lopez-Gimenez et al., 2007). Conversely, a CCR5 receptor mutant that is unable to dimerize is reported to maintain the capacity to traffic to the plasma membrane (Hernanz-Falcon et al., 2004).

These findings demonstrate that when co-expressed, mutant receptors can trap wild-type receptors in the ER and thus provide evidence that GPCR dimerization occurs early in biogenesis. These results also demonstrate that various receptor mutations can inhibit both dimerization and trafficking, however, they do not show that trafficking is explicitly dependent on dimerization. In fact, evidence from the CCR5 receptor argues the opposite. Mutagenesis as a technique has limitations and it is experimentally difficult to disrupt dimeric interactions exclusively, without otherwise altering receptor properties. For example, the mutations made in the β_2-AR that inhibited dimerization may have affected receptor conformation to the point that many receptor processes were defunct, including membrane targeting. Alternatively mutations made to CCR5 that inhibited dimerization might have also allowed it to mistakenly pass through ER quality control. Thus it is difficult to conclusively determine whether trafficking

is actually dependent upon dimerization or if dimerization is a phenomenon that simply happens to occur prior to membrane targeting.

For heterologously-expressed ORs, homodimerization alone is clearly insufficient to achieve proper trafficking. Furthermore, co-expressing POGR with PSGR did not result in any synergistic enhancement of surface expression, suggesting OR/OR heterodimerization also is not sufficient for membrane targeting (data not shown). While OR/OR dimerization events are obviously not sufficient for surface expression, dimerization might still be a necessary component of a multi-step trafficking system that functions to properly process and shuttle ORs to the plasma membrane. In addition to non-OR GPCRs, other accessory proteins including receptor transporting protein 1 and 2 (RTP1 and RTP2), receptor expression enhancing protein 1 (REEP1) and a heat shock protein called Hsc70t have also been demonstrated to increase the surface targeting of heterologously-expressed ORs (Saito et al., 2004). HEK-293T cells stably expressing RTP1, RTP2, and REEP1 together with a cyclic AMP response element-luciferase reporter have been used to successfully generate the rough odorant response profiles for 11 ORs, including 7 orphans (Saito et al., 2004). Co-expression of the OR 17-4 with Hsc70t resulted in enhanced 17-4 functionality (Neuhaus et al., 2006). Such findings demonstrate that ORs reaching the plasma membrane via interactions with RTP, REEP, and Hsc70t proteins are functionally active. However, it is currently unknown by what mechanisms these accessory proteins exert their effects on OR surface targeting.

One possibility is that OR interactions with accessory proteins could be related to OR dimerization events. Accessory proteins might plausibly play a role in receptor trafficking by regulating the formation of OR dimers or preferentially interacting with OR dimers versus monomers. In fact, growing evidence appears to support a broad role for RTP and REEP family proteins in GPCR trafficking. In addition to their interactions with ORs, specific RTPs and REEPs have been demonstrated to enhance the surface localization of bitter taste receptors (Behrens et al., 2006). Moreover, RTP 4 is currently under investigation in the regulation of opioid receptor trafficking (personal communication, Lakshmi Devi, New York University). An initial means to explore the potential relationship between OR dimerization and accessory proteins would be to examine POGR/PSGR trafficking and functional interactions in the presence and absence of RTP1/RTP2/REEP1. Results from such an experiment might shed light on potential overlap between OR dimerization events and interactions with accessory proteins.

In addition to potential consequences for receptor trafficking, another potential outcome of OR/OR dimerization is the potential to generate a huge number of OR receptor combinations, each with its own functional characteristics. This is an intriguing scenario given that mammals have only a small number of intact OR genes relative to the many thousands of odors that can be detected. Different dimerization combinations of ORs could potentially create thousands of unique odorant binding pockets. The existence of specific dimer ligand binding sites has previously been proposed to explain action of a

dimer-specific agonist of the δ-κ opioid receptor dimer (Waldhoer et al., 2005). Furthermore, similar to dimerization with non-OR GPCRs, OR/OR interactions could potentially influence G protein coupling and downstream signaling mechanisms creating further variety in odorant perception. A caveat of this idea is the current dogma that a given OSN expresses only one OR, which if true, would diminish the potential for OR/OR heterodimerization *in vivo*. However, as mentioned previously, the one OR per OSN hypothesis has not been conclusively demonstrated and even the possibility that each OSN expresses only two or a small number of ORs could allow for extensive heterogeneity of receptor combinations and signaling. Moreover, as OR expression is not limited to OSNs, dimerization might be uniquely important for the function of specific ectopically-expressed ORs, like POGR and PSGR.

