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Jill Harley Dunham Date

Trafficking and Signaling of Parkin-Associated Endothelin-Like Receptor GPR37

By

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B.S. The University of Georgia, 2003

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TRAFFICKING AND SIGNALING OF THE PARKIN-ASSOCIATED ENDOTHELIN-LIKE RECEPTOR GPR37

By

Jill Harley Dunham

B.S., University of Georgia, 2003

Advisor: 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 requirements for the degree of Doctor of Philosophy

Molecular and Systems Pharmacology

Graduate Division of Biological and Biomedical Sciences

2009

Abstract

Trafficking and Signaling of Parkin-Associated Endothelin Like Receptor GPR37

Jill Harley Dunham

Dopaminergic neuronal cell death is a hallmark of Parkinson's disease (PD), believed at least in part to be due to protein aggregation. This cell death leads to a major disruption of the dopaminergic system, which is involved in many different aspects of behavior, such as movement, cognition, motivation, and pleasure. GPR37, also known as parkin-associated endothelin-like receptor (Pael-R), is an orphan G protein-coupled receptor (GPCR) that exhibits poor plasma membrane expression when expressed in most cell types. Due to the association of GPR37 with the PD-associated gene parkin, GPR37 is considered as a potential target for novel PD therapies. Thus, we sought to find ways to enhance GPR37 trafficking to the cell surface in order to facilitate studies of GPR37 functional activity in heterologous cells. In truncation studies, we found that removing the receptor's N-terminus (NT) dramatically enhanced the receptor's plasma membrane insertion. Further studies on sequential NT truncations revealed that removal of the first 210 amino acids increased surface expression nearly as much as removal of the entire NT. In studies examining the effects of co-expression of GPR37 with a variety of other GPCRs, we observed significant increases in GPR37 surface expression when the receptor was co-expressed with the adenosine receptor $A_{2A}R$ or the dopamine receptor D_2R. Co-immunoprecipitation experiments revealed that full-length GPR37 and, to a greater extent, the truncated GPR37 were capable of robustly associating with D_2R, resulting in modestly-altered D_2R affinity for both agonists and antagonists. In studies examining potential interactions of GPR37 with PDZ scaffolds, we observed a specific interaction between GPR37 and syntenin-1, which resulted in a dramatic increase in GPR37 surface expression in HEK-293 cells. These findings reveal three independent approaches – N-terminal truncation, co-expression with other receptors and co-expression with syntenin-1 – by which GPR37 surface trafficking in heterologous cells can be greatly enhanced to facilitate functional studies and drug discovery efforts focused on this orphan receptor.

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List of Abbreviations

4-phenylbutyrate.....4-PBA

Adenylyl Cyclase.....AC

Adrenergic Receptor.....AR

Angiotensin.....AT

Autosomal Dominant Retinitis Pigmentosa..... ADRP

Autosomal Recessive Juvenile Parkinson's Disease.....AR-JP

Bilateral Frontoparietal polymicrogyria.....BFPP

Bovine Serum Albumin.....BSA

Calcitonin Receptor-Like Receptor.....CRLR

Cannabinoid .....CB

C-terminus.....CT

Diacylglycerol.....DAG

Dopamine Receptor.....DR

Dopamine Transporter.....DAT

Dopaminergic.....DA

Dulbecco's Modified Eagle Medium.....DMEM

Endoplasmic Reticulum.....ER

Endothelin Receptor.....ETR

Extracellular.....EC

Extracellular Signal-Regulated Kinase.....ERK

Fetal Bovine Serum.....FBS

γ-Aminobutyric Acid.....GABA

Glutathione S-transferase.....GST

Gonadotropin-Releasing Hormone Receptor.....GnRHR

G-protein coupled receptor.....GPCR

GPCR kinase.....GRK

GPR37-Like 1.....GPR37L1

Head activator.....HA

Human Embryonic Kidney.....HEK

Hypogonadic hypogonadism....HH

Inositol 1,4,5-triphosphate.....IP_3

Knockout.....KO

Major Histocompatibility.....MHC

Melanocortin Receptor.....MCR

Melanocortin Receptor Accessory Protein.....MRAP

Metabotropic Glutamate Receptor..... mGluR

Na(+)/H(+) Exchanger Regulatory Factor.....NHERF

Nephrogenic Diabetes Insipidus...NDI

Neuropeptide Y Receptor .....NPYR

N-terminus.....NT

Odorant Response abnormal.....ODR

Olfactory Receptor.....OR

Paraformaldehyde.....PFA

Parkin-associated endothelin-like Receptor.....Pael-R

Parkinson's disease.....PD

Phosphate Buffered Saline.....PBS

Postsynaptic density protein, Disc large and Zonula Occludens.....PDZ

Receptor activity modifying protein.....RAMP

Receptor expression enhancing protein.....REEP

Retinitis Pigmentosa.....RP

Rhodopsin 1 receptor.....Rh1

Room Temperature.....RT

Serotonergic Receptor.....5-HTR

Taste Receptor.....TR

Transgenic.....Tg

Transmembrane.....TM

Vasopressin Receptor.....VR

Wild Type.....wt

CHAPTER I: Introduction

1.1 G-protein coupled receptors (GPCRs)

Multicellular organisms possess a plethora of receptors that detect extracellular stimuli. Members within the largest class of cell-surface receptors possess seven transmembrane spans and exhibit characteristic coupling to G-proteins to elicit intracellular responses upon activation. Thus, the members of this family have been dubbed G-protein coupled receptors (GPCRs), seven-transmembrane receptors, or heptahelical receptors.