A limitation of these findings is that POGR/PSGR heterodimerization and homodimerization were observed using an over-expression approach and the actual occurrence of such interactions in prostate cells or OSNs where the receptors are natively expressed will be difficult to confirm. This is a common challenge for GPCR/GPCR interactions and will be addressed more thoroughly in the upcoming discussion section. However, for POGR and PSGR at least, it is feasible that co-immunoprecipitation studies could be performed in a more native system in the near future. PSGR antibodies are currently available and the development of POGR-specific antibodies could potentially allow for co-immunoprecipitation from the LNCaP prostate cancer cell line that reportedly expresses both receptors (Weigle et al., 2004; Xu et al., 2000). In general,

LNCaP cells may represent a more physiologically relevant system in which to perform further studies on POGR and PSGR. Identification of endogenous ligands for POGR and PSGR may also expedite exploration of the physiological significance of POGR/PSGR dimerization events.

CHAPTER 5:

Discussion

5.1 Can olfactory receptors interact with other receptors?

The data presented in Chapters 2, 3, and 4 of this dissertation demonstrate that ORs have the capacity to interact with other GPCRs in heterologous cells. Specifically, we demonstrate that two members of the olfactory receptor subfamily 171, M71 (171-2) and 171-4 are able to associate with at least four non-OR GPCRs including the β_2-AR and the P2Y1, P2Y2, and A2A receptors. We furthermore demonstrate that ORs are also able to associate with other ORs in heterolgous cells. Specifically, we show physical association between two closely related prostate-overexpressed ORs, POGR and PSGR. These are the first reports of receptor dimerization involving olfactory receptors.

Like many other examples of GPCR heterodimerization, the associations summarized above were observed via studies of overexpressed proteins in HEK-293 cells. As such, these studies harbor certain limitations. One concern of overexpression studies is that overexpressed proteins may nonspecifically aggregate. Massive overexpression of a protein or proteins can overwhelm the processing machinery of a cell, allowing buildup of proteins in the ER and creating the potential for nonspecific aggregation. Furthermore, co-immunoprecipitation of ORs from cell lysates requires solubilization of membrane bound receptors, which may also lead to nonspecific aggregation. We attempted to control for nonspecific interactions by demonstrating that M71 did not co-immunoprecipitate with all co-expressed receptors; two GPCRs that did not influence M71 surface expression, the α_{1B}-AR and the δ-opioid receptor, also

were unable to co-immunoprecipitate with M71 despite identical experimental conditions. These results suggest that the associations we identified via co-immunopreicipitation are not an artifact of overexpression or experimental protocol and instead represent true specificity for binding partners.

Massive protein overexpression and subsequent overwhelming of cellular processes might also lead to altered subcellular localization of proteins. Thus it could be argued that increases in M71 at the plasma membrane might simply have resulted from additional strain on the cell due to co-expression of another GPCR. Again, however, the specificity of our findings speaks against this. In studies co-expressing M71 with 42 different GPCRs, only co-expression with the β_2-AR, P2Y1, P2Y2, or A2A receptors elicited enhanced M71 membrane localization, whereas co-expression with 38 other receptors did not enhance M71 surface distribution.

A desirable approach in studying physical interactions between proteins is to perform co-immunoprecipitation from native tissues. This assures that proteins are expressed at physiological levels and the cellular machinery is working in a normal manner. In the case of ORs, however, biochemical studies on native tissues are difficult because most ORs are expressed in only a small subset of OSNs. Thus, studies on native tissue require larger amounts of tissue than are feasible. A further complication of native immunoprecipitation studies is the current lack of OR-specific antibodies. Some OR antibodies have been successfully developed (Strotmann et al., 2004; Vanderhaeghen et al., 1993); however, for the majority of ORs, no antibodies are available at the present time.

Most ORs have relatively short N- and C-termini and it might be difficult to identify suitable epitopes to use as antigens. Furthermore, some ORs are nearly 99% homologous, such as M71 and M72, and specific antibodies might not be realizable in these instances.

ORs expressed outside of the OE might be more amenable to native immunoprecipitation studies because of tissue availability. As suggested in the discussion section of Chapter 4, it seems reasonable that POGR and PSGR could be co-immunoprecipitated from either prostate tissue or cells from a prostate cancer derived cell line, assuming availability of good antibodies. This might be a feasible approach to examine potential protein-protein interactions for spermatid, heart, and other ectopically-expressed ORs as well.

An additional limitation of our observations of OR associations with other receptors is the inability to definitively conclude whether these associations represent direct physical interactions between the proteins. Because our co-immunoprecipitation studies were carried out using proteins from cell lystates, we cannot exclude the possibility that ORs interact with other receptors indirectly, as part of a multi-protein complex. An ideal solution to this dilemma would be to perform *in vitro* pull-down studies using purified proteins. Direct association between the $GABA_B$ R1 and $GABA_B$ R2 receptors was established in this manner (Kammerer et al., 1999). For GPCRs, experiments of this type are generally performed using purified portions of the receptor N or C termini or loop regions, as the transmembrane domains are not good candidates for bacterial fusion protein purification owing to their hydophobicity. Some GPCRs dimerize through

interactions of their hydrophilic regions. For instance, metabotropic glutamate receptor and $GABA_B$ receptor dimerization events are believed to occur via regions in their N- and C-termini, respectively (Tsuji et al., 2000; White et al., 1998). Dimerization interfaces for several other GPCR dimers, however, are reported to be in the receptor transmembrane domains (reviewed in Bush et al., 2007), which again are regions that are likely unsuitable to generate as fusion proteins. At the present time, with the exception of involvement of the 2^{nd} transmembrane domain of the β_2-AR (Figure 3.9), we do not know what receptor regions mediate OR interactions with other receptors. Thus initially it might be most effective to determine the regions of importance for OR dimers, using receptor chimeras, protein modeling, or peptide blocking as possible experimental techniques. A caveat of such approaches is that multiple receptor regions may be involved in protein interaction events and therefore it may not be possible to pinpoint particular residues that are critical to OR heterodimerization. Still, given identification of hydrophilic receptor regions that are deemed to be important for receptor dimerization, subsequent pull-down experiments could demonstrate that the associations we have observed in a cellular context represent direct physical interaction between the receptors. A negative pull-down result would be more difficult to interpret, as certain receptor regions might be necessary, but not sufficient to mediate receptor-receptor association.

In summary, while our findings represent the first reports of OR dimerization with other GPCRs, further studies are required to validate and clarify these findings. Co-immunoprecipitation experiments from native tissue would

help affirm the interaction results we have observed in overexpression studies in heterolgous cells. Furthermore, it seems likely that OR heterodimerization with other receptors might occur via direct physical interaction based on precedent from other heterodimer pairs and our observations that OR functionality is altered depending on its dimer partner. This speculation could be substantiated upon identification of regions mediating OR dimerization events followed by fusion protein pull-downs using purified receptor regions.

5.2 Do olfactory receptors interact with other receptors in olfactory sensory neurons?

As discussed above, our findings that ORs interact with other receptors came from observations using overexpressed proteins in heterologous cells and as such do not reveal whether similar associations occur between endogenous receptors in native tissue. As mentioned, co-immunoprecipitation studies from native tissue would lend credence to our present findings. Additionally, conclusive determination of whether β_2-adrenergic, P2Y1, P2Y2, and A2A receptors are expressed at physiological levels in OSNs will also be critical to assess whether ORs interact with these receptors *in vivo*. A recent GeneChip expression profiling study of mouse OSN-expressed genes did not reveal significant expression of β_2-AR, P2Y1R, P2Y2R, and A2AR (Sammeta et al., 2007). However, *in situ* hybridization and immunohistochemical studies presented here and elsewhere have provided evidence for the expression of

these receptor subtypes in the OE (Bush et al., 2007; Gayle and Burnstock, 2005; Hague et al., 2004b; Hegg et al., 2003). As it stands, in the absence of electron microscopy analyses it is difficult to determine conclusively whether the receptors are truly expressed in OSNs or rather in surrounding cells.

Transgenic animals could seemingly represent an ideal approach to clarify whether OR dimerization is an *in vitro* artifact or rather has *in vivo* significance. For instance, studies in *Drosophila* demonstrated that deletion of a single gene, OR83b, results in deficient trafficking and functionality of all fly ORs (Larsson et al., 2004). Similarly, mice lacking a receptor found to interact with the M71 OR might be expected to have altered M71 function resulting in a distinct odorant-related phenotype that might be revealed upon behavioral studies. In line with this thinking, preliminary studies were carried out at the onset of this project to determine whether β_2-AR knock-out (KO) animals showed abnormal olfactory responses to the M71 ligand acetophenone. However, we observed no difference between KO and wild-type animals in multiple behavioral assays that tested olfaction (data not shown). A potential explanation of this result is that the β_2-AR plays no role in M71 recognition of acetophenone, potentially due to lack of *in vivo* interactions between the two receptors. An alternative possibility is that M71 does interact with the β_2-AR *in vivo*, but because of additional interactions with P2Y1R, P2Y2R, and A2AR, no olfactory deficit is detected in β_2-AR KO animals. Moreover, M71 may interact with receptors beyond the four that we have identified thus far. The ability of M71 to interact with multiple partners

therefore makes it unfeasible to examine the physiological relevance of such receptor-receptor interactions using transgenic animals.

Evidence for receptor heterodimerization *in vivo* can also come from pharmacological approaches. For example, existence of a heterodimer specific ligand (6-GNTI) was reported for the κ-δ opioid receptor dimer. 6-GNTI is an δ-opioid receptor antagonist and exhibits agonist activity at the κ-opioid receptor. 6-GNTI shows analgesic properties *in vivo* that cannot be recapitulated upon co-treatment with an κ agonist and δ antagonist, suggesting its effects might be elicited specifically through binding to receptor dimers (Waldhoer et al., 2005). Pharmacological heterodimer evidence also exists for the CCR2 and CCR5 dimer *in vivo*. In lymphoblasts, which express both receptor subtypes, treatment with a CCR5 specific ligand was observed to partially inhibit binding of a CCR2 radioligand (El-Asmar et al., 2005). These results demonstrate pharmacological approaches to evaluate *in vivo* dimerization events. Theoretically, similar dimer specific ligands or ligand cooperativity could be assessed for other receptor pairs such as ORs and their interacting partners (Zhang et al., 2007).