Most vertebrate genomes encode approximately 1000 GPCRs, making the GPCR superfamily the largest group of cell surface receptors (Hill 2006). They are also the most diverse group of cell surface receptors, in that they can be activated by any number of small molecules, including peptides, biogenic amines, lipid mediators, hormones, and amino acids, as well as sensory stimuli as in the case of olfactory, taste, and visual GPCRs (Civelli 2005). Each of these activators, also known as ligands, are either already present in the environment or are released from one cell to then carry a message to another cell (Civelli et al 2006). GPCRs sometimes exhibit specificity for specific ligands, although in many cases a given GPCR may bind to multiple ligands. In other cases, one ligand may activate multiple receptors. GPCRs activated by related ligands generally share significant sequence homology, such that they are considered as members of a subfamily.

Broad GPCR subfamilies have been created via division of the GPCR superfamily into six subclasses, typically dubbed classes A-F, which are then often broken down even further into smaller, more specifically defined groups. Classes D-F are represented only among non-mammalian species (Kristiansen 2004), so the focus here will be on classes A-C. The largest of the classes is class A, also referred to as the *Rhodopsin*-like family. It

includes over 650 human receptor proteins, including the olfactory receptors(ORs), which compose the largest GPCR subgroup (Lagerstrom & Schioth 2008). Class A receptors typically have shorter N-termini (NT) than class B or C receptors and contain specific highly-conserved sequence motifs within their transmembrane (TM) regions (Insel et al 2007). The ligands of class A receptors are varied, from small peptides, biogenic amines and purines to chemosensory signals, so class A is oftentimes subdivided further—α, β, γ, and δ—based upon the size and chemical nature of the ligands that activate them (Gurrath 2001; Lagerstrom & Schioth 2008). Interestingly, the mode of activation of each subgroup is the same for all but a select few, with ligand interaction at the TM regions and extracellular (EC) loops initiating receptor activation (Gurrath 2001; Ji et al 1998).

Class B is a much smaller family of GPCRs but is also subdivided into subgroups. One group of GPCRS, the *Secretin* receptors, possesses an EC binding domain that binds peptide hormones. These receptors contain conserved cysteine residues, which likely form disulfide bonds, in the first and second extracellular loops of the TM regions (Lagerstrom & Schioth 2008). *Adhesion* receptors make up the second subfamily of Class B GPCRs; extracellular matrix molecules are likely to be their natural ligands. Only 48 identified receptors belong to class B and they vary most at their long N-terminal (NT) regions, the region most crucial for ligand interactions of receptors in this family. The ligand is believed to activate the receptors by bridging the NT and the TM segments or the EC loops (Lagerstrom & Schioth 2008).

Class C includes only 22 human receptors, composed mainly of metabotropic glutamate receptors (mGluRs) and taste receptors (TRs). Most of these receptors bind their ligands within the NT region, which is often compared to a Venus flytrap due to its folded formation. However, allosteric modulators, which activate the receptor by

binding somewhere other than the active ligand binding site, are common for many of these receptors and seem to interact with various TM regions of the GPCRs.

1.2 Clinical relevance of GPCRs

GPCRs participate in many different physiological responses. Most cells express at least a handful of GPCRs and one cell may express dozens of different GPCRs, each with their own distinct specificity for one or several G-proteins (Civelli et al 2006; Hardman 2001). This widespread importance in the human body is partly the reason that GPCRs currently comprise 30-45% of all clinical drug targets and have been the number one target for drug discovery for some time (Kristiansen 2004; Lagerstrom & Schioth 2008). The main focus of drug discovery for GPCRs, however, has been on those therapeutics acting at class A biogenic amine-binding receptors. In fact, only about 30 GPCRs are currently clinically targeted, with just a few of those being non-class A targets (Gurrath 2001; Mustafi 2009). However, there is great potential in other GPCRs, such as those that bind peptides, because they bind to a more limited number of ligands than others. In addition, many of these peptide-activated GPCRs are involved in pain sensation, the immune system, and body weight regulation, and therefore are potential targets for treating diseases that affect everyone, regardless of race, age, or gender (Lagerstrom & Schioth 2008).

Every GPCR depends upon a relay chain of intracellular signaling, with the most classic example beginning with coupling to G-proteins. In visual transduction, for example, a single activated rhodopsin molecule catalyzes the activation of hundreds of molecules of transducin at a rate of about 1000 transducin molecules per second. Each

one of those molecules then activates a molecule of cyclic GMP phophodiesterase, which each hydrolyze about 4000 molecules of cyclic GMP per second. This cascade only lasts about 1 second, but results in the hydrolysis of over 10^5 cyclic GMP molecules and the closure of hundreds of Na^{2+} channels in the plasma membrane (Alberts 2002). Therefore, a single extracellular signal molecule activates a single GPCR, but has the capacity to influence an entire system's equilibrium. A cascade such as this, though, with amplification of stimulatory signals, requires a compensating mechanism at every step to restore the system to its resting state when stimulation ceases. All cells possess efficient mechanisms for rapidly degrading cyclic nucleotides, buffering and removing cytosolic Ca^{2+}, and inactivating responding enzymes and ion channels. The speed, extent and relative brevity of GPCR-initiated downstream signaling are the major reasons as to why GPCRs are valuable drug targets.

A variety of additional factors also help to explain why GPCRs have proven to be such valuable targets in drug discovery. The location of GPCRs on the cell-surface makes them easily accessible, especially to drugs which cannot pass through the plasma membrane. Furthermore, a key part of drug discovery is to create drugs that will selectively act on a particular receptor in a particular tissue, sparing those in other tissues to minimize side effects. The discrete tissue distribution of GPCRs allows for such desired selectivity (Insel et al 2007). Finally, lead compounds are the starting points for molecular optimization of drugs that may target and activate or block a given receptor; endogenous ligands are great leads for developing drugs to target individual receptors. In addition, each well-characterized GPCR possesses a defined ligand-binding pocket that can be targeted by small molecular weight compounds (Levoye & Jockers 2008). For instance, the well-defined adrenergic receptors have been particularly useful as drug targets for treatments of disorders ranging from prostatic hyperplasia and

congestive heart failure to asthma and the delay of preterm labor (Insel et al 2007; Ram & Sestini 2003; Squire & Barnett 2000). This is true in part because the structures of the endogenous ligands epinephrine and norepinephrine are well-understood. The use of these receptors as targets may further increase, too, since crystal structures of two of the β-adrenergic receptors bound to inverse agonists were recently determined (Cherezov 2007; Rasmussen 2007; Rosenbaum 2007). This type of structural data creates a framework for designing and testing potential models of transformation from inactive to active receptor signaling states and for initiating rational drug design, which is likely the next step in targeting GPCRs clinically.