In conclusion, further studies are required to determine whether ORs interact with other receptors in OSNs. Co-localization of M71 with β_2-AR, P2Y1R, P2Y2R, and A2AR at the electron microscopy level and co-immunoprecipitation of these receptor from native tissue would help clarify our findings from heterologous cells. However, even if these receptor subtypes are not highly-expressed in OSNs, the observations that these receptors associate with ORs and drive OR surface expression in transfected heterologous cells still

demonstrate that ORs have the capacity to form dimers (or higher order oligomers) and that such oligomerization can influence OR trafficking.

5.3 Can olfactory receptors homodimerize and do they homodimerize in olfactory sensory neurons?

Evidence presented for the first time in this thesis demonstrates that, in addition to their capacity to heterodimerize with other receptors, ORs are also able to homodimerize. Specifically we demonstrate homodimerization of three different ORs, POGR and PSGR, both of which are highly expressed in human prostate, and rat I7, which is localized to OSNs. As our observations were made using co-immunoprecipitation of differentially tagged receptor constructs transiently transfected in HEK-293 cells, these findings are limited by the same caveats discussed in the sections above. However, in terms of the potential for native ORs to homodimerize, there is precedent from other GPCRs that suggests homodimerization is a physiologically relevant occurrence.

As mentioned above, a caveat of co-immunoprecipitation studies is that receptors must be solubilized from the membrane, potentially leading to nonspecific aggregation of proteins. Such aggregations could potentially occur between proteins of the same type and thus be observed as homodimerization. Bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) are techniques that enable observation of protein-protein interactions in living cells, thus circumventing solubilization-related artifact concerns. Evidence of dimerization from BRET and FRET assays now exists for a number of GPCR homodimers some of which are the β_2-

adrenergic (Angers et al., 2000), neuropeptide Y-Y4 (Berglund et al., 2003), A2A (Canals et al., 2004), galanin type 1 (Wirz et al., 2005), α_{2A} and α_{2C}-adrenergic (Small et al., 2006), and 5-HT_{2C} (Herrick-Davis et al., 2007) receptors. In several cases, BRET and FRET analyses have helped to substantiate previous co-immunoprecipitation results that initially suggested homodimerization. Even so, BRET and FRET analyses still employ overexpression of receptors in heterologous cells and artifactual BRET/FRET signals could potentially result from overcrowding of receptors. Speaking against this, however, are results from a study that demonstrated that the BRET signal for β_2-AR stays nearly the same at even 100-fold increases in receptor expression levels (Mercier et al., 2002). Finally, both co-immunoprecipitation and BRET/FRET analysis of homodimers requires that receptors be differentially tagged. Sometimes, particularly in the case of BRET/FRET, these tags can be quite large. A reasonable concern in these studies could be that addition of such tags might alter the conformations of GPCRs, leading to nonspecific binding events. Presumably, however, such aggregates would not be expected to localize at the plasma membrane, which can be specifically assessed by various biotinylation, microscopy, and FRET applications. Biophysical evidence from techniques such as BRET or FRET would help affirm the case for OR homodimerization.

As mentioned previously, because of the numerous confounds associated with *in vitro* analysis of GPCR dimerization events, it would obviously be advantageous to be able to examine GPCR dimerization in native tissue. The most compelling way to evaluate OR homodimerization might be via a transgenic

model. For instance, by mutating an OR gene such that protein homodimerization is impaired, and creating a “knock-in” of this gene, one could look for alterations in olfactory behavior related to the knocked-in gene. The obvious problem with this idea is creating a receptor in which homodimerization is the only receptor property that is affected. Because dimerization can be dependent on multiple receptor regions rather than a single residue (Hernanz-Falcon et al., 2004), such an approach may not be realizable in many instances. Thus at the present time it may be more valuable to consider evidence from other types of experimentation.

Some of the clearest evidence in support of GPCR homodimerization arises from studies on rhodopsin. Rhodopsin is the prototype of the class I group of GPCRs, to which ORs also belong. Until very recently, rhodopsin was also the only GPCR for which a high-resolution crystal structure had been achieved. In initial crystallography reports, rhodopsin was crystallized as a non-physiological dimer, with the extracellular face of one receptor and the cytoplasmic face of another receptor pointed in the same direction (Palczewski et al., 2000). This odd orientation made it unclear how to interpret the association of the receptors. Subsequently, however, high-resolution atomic force microscopy revealed the highly ordered oligomeric structure of rhodopsin in mouse rod outer segment discs (Fotiadis et al., 2003; Liang et al., 2003). These studies clearly show rhodopsin dimers packed closely together in native membranes and intimate that homodimerization is an important part of endogenous rhodopsin biology.