1.3 Signaling through GPCRs

Upon activation by agonists, the majority of GPCRs couple to heterotrimeric G-proteins. G-proteins are composed of three subunits—α, β, and γ—and are divided into 4 major families, based upon the degree of primary sequence similarities of their α subunits (Kristiansen 2004). All of the major families, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$, are associated with different biological outputs—activation of adenylyl cyclase, inhibition of adenylyl cyclase, activation of PLCβ, and regulation of the RhoGEFs, respectively—but they all follow similar activation and inactivation cycles. These cycles include GDP binding to the α subunit and subsequent association with the βγ subunits, forming the inactive heterotrimer. Agonist binding to a receptor induces a conformational change that promotes the receptor's interactions with G-proteins, which induces guanine nucleotide exchange on the α subunit: GDP is rapidly released from its binding site, GTP instantly replaces it, and the heterotrimer dissociates, resulting in the aforementioned

biological outputs. Inactivation occurs when GTP gets hydrolyzed back to GDP by GTPase and/or when the G-proteins are inactivated by GTPase activating proteins and the heterotrimer reassociates.

Though the majority of GPCRs exhibit the capacity for G-protein coupling, they are also capable of signaling independently of the heterotrimeric G-proteins. By associating with scaffolding, chaperone, or signaling proteins, GPCRs can organize various signaling complexes to generate a variety of responses. For instance, activated GPCRs can be phosphorylated by GPCR kinases (GRKs), increasing their affinity for β-arrestins, which can then initiate a round signaling via mitogen-activated protein kinase cascades, promoting ERK1/2 phosphorylation (Gurevich & Gurevich 2008; Lefkowitz 1998). Via association with the Na^+-H^+ exchanger regulatory factor (NHERF) , the β_2-adrenergic receptor (AR) can also function without coupling to a Gα-protein to induce inhibition of renal Na^+-H^+ exchangers (Hall et al 1998a; Hall et al 1998b). Additionally, metabotropic glutamate receptors (mGluRs) can associate with the endoplasmic reticulum (ER)-associated IP_3 receptor via physical interaction with Homer to induce release of intracellular Ca^{2+} (Brzostowski & Kimmel 2001; Tu et al 1998). GPCRs can also signal through pathways not traditionally associated with G-protein coupling, such as Jak/STAT cascades, as demonstrated by experiments with angiotensin II (AT) receptors and serotonergic (5-HT) receptors. Specifically, STAT3 becomes phosphorylated in response to stimulation of 5-HT_{2A} receptors and interaction of the AT_1 receptor with JAK 2 occurs upon angiotensin II stimulation (Ali et al 1997; Guillet-Deniau et al 1997). By associating with GPCRs, scaffolding proteins like NHERF, and signaling proteins, such as β-arrestins, JAK and STAT, are able to induce intracellular effects independent of G-protein coupling or activation of another type of receptor.

1.4 Orphan GPCRs

The concept of ligands binding to putative receptors dates back to the work of Paul Ehrlich in Germany, who studied the interaction of dyes with biological structures (Hill 2006). A better understanding of the differentiation of ligands and the progression into labeling them as agonists, partial agonists, and antagonists was then developed in the mid 1900's. However, it was not until the development of radioligand-binding studies in the late 1960's that the molecular properties of GPCRs could begin to be deciphered. The purification and subsequent cloning in the 1980s of rhodopsin and several ARs—β_2, α_{2A}, and α_{1B}—opened up the field of GPCRs to what we now know today, which is the recognition of many more predicted receptors than currently identified ligands (Lefkowitz 2004).

While many GPCRs have been matched to a known endogenous ligand, more than 100 still have not; these have been termed orphan GPCRs. Initial discoveries of GPCRs through homology screening, low stringency hybridization, and PCR-derived approaches left their pharmacological properties a mystery (Civelli et al 2006). For a period of time, 'deorphanization' of these receptors was occurring at a rate of about 7-8 matches per year (Civelli 2005; Civelli et al 2006). In the past five years, however, the rate of deorphanization has been reduced to about half of that. A few reasons for this severe cut in deorphanizing receptors have been proposed. First, it has been suggested that some of these so-called orphan receptors may not have significant roles in mammals as ligand-binding receptors. Instead, orphan GPCRs are suggested to possibly act as transporters or chaperones, whereby receptors might assist in the trafficking of other receptors to the cell surface but are not needed to bind ligand. Similarly, it has been suggested that perhaps orphan receptors function simply to regulate pharmacological

properties of non-orphan GPCRs, as has been argued for the Mrg GPCR pair, MrgD (non-orphan; activated by β-alanine) and MrgE (orphan). It has not been definitively determined that MrgE does not have a ligand; however, in the context of the heterodimer, MrgE is observed to regulate both signaling and trafficking of MrgD (Milasta et al 2006). In addition, identification of constitutively active orphan receptors, such as the human herpesvirus-8-encoded receptor ORF74 and the Epstein-Barr virus-induced receptor 2, has raised the issue that some orphan receptors may not need to bind ligand to exert effects on cellular physiology (Levoye et al 2006). While these are all valid points, it is still widely expected that most orphan GPCRs will be found to bind endogenous ligands.

The primary way to determine the endogenous ligand of orphan GPCRs has typically been to express the receptors in heterologous cells, screen them against a variety of peptides and other ligands and look for some sort of second messenger response such as the previously listed biological outputs such as AC activation, cAMP production, or IP_3 production. Since most GPCR ligands are not membrane-permeable, it is imperative that the receptor be situated at the cell surface in these screens, such that the ligand has full access to its binding site, which is possibly a key reason as to why certain receptors have not been deorphanized. Many of the receptors that remain orphans may be GPCRs that are not well-expressed at the cell surface when transfected into heterologous cells.

1.5 Trafficking of GPCRs

Cell surface localization of GPCRs relies on two principal mechanisms: receptor delivery to a site and retention at that site (Tan et al 2004). Many factors are involved in

the process of receptor delivery to the plasma membrane. One key factor is endoplasmic reticulum (ER) quality control. The ER is the primary location for protein folding and maturation, which must proceed properly for a receptor to be released out of the ER. Several co-translational and post-translational modifications take place in the ER, such as disulfide-bond formation, signal-peptide cleavage, and N-linked glycosylation, which are all imperative for proper protein folding (Ellgaard & Helenius 2003). Chaperones and folding enzymes are present in the ER lumen in high concentrations and play a part in all of these processes. If problems occur in these steps, the strict quality control system within the ER will prevent the transport of receptors and other proteins from the ER to the Golgi, which then prevents eventual trafficking to the cell surface. This both extends the exposure of substrates to the folding machinery to improve the chance for appropriate maturation, and also ensures that proteins are not dispatched to terminal compartments when they are still incompletely folded, which potentially could be toxic to the cell.

For instance, various amino acid motifs have been found on the NT or C-terminus (CT) of GPCRs, that play a variety of roles in intracellular trafficking, including anterograde trafficking inhibition, like the RSRR motif on the CT of $GABA_BR1$, discussed later (Margeta-Mitrovic et al 2000). It is known, however, that $GABA_BR1$ still escapes from the ER *in vivo*, as it is necessary to create a functional $GABA_BR$. It seems that motifs such as this one can be masked from the quality control system via oligomerization with other GPCRs or association with other proteins that play roles as trafficking chaperones. This information became pharmacologically relevant when experiments using antagonists or other proteins, targeted at GPCRs retained in the ER in disease states such as nephrogenic diabetes insipidus (NDI) and hypogonadic

hypogonadism (HH), allowed the receptors to bypass the quality control system of the ER and reduced the disease-related symptoms (Bernier et al 2004a).

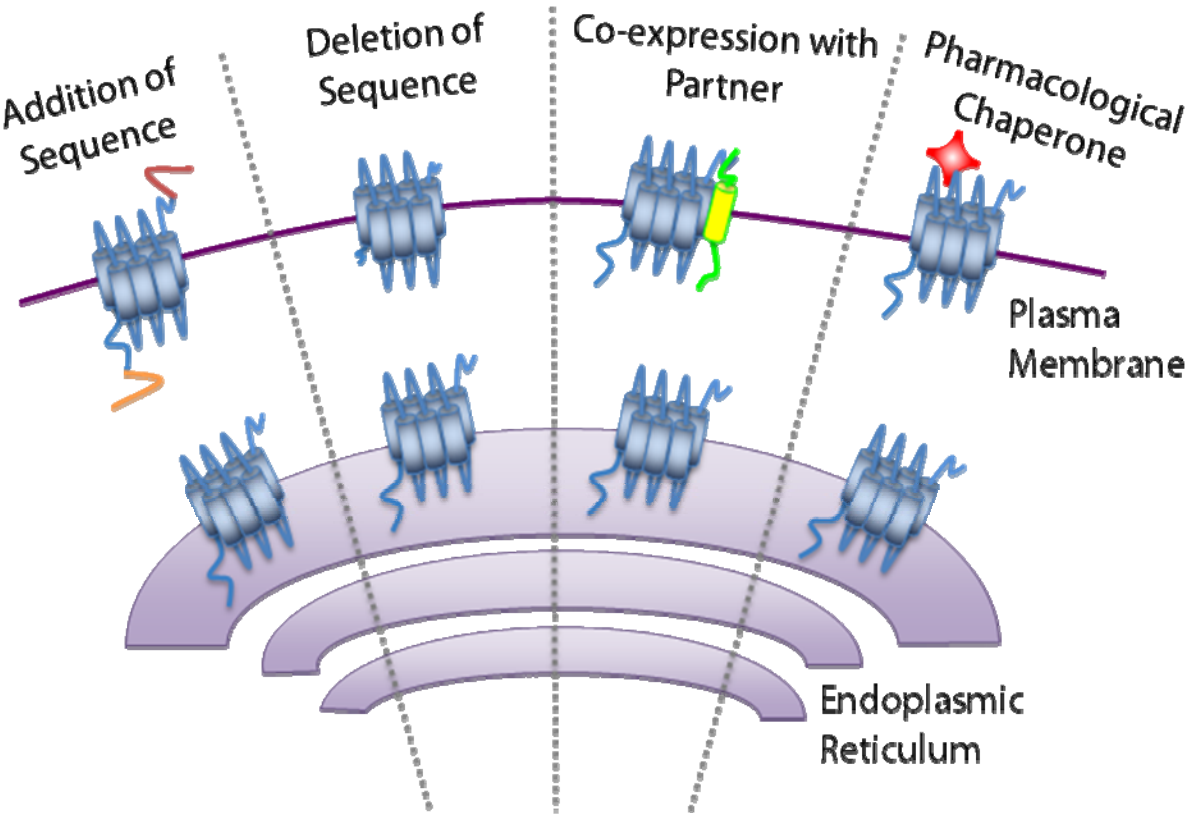
GPCRs often experience such trafficking woes when expressed in heterologous cell lines, preventing them from expressing at the plasma membrane and hindering successful functional studies. However, for structure-function studies and drug screening efforts, it is critically important to be able to express receptors in cells that do not endogenously express the receptor of interest. Therefore, it is essential for receptor characterization, deorphanization and, most importantly, drug discovery, that orphan receptors' trafficking in heterologous cells be understood and controlled. Several different methods that have been employed for enhancing receptor trafficking are shown in Figure I-1.