Very recently, the crystal structure of another class A GPCR, the β_2-AR, was realized. Interestingly, crystal structures obtained via two independent strategies both show β_2-AR as a monomer (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007). This is in stark contrast to numerous reports from overexpression studies that suggest β_2-AR readily homodimerizes (Hebert et al., 1998; Hebert et al., 1996; Salahpour et al., 2004). As crystallization of GPCRs is an arduous task complicated by limited amounts of available protein, conformational heterogeneity, and limited polar surface area (Shukla et al., 2008) certain modifications had to be made to β_2-AR to achieve suitable crystals. These modifications included truncation of the receptor C-terminus along with co-crystalization of an antibody fragment to the 3rd intracellular loop in one instance (Rasmussen et al., 2007)and replacement of the 3rd intracellular loop with T4 lysozyme (a readily crystallized protein) in the second strategy (Cherezov et al., 2007; Rosenbaum et al., 2007). It is plausible that alteration of these regions of the receptor may have interfered with β_2-AR dimerization and thus further structural analyses are required to evaluate this point.

Crystalization of the β_2-AR has supplied a wealth of structural information that can now be correlated with the large body of β_2-AR mutagenesis and biophysical data accumulated over the years. Moreover, the crystal structures of rhodopsin and the β_2-AR can be compared with one another to identify similarities and differences that might be applicable to other GPCRs. Successful crystallization of the β_2-AR will likely speed identification of additional GPCR crystal structures. Identification of an OR crystal structure would significantly

enhance our understanding of these receptors, particularly in relation to their structural conformation and could shed light on their preferred dimerization state.

5.4 Which G proteins do olfactory receptors signal through?

As outlined in Figure 1.2, the canonical pathway associated with OR signaling involves receptor coupling to $G\alpha_{olf}$. $G\alpha_{olf}$ is highly enriched in the OE compared to many other tissues, and within the OE $G\alpha_{olf}$ appears to be expressed specifically in OSNs (Jones and Reed, 1989). Furthermore, knocking out the $G\alpha_{olf}$ gene in mice results in anosmia (Belluscio et al., 1998). These findings, in conjunction with odorant-evoked accumulation of cAMP in OSNs, have led to a widespread belief that $G\alpha_{olf}$ is the main mediator of OR signal transduction.

Additionally, it has long been speculated that ORs might also signal through a secondary mechanism mediated by phospholipase C (PLC) action on phosphatidyl inositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5-trisphophate (IP_3). Much of the evidence implicating OR induction of IP_3 signaling comes from work on non-mammalian species including catfish (Restrepo et al., 1990), salamander (Firestein et al., 1991), frog (Kashiwayanagi et al., 1996), and lobster (Fadool and Ache, 1992). The importance of the IP3 pathway in mammals is more controversial (Brunet et al., 1996). Recently, however, activation of the rat I7 OR stably expressed in HEK-293 cells was demonstrated to stimulate both the cAMP and IP_3 pathways, with IP_3 activation

occurring at high concentrations of odorant (≥ octanol 10^{-4}) (Ko and Park, 2006). No exogenous G proteins were transfected in this study, indicating that the rat I7 receptor is capable of signaling through those found endogenously in HEK-293 cells, potentially $G\alpha_s$ and $G\alpha_q$. Additional functional studies in heterologous cells have also suggested the capacity of ORs to signal through G proteins other than $G\alpha_{olf}$, specifically $G\alpha_s$ and $G\alpha_{15/16}$ (Kajiya et al., 2001). Such findings suggest that the IP_3 signaling pathway may indeed be relevant to mammalian ORs and further imply that ORs can couple to multiple G-proteins.

$G\alpha_s$/$G\alpha_{olf}$ and $G\alpha_{o-1}$/$G\alpha_{o-2}$ show enriched expression in rat olfactory cilia preparations compared to whole olfactory epithelium as assessed by Western blot analysis. Conversely, $G\alpha_{i-l}$/$G\alpha_{i-2}$/$G\alpha_{i-3}$ and $G\alpha_q$ do not appear to be enriched in the cilia (Schandar et al., 1998). Moreover, $G\alpha_s$/$G\alpha_{olf}$ and $G\alpha_{o-1}$/$G\alpha_{o-2}$ antibody application to cilia preparations followed by stimulation with various odorants was shown to result in a significant dose dependent decrease in accumulation of cAMP or IP_3, respectively (Schandar et al., 1998). PLC activation is established to occur in response to receptor activation of multiple G proteins, however as opposed to the α subunit of G_q, it is the $\beta\gamma$ subunits of G_i and G_o that are typically thought to be responsible for activation of PLC (Exton, 1997). Accordingly, pretreatment with a general $G\beta$ antibody was shown to inhibit IP_3 accumulation in cilia preparations. These findings further suggest that the IP_3 pathway plays a role in mammalian olfaction and offer evidence that IP_3 signaling in native OSNs may result from OR activation of an isoform of $G\alpha_o$, mediated by the $G\beta$ subunit specifically.