1.5.1 Domains enhancing forward trafficking

One major approach that has been utilized to enhance the plasma membrane expression of GPCRs in heterologous cells is the addition of sequences to the amino-termini of receptors. The first example of this approach was the engineering of an artificial signal sequence onto the N-terminus of the β_2-adrenergic receptor (Guan et al 1992), which resulted in a several-fold increase in insertion of the receptor into the plasma membrane. Artificial signal sequences have subsequently been used to enhance the surface expression of other GPCRs, notably the CB1 cannabinoid receptor (Andersson et al 2003; McDonald et al 2007). It is presumed that signal sequences facilitate receptor interactions with the signal recognition particle (SRP) and SRP receptor, which promote more efficient receptor targeting through the ER and membrane insertion (Hegde & Kang 2008).

Other types of sequences, beyond traditional signal sequences, have also been grafted onto the N-termini of certain GPCRs in order to enhance their surface

Figure I-1. Experimental approaches for enhancement of G protein-coupled receptor surface expression. When expressed in heterologous cells, many GPCRs exhibit poor plasma membrane trafficking, which can be enhanced using a variety of methods. For instance, addition or deletion of receptor sequences can in some cases greatly improve receptor surface expression. In other situations, co-expression with specific receptor-interacting partners can strongly promote proper surface trafficking. These receptor-interacting partners can either be transmembrane proteins, as illustrated in this schematic figure, or cytoplasmic proteins that associate with receptors' intracellular domains. Finally, pharmacological chaperones can release certain misfolded receptors from the endoplasmic reticulum and allow their enhanced trafficking to the plasma membrane.


Addition of Sequence
Deletion of Sequence
Co-expression with Partner
Pharmacological Chaperone
Plasma Membrane
Endoplasmic Reticulum

expression. For example, in the case of olfactory receptors (ORs), which are the largest subfamily of GPCRs with more than 300 members in humans and approximately 1000 members in rodents, a variety of N-terminal sequences have been utilized to enhance plasma membrane targeting. Most ORs are inefficiently trafficked to the plasma membrane in heterologous cells (Bush & Hall 2008), but the additions of N-terminal sequences from the serotonin 5-HT3 receptor (Wellerdieck et al 1997; Wetzel et al 1999) or rhodopsin (Katada et al 2004; Krautwurst et al 1998) have been shown to markedly enhance heterologous surface expression of many ORs. The use of ORs with modified N-termini has allowed for significant advances over the past few years in defining the pharmacological and signaling properties of this large and diverse family of GPCRs (Bush & Hall 2008).

While most sequences that enhance GPCR trafficking have been added to the N-terminal regions of the receptors, there are also examples of C-terminal additions that enhance receptor surface expression. For example, the rat gonadotropin-releasing hormone receptor (GnRHR) has a very short C-terminal tail and exhibits poor surface expression in most heterologous cells, whereas the catfish GnRHR has a much longer C-terminal region and exhibits robust surface expression in most cell types (Lin et al 1998). Addition of the catfish GnRHR C-terminus onto the rat GnRHR results in a striking improvement in the surface trafficking of the rat version of the receptor (Lin et al 1998). Interestingly, the GnRHR C-terminus is highly variable between species, suggesting that this receptor region may have been subject to intense evolutionary selection pressure as a mechanism for controlling GnRHR expression and functionality (Ulloa-Aguirre et al 2006).

1.5.2 Domains preventing forward trafficking

Following the cloning of $GABA_BR1$ (Kaupmann et al 1997), it was widely recognized that the receptor was poorly-trafficked and largely non-functional when expressed in heterologous cells (Couve et al 1998). Truncations of the $GABA_BR1$ CT, or mutation of a specific CT motif (RSRR), were found to relieve ER retention of $GABA_BR1$ and allow for robust plasma membrane expression of the receptor (Calver et al 2001; Margeta-Mitrovic et al 2000; Pagano et al 2001). Interestingly, the critical RSRR motif on the $GABA_BR1$ CT is similar to ER retention motifs that have been identified on certain ion channels and other transmembrane proteins (Michelsen et al 2005). However, despite the improved trafficking of a truncated $GABA_BR1$, which had the RSRR motif deleted, the mutant receptor remained incompetent in G protein coupling unless it was co-expressed with a related receptor, $GABA_BR2$. This second subunit seems to be a required heterodimer partner of $GABA_BR1$ in order to achieve the formation of functional $GABA_B$ receptors, a relationship that will be discussed in more detail later (Couve et al 2001; Margeta-Mitrovic et al 2001a; b; Pagano et al 2001).

Analogous to the removal of CT sequences from $GABA_{B1}R$, removal of NT sequences from GPCRs has in some cases proven to be effective in enhancing receptor surface expression. For example, truncation of 79 amino acids from the NT of the α_{1D}-adrenergic receptor (α_{1D}-AR) was found to dramatically enhance expression of the receptor binding sites (Pupo et al 2003) and plasma membrane localization (Hague et al 2004a). In contrast, grafting the α_{1D}-AR NT onto the related α_{1A}-AR or α_{1B}-AR was found to markedly impair surface expression of these receptors in heterologous cells (Hague et al 2004a), suggesting that the α_{1D}-AR NT either possesses an ER retention motif or has difficulty in folding properly. Similar findings have been made for the CB1 cannabinoid receptor, for which it has been shown that truncations to the receptor's long NT are

capable of greatly enhancing receptor surface expression (Andersson et al 2003). It is possible that this truncation of the CB1-NT occurs endogenously in some cells to remove a possible retention motif, allowing CB1 to reach the plasma membrane and bind to ligands (Nordstrom & Andersson 2006). For both α_{1D}-AR and CB1, it has been shown that the NT truncations that enhance trafficking do not alter ligand binding (Andersson et al 2003; Hague et al 2004a), and that these truncated mutants are thus useful for achieving enhanced surface expression of functional receptors in heterologous cells.

1.5.2 Receptor-receptor interactions that assist in proper trafficking

Growing evidence of GPCR oligomerization is shifting the one-time belief that GPCRs exist and act purely as monomers (Prinster et al 2005). With some receptors, heterodimerization may be an artifact of overexpression in heterologous cells. In other cases, however, oligomerization can change the functionality of the receptor by changing the affinity of the ligand or switching the G-protein coupling of the receptor (Franco et al 2007). And as mentioned before, dimerization, or multimerization, can sometimes strongly modulate membrane-directed trafficking.

The first widely accepted example of the effect of heterodimerization on trafficking was the aforementioned $GABA_BR$ pair (White et al 1998). As noted above, when $GABA_{B1}R$ is expressed alone in most heterologous cell types, an ER retention motif prevents efficient receptor trafficking to the plasma membrane (Couve et al 1998; Margeta-Mitrovic et al 2000). However, co-expression of $GABA_BR1$ with $GABA_BR2$ results in a massive enhancement in $GABA_BR1$ surface expression (Marshall et al 1999; White et al 1998). One result of the heterodimerization is believed to be the masking of the aforementioned ER retention motif present on the $GABA_BR1$ CT (Margeta-Mitrovic et al 2000), providing an example of how deletion of a sequence and co-expression with an appropriate partner can sometimes enhance the surface targeting of a given GPCR via

a common mechanism. There is strong evidence that the interaction between $GABA_BR1$ and $GABA_BR2$ is also essential for $GABA_BR1$ trafficking *in vivo*, since the brains of $GABA_BR2$ knockout mice exhibit a striking redistribution of $GABA_BR1$ and substantial loss in $GABA_B$ receptor functional activity (Gassmann et al 2004).

In addition to $GABA_B$ receptors, there are a number of other GPCRs that have been found to exhibit enhanced surface expression in heterologous cells upon co-expression and association with other GPCRs (Prinster et al 2005). The AR family also presents a key example of heterodimerization. As previously mentioned, the α_{1D}-AR shows little to no functional activity when expressed alone in most heterologous cells (Chalothorn et al 2002; Hague et al 2004a; Hirasawa et al 1997; Theroux et al 1996). However, when α_{1D}-AR is co-expressed with α_{1B}-AR (Hague et al 2004c; Uberti et al 2003) or β_2-AR (Uberti et al 2005), it heterodimerizes in a manner that strongly promotes α_{1D}-AR cell surface trafficking in heterologous cells. β_2-AR endogenously expresses at low levels in HEK 293 cells, so it is likely that the slight cell surface expression and functioning observed in cells with α_{1D}-AR expressed alone is enabled via heterodimerization (Chalothorn et al 2002).

Olfactory receptors (ORs) make up the largest family of GPCRs in mammalian genomes. Despite that, characterization of their pharmacological and signaling properties has been restricted, because many remain orphans and most are unable to efficiently traffic to the cell surface when expressed in heterologous cells (Bush & Hall 2008). Mammalian olfaction begins at the plasma membrane of olfactory sensory neuron cilia to bind and activate GPCRs, so ORs must be at the cell surface for accurate functional analysis. Screens of non-OR GPCRs co-expressed with the poorly trafficked OR M71 have been performed to determine if heterodimerization can also assist in OR localization. Indeed, co-assembly of certain members of the adrenergic and purinergic receptor families with M71 can enhance its surface expression (Bush et al 2007; Hague et

al 2004b). M71 remained functional when coexpressed with β_2-AR, inducing an increase in cAMP accumulation and co-internalization (Hague et al 2004b). In addition, when M71 associated with P2Y1 or P2Y2, the mitogen-activated protein kinase pathway was activated via $G\alpha_{i/o}$ signaling, demonstrating that the G-protein coupling preferences of ORs can be malleable depending on OR interactions with other receptors (Bush et al 2007).

Other receptors that exhibit dimerization are in the taste receptor (TR) family. T1R1 and T1R2 were initially cloned and found to express in distinct taste bud regions, but neither were responsive to sweet stimuli when expressed alone in heterologous cells. However, a gene encoding a GPCR with extensive homology to both T1R1 and T1R2 was discovered. This new receptor T1R3 also could not be activated when expressed alone, but co-expression with T1R2 allowed for activation by sweet tastants and co-expression with T1R1 allowed for activation by umami tastes, e.g. amino acids (Prinster et al 2005). This enhanced functionality is believed to correlate with improved surface expression, although most of the work undertaken in this area so far has focused more on assessing changes in receptor activity and pharmacology following heterodimerization than on addressing any changes in receptor trafficking (Bachmanov & Beauchamp 2007). Evidence with the ORs, ARs, and GABARs, suggest that many other GPCRs may require dimerization, too, but have yet to be studied in that manner.

Just as hetero-oligomerization has proven to play a significant role in regulating the ER export and plasma membrane trafficking of some receptors, homodimerization may also play an important role in controlling GPCR trafficking. For example, studies on β_2-AR homodimerization have revealed that this receptor likely requires homodimerization for its robust surface expression. Salahpour *et al.* compared β_2-AR mutants that retained the capacity to dimerize to β_2-AR mutants that could not dimerize

and observed that disruption of the putative dimerization motif of the receptor prevented normal surface trafficking (Salahpour et al 2004). It was also noted that dimerization of wt β_2-AR with a β_2-AR mutant lacking an ER-export motif or one harboring an ER-retention signal inhibited trafficking of the wild-type receptor (Salahpour et al 2004). With previous reports that homodimerization likely occurs as early as during ER processing (Bouvier 2001), it seems probable that β_2-AR, as well as a number of other GPCRs, need to homodimerize to properly traffic to the plasma membrane.