Interestingly, results from this dissertation also suggest that ORs can couple to G proteins beyond $G\alpha_{olf}$, including $G\alpha_o$. Studies with M71 co-expressed with the β_2-AR resulted in accumulation of cAMP via a G protein endogenous to HEK-293 cells, presumably $G\alpha_s$. Conversely, while M71 co-expressed with P2Y1, P2Y2, and A2A receptors localized at the plasma membrane, stimulation with acetophenone did not result in detectable cAMP generation. In accordance with reports that heterodimerization partners can influence G protein coupling specificity (George et al., 2000), we wondered whether M71 co-expressed with the purinergic receptors might signal more effectively through a G protein other than $G\alpha_s$. Subsequently we identified that co-expression of $G\alpha_o$ with M71 plus P2Y1R and P2Y2R resulted in significant phosphorylation of ERK that was pertussis-toxin sensitive. The $\beta\gamma$ subunits of various G proteins are previously established to activate MAPK pathways (George et al., 2000). Furthermore, both P2Y1 and P2Y2 receptors are known to couple to $G\alpha_{i/o}$ (Chen and Chen, 1998; Filippov et al., 1998; Vasquez, 2002) and thus it seems reasonable that OR heterodimerization with these receptors might influence the preference for particular G protein partners. As M71 co-expression with the A2AR did not result in either cAMP generation or phosphorylation of ERK, it would be interesting to examine the functionality of this dimer pair in the presence of other G proteins, such as $G\alpha_{15/16}$ to identify whether this dimer complex is indeed functional.

The data from heterologous cells outlined above, along with studies from native cilia preparations, suggest that some ORs may couple to $G\alpha_o$.

Interestingly, $G\alpha_o$ knockout mice exhibit compromised olfactory behavior (Luo et al., 2002). While $G\alpha_o$ deficiency could very well affect olfactory processing in higher brain regions rather than at the chemodetection level, histological examination showed no gross structural alterations to the olfactory bulb (Luo et al., 2002). Similar to the experiments carried out by Schander et al., it would be interesting to examine whether the absence of $G\alpha_o$ affects IP_3 accumulation in native cilia preparations from the knockout mice.

In conclusion, the evidence outlined above supports the likelihood that ORs can couple to G proteins other than $G\alpha_{olf}$ and such coupling may result in activation of signaling pathways beyond cAMP accumulation. Such signaling heterogeneity may help in the encoding of the large number of detectable odorants relative to the pool of available receptor types. More detailed physiological and behavioral olfactory analyses of specific G protein knockout mice may help pinpoint the *in vivo* relevance of the findings discussed here. Notably, however, variable G protein coupling would not represent an ability unique to ORs, as many other types of GPCRs are also recognized to couple to multiple G proteins.