1.5.3 Associating proteins assist in proper trafficking

A variety of other protein interactions, beyond receptor-receptor associations, have also been identified as key regulators of GPCR trafficking. Some of these GPCR-interacting partners have been identified in genetic screens. One such example is the discovery of the cyclophilin-related protein Nina A, partnering with the Rhodopsin 1 receptor (Rh1) in the *Drosophila melanogaster*, for proper receptor folding and transport (Baker et al 1994; Shieh et al 1989). In flies lacking *nina A*, the gene encoding for the photo-receptor specific integral membrane glycoprotein, protein levels of Rh1 were reduced tenfold and binding activity was also significantly decreased (Shieh et al 1989). In addition, immaturely glycosylated Rh 1 accumulated in the ER of photoreceptor cells of these mutant flies, indicating the protein Nina A is required for Rh1 cell surface expression (Colley et al 1991). Subsequent work has revealed that RanBP2, the vertebrate homolog of Nina A, associates with vertebrate opsins to regulate their folding, trafficking and surface expression (Ferreira et al 1996).

Similarly, in screens for mutations that affect chemosensory signaling in *C. elegans*, the protein odorant response abnormal 4 (ODR-4) was identified and shown to associate with certain olfactory receptors (Dwyer et al 1998; Gimelbrant et al 2001). It is not known, however, if the vertebrate ortholog of ODR-4 plays a comparable role for any

vertebrate receptors (Lehman et al 2005). Other GPCR-interacting partners that promote receptor trafficking include GEC1, which promotes surface expression of mammalian κ-opioid (Chen et al 2006) and prostaglandin EP3 receptors (Chen et al 2009), RACK1, which enhances trafficking of thromboxane A_2 receptors (Parent et al 2008), Usp4, which increases plasma membrane expression of adenosine A_{2A} receptors (Milojevic et al 2006), ATBP50, which regulates the transport of angiotensin AT_2 receptor to the cell surface (Wruck et al 2004), and Drip78, which enhances angiotensin II AT_1 receptor expression on the cell surface (Leclerc et al 2002).

A variety of transmembrane proteins, including receptor activity modifying proteins (RAMPs), receptor transporting proteins (RTPs), receptor expression enhancing proteins (REEPs), melanocortin receptor accessory proteins (MRAPs), and the M10 family of major histocompatibility (MHC) proteins have been identified during the past decade as GPCR-interacting proteins that can also promote the surface expression of specific subsets of GPCRs. The RAMPs were first identified as key regulators of the trafficking and functionality of the calcitonin receptor-like receptor (CRLR), an orphan receptor that had proven difficult to study until the realization that associations with RAMPs were required for its efficient plasma membrane localization (McLatchie et al 1998). The three members of the RAMP family are now known to interact with several Class B GPCRs, as well as a Class C GPCR, the calcium-sensing receptor (Bouschet et al 2005), to influence receptor trafficking and pharmacology (Hay et al 2006). The RTP and REEP proteins were first identified in screens for proteins that enhance olfactory receptor functionality (Saito et al 2004). RTP1 and RTP2 are selectively expressed in the olfactory epithelium (Saito et al 2004), and their role in controlling OR trafficking have shed light on the underlying reasons why ORs are efficiently targeted to the plasma membrane in olfactory sensory neurons but not in heterologous cells. Other members of

the RTP and REEP families have wider tissue distribution patterns and have been shown to promote the surface expression in heterologous cells of T2R bitter taste receptors (Behrens et al 2006) and mu-delta opioid receptor heterodimers (Decaillot et al 2008). MRAP and MRAP2 have been shown to associate with the melanocortin 2 receptor (MC2R) and dramatically enhance surface expression of this receptor in a variety of cells (Hinkle & Sebag 2009; Metherell et al 2005; Roy et al 2007; Sebag & Hinkle 2009). Naturally-occurring mutations to MRAP cause defects in the trafficking and functionality of MC2R, resulting in an inherited disorder known as familial glucocorticoid deficiency type 2 (Metherell et al 2005). MRAPs also have been shown to associate with MC3R and MC4R to reduce the signaling activity and/or surface expression of these receptors (Chan et al 2009), so MRAP effects on receptor functionality appear to be receptor-specific. Finally, V2R vomeronasal receptors proved difficult to study in heterologous cells until the finding that co-expression and interactions with M10 MHC molecules and β_2-microglobulin were capable of dramatically enhancing V2R surface expression in heterologous cells (Loconto et al 2003).

The HSP70 heat shock protein Hsc70t is also viewed as a sort of escort for ORs. The HSP70 family is a group of molecular chaperones that interact and assist in correct folding of improperly folded proteins (Young et al 2003), and Hsc70t, constitutively expressed in the olfactory epithelium of humans and mice, has been observed to significantly increase the functional response of human OR 17-4 in HEK293 cells, suggesting higher levels of OR on the cell surface (Bush & Hall 2008; Neuhaus et al 2006).

1.5.4 GPCR trafficking defects in human disease

Clearly, GPCR localization at the plasma membrane is necessary, in most cases, for proper functioning and signaling of the receptor. Interestingly, a number of

naturally-occurring GPCR mutations have been identified that cause human disease by impairing normal receptor trafficking. Thus, there has been tremendous interest over the past few years in identifying small molecules that can bind to poorly-trafficked, disease-causing GPCRs with the aim to enhance the surface expression and functionality of these receptors. Such molecules are often referred to as "pharmacological chaperones," "pharmacochaperones" or "pharmacoperones" (Bernier et al 2004a; Conn et al 2007).