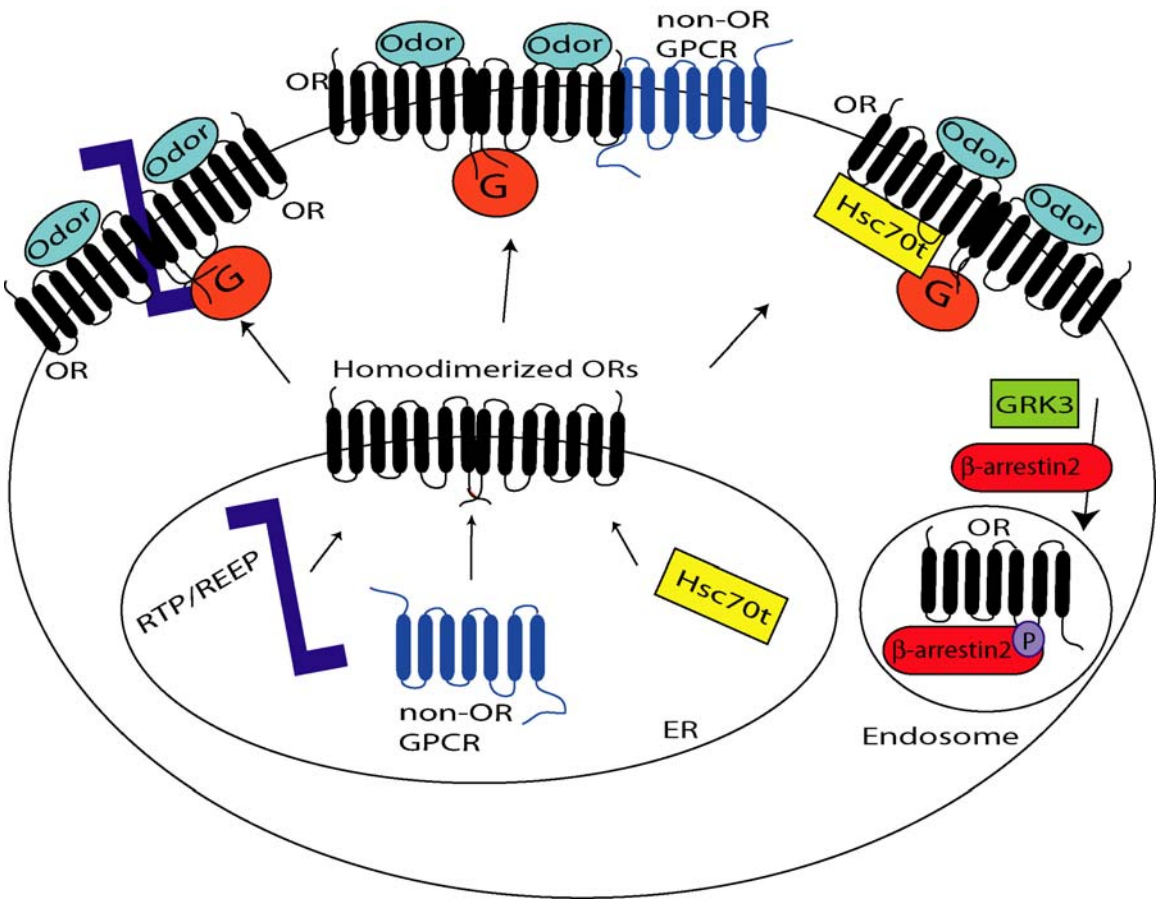
5.5 How do the findings of this dissertation interplay?

The major theme of this dissertation is that in heterologous cells, ORs participate in dimerization events that can influence their trafficking and signaling prerogatives. We envision that ORs expressed alone in heterologous cells

homodimerize in the ER, but are still unable to be exported to the plasma membrane. ER-trapped OR homodimers can further associate with co-expressed accessory proteins included particular non-OR GPCRs, RTPs, REEPs, or Hsc70t. Association with these specific interacting partners alters the conformation of the ORs such that they can achieve successful export from the ER (Figure 5.1). We believe OR heterodimerization with non-OR GPCRs persists at the plasma membrane, owing to observations of differential signaling mechanisms depending on the dimer partner. It is presently not known whether RTP, REEP, or Hsc70t association with ORs persists at the cell surface or influences functionality. Once localized correctly at the plasma membrane, ORs respond to odorant stimulation by activating G protein partners that may be in part determined by their associated accessory proteins. Finally, activated ORs undergo regulation by kinases and arrestins.

Certain non-OR GPCRs, RTPs, REEPs, and Hsc70t all exhibit the capacity to facilitate appropriate localization and functionality of particular ORs in HEK-293 cells. However, it is not clear at present if any of these proteins play a comparable role in influencing the trafficking of endogenous ORs in OSNs. Moreover, the specific mechanisms by which these accessory proteins enhance cell surface expression are presently not understood. Non-OR GPCRs, RTPs, REEPs, and Hsc70t do not share significant amino acid sequence similarity with each other, and thus it is possible that each unique accessory protein exerts its effects by different means. These accessory proteins may potentially carry out complementary functions, each acting at a particular step in the maturation and

Figure 5.1. Olfactory receptor localization at the plasma membrane can be facilitated by co-expression with multiple accessory proteins. Olfactory receptors (ORs) can homodimerize, likely at a point early in biogenesis. When expressed alone in heterologous cells, ORs typically exhibit endoplasmic reticulum (ER) retention despite homodimerization. However, enhanced cell surface localization and functionality of ORs can be observed upon co-expression with specific RTPs, REEPs, non-OR GPCRs, and the heat shock protein Hsc70t. Whether the co-expressed accessory proteins maintain association with ORs at the plasma membrane is not clear, but seems plausible. Plasma membrane-bound ORs are activated by odorants to elicit coupling to G proteins, which might be determined in part by associated proteins. Activated ORs are phosphorylated at specific residues by GRK3 and other protein kinases, causing desensitization. Desensitized ORs may then undergo clathrin-mediated internalization into endosomes via association with β-arrestin 2.


non-OR
GPCR
Odor
Odor
OR
G
OR
Odor
Odor
OR
Hsc70t
Odor
Odor
G
G
OR
Homodimerized ORs
GRK3
β-arrestin2
OR
RTP/REEP
Hsc70t
β-arrestin2
P
non-OR
GPCR
ER
Endosome

trafficking of ORs. Indeed, multiple mechanisms may be required to regulate the targeting of a receptor family as large and diverse as that of the ORs.

5.6 What are the implications of these results for future understanding of olfactory receptors and olfaction?

The human OR family is comprised of only ~350 functional proteins, yet can detect several thousand odors. The majority of ORs have evolved to identify volatile chemicals in the environment, yet some ORs are expressed deep within internal tissues. ORs congregate at the plasma membrane of olfactory sensory neuron cilia, but remain locked inside heterologous cells. These are but a few of the perplexities surrounding this mysterious family of receptors. Adding to the complexity of OR biology are novel findings from this dissertation indicating that ORs can heterodimerize with non-OR GPCRs as well as with other ORs and also that ORs can homodimerize. The true extent to which such dimerization events influence OR biology and olfactory perception remains to be seen. At a minimum, OR heterodimerization with non-OR GPCRs represents another example whereby the maturation and trafficking of GPCRs can be influenced by dimerization events. On a broader scale, the capacity for ORs to dimerize may become a necessary consideration for all OR related research in the future.

In relation to human health, olfaction is often considered a trivial matter, particularly in comparison to other senses like vision or hearing. But lack of the

ability to smell can be dangerous, as people are unable to detect fire, gas leaks, or spoiled food. These types of severe anosmias generally result from damage to OSNs or the olfactory nerve, rather than problems with the ORs themselves. Conversely, milder general variability of odor detection/perception is widespread across the population and has recently been determined to result from genetic variability among OR genes; single nucleotide polymorphisms in the human OR7D4 have been shown to correlate with impaired OR function *in vitro* and variable perception of the odorous steroid androstenone in humans (Keller et al., 2007). One possibility is that such mutations could affect OR ability to dimerize and that altered dimerization leads to variability in perception of particular odors.

Beyond odor perception, there are additional areas of human biology where OR involvement is just beginning to be explored. One example is in the study of cancer. As discussed at various points throughout this dissertation, POGR and PSGR are two ORs with high expression in the human prostate. Interestingly, these receptors show greatly upregulated expression in prostate cancer (Weigle et al., 2004; Xia et al., 2001; Xu et al., 2000). Consequently there is an obvious interest in identifying the roles of these ORs and how they could potentially be involved in the mechanisms of prostate cancer. Another example of OR relevance to human health is spermatid-expressed ORs, which are suggested to function in sperm chemotaxis and thus might be a critical component of reproductive biology (Spehr et al., 2003; Spehr et al., 2006b). Spermatid-expressed ORs might present a potential target for future therapeutics. OR antagonists could plausibly inhibit ability of sperm to locate the

egg, and thereby serve as a novel form of birth control. To further advance our understanding of OR roles in these fields, it is critical to appreciate the mechanisms by which OR trafficking and signaling occur. The findings from this dissertation have shed light on these mechanisms.

Outside of medical research, ORs are also important in the research and development of consumable products such as perfumes and foods. In the perfume industry, various odorants (synthetic and natural) are mixed in an attempt to achieve unique pleasant scents. *In vitro* expression systems of human ORs might present a simple high-throughput means to preliminarily assess the olfactory power of certain scent combinations. For instance, perfume activation of the human OR7D4 (discussed in the paragraphs above) might be undesirable, as activation of this receptor by adrostenone is perceived as a unpleasant urinous scent by a large proportion of people. Conversely, it would be beneficial to identify scent combinations that resulted in activation of ORs associated with pleasant odors.

In the development of new food products, various companies employ patented taste receptor technology (such as binding assays or heterolgous expression systems) for the identification of novel ingredients (Marketwire, 2008). Oftentimes the goal is to create an ingredient that may be “healthier” in terms of lower fat or lower calorie content than the ingredient that is currently in use. Presently, work is under development on compounds that “enhance” sucrose (table sugar), such that the amount of sugar added to products can be reduced without altering taste. Using less sugar would ideally equate with a lower calorie

product. Umami receptor assays have helped in the development of new ingredients that have already been incorporated into food products marketed by Nestlé (Marketwire, 2008). Taste receptors only detect sweet, salty, savory, bitter, and sour tastes. It is actually the sense of smell that is the larger determinant of food flavors. Therefore, it would be reasonable that incorporation of *in vitro* OR assays might also help in the development of food products. Again, to further pursue such avenues of research, it is necessary to understand the trafficking and signaling mechanisms of ORs.

In summary, the results reported in this dissertation may help in our overall understanding of OR biology and have implications for multiple areas of human health. Unraveling the mechanisms of OR trafficking and signaling may allow for the development of potential new methods of birth control, aid in development of novel food products that are healthier for our population, and further our understanding of specific disease states such as prostate cancer. In a broader context, the results reported in this dissertation contribute to the rapidly expanding field of GPCR biology as a whole, particularly in the area of receptor dimerization events. As GPCRs are currently the most common target for pharmacological therapies, increased understanding of GPCR dimerization will fuel the pursuit of novel therapeutics, such as dimer-specific ligands.

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