1.5.4.1 Vasopressin and Nephrogenic Diabetes Insipidus

NDI is a rare X-linked disease characterized by loss of anti-diuretic response to the hormone arginine-vasopressin, resulting in an inability to concentrate urine (Bernier et al 2004a; Morello & Bichet 2001; Tan et al 2004). If left untreated or unnoticed, as often is the case in infants, severe dehydration can occur, leading to growth retardation and mental retardation or death in the most extreme cases. Vasopressin V_2 receptors (V_2Rs) are found in the vascular endothelium and the principal cells of renal collecting and connecting tubules (Greenberg & Verbalis 2006). NDI has been linked to over 175 different mutations in V_2Rs, with the majority of these mutations causing V_2R to be retained in the ER and degraded (Bernier et al 2004a). Treatment of cells with certain membrane permeant V_2R antagonists SR121463 and VPA-985 has been shown to restore cell surface expression of ER-retained V_2R mutants (Bernier et al 2004b; Morello et al 2000; Robben et al 2007; Wuller et al 2004). These findings are believed to be due to binding of the antagonists to misfolded V_2R in the ER, resulting in the stabilization of receptor structure and trafficking of V_2R to the plasma membrane. Clinical studies have indeed provided proof-of-concept evidence that vasopressin receptor-targeted pharmacological chaperones can have beneficial effects in patients suffering from NDI (Bernier et al 2006).

1.5.4.2 Rhodopsin and Retinitis Pigmentosa

Retinitis Pigmentosa (RP) is another disease caused by mutations to a GPCR. Characterized by progressive photoreceptor degeneration and eventual retinal dysfunction, RP has been linked to a number of mutations in various gene products encoding nearly all of the components of the visual signaling pathway, including rhodopsin (Dejneka & Bennett 2001). Rhodopsin naturally exists in ordered, paracrystalline dimeric arrays at the cell surface where its signal transduction cascade is initiated by multiple electronic isomerizations of 11-*cis*-retinal attached to its GPCR, opsin (Fotiadis et al 2003; Liang et al 2003; Tan et al 2004). Mutations within the gene encoding rhodopsin are observed in one particular form of RP, autosomal dominant RP, and result in a mutated receptor molecule that is retained intracellularly with no 11-*cis*-retinal binding. The majority of the gene mutations observed are rare, with the exception of the P23H Class III mutation, which constitutes ~10% of all autosomal dominant cases of RP (Dryja et al 1990; Garriga et al 1996; Sung et al 1991). P23H rhodopsin molecules are observed as aggregates, including when in complex with ER chaperones (Anukanth & Khorana 1994). However, P23H rhodopsin mutants can be rescued with the 11-*cis*-retinal analog, 11-*cis*-ring-retinal, which results in restoration of receptor surface expression (Noorwez et al 2003) in a manner that is analogous to the above-described rescue of V_2R by V_2R antagonists.

1.5.4.3 Gonadotropin-Releasing Hormone Receptor and Hypogonadic Hypogonadism

GnRHR is activated by GnRH, which is secreted from the hypothalamus to induce hormonal gonadotropin synthesis and is also released from the pituitary gland gonadotropes to trigger the sexual organs to release additional hormones responsible for proper sexual development. Disruption in this hormonal pathway results in sexual

dysfunction and can impair sexual maturation (Tan et al 2004), as observed in hypogonadotropic hypogonadism. Mutations to GnRHR can result in misfolded receptors with an increased predisposition to be targeted for degradation or intracellular retention, resulting in HH. As in the above-described cases of pharmacological chaperones for V_2R and rhodopsin, selective non-peptidic GnRHR antagonists have been found to be capable of rescuing surface expression and signaling activity in the majority of mutant GnRHRs (Bernier et al 2004a; Janovick et al 2003; Janovick et al 2002; Leanos-Miranda et al 2002; Ulloa-Aguirre et al 2003).

1.5.5 Trafficking of orphan GPCRs

Many orphan GPCRs have also been observed to exhibit similar problems with trafficking *in vivo*, and a handful of these orphans have been characterized well enough to show that their trafficking can also be aided by some of the same techniques described above. For instance, bilateral frontoparietal polymicrogyria (BFPP) is linked to mutations in the orphan receptor GPR56. GPR56 is an adhesion GPCR that exhibits selective expression in hematopoietic stem cells and neural progenitors, revealing a potentially important role in multipotent cell identity and tissue development (Guerrini & Marini 2006; Jin et al 2007). GPR56 was also the first gene implicated in the development of BFPP, a malformation of cortical development in which the brain surface is irregular and normal gyral pattern is replaced by excessive number of small and partly fused gyri separated by shallow sulci (Jansen & Andermann 2005). Global developmental delay, a dysconjugate gaze (esotropica), language impairment, severe epilepsy and mental retardation are all characteristic of BFPP patients. Many different mutations of GPR56 have been linked to BFPP, most of which cause improper trafficking of the receptor and ER retention. Although no ligands have been identified for GPR56, treatments with thapsigargin, an ER calcium pump inhibitor that depletes ER calcium

stores, allowing for ER export, or 4-phenylbutyrate (4-PBA), which reduces mRNA and protein levels of heat shock protein Hsc70, were both able to rescue cell surface expression of GPR56 (Jin et al 2007). These types of treatment are likely successful due to a global disruption of ER function as opposed to specific assistance of a particular receptor's trafficking, but nonetheless, such approaches can still be utilized to study the signaling of trafficking-defective receptors.

Another orphan receptor exhibiting poor surface expression in heterologous cells is GPRC6A (Wellendorph & Brauner-Osborne 2004; Wellendorph et al 2005). An RKR motif, similar to that seen in $GABA_BR1$, was identified seven amino acids upstream of the CT of GPRC6A. However, unlike $GABA_BR1$, mutation of the motif to all alanines had no effect on GPRC6A cell surface expression. Given the receptor's similarity to metabotropic glutamate receptors and $GABA_BRs$, it has been thought that the NT of GPRC6A is most likely the receptor's primary site of ligand binding. In addition, GPRC6A is very similar to a goldfish receptor known as 5.24, which happens to be trafficked well to the plasma membrane in heterologous cells. Thus, a chimera was created in which the NT GPRC6A was fused to the TM and CT regions of 5.24 (Wellendorph et al 2005). This mutant receptor exhibited efficient trafficking to the plasma membrane, allowing for the identification of positively-charged amino acids, such as arginine, as putative ligands for GPRC6A (Wellendorph et al 2005).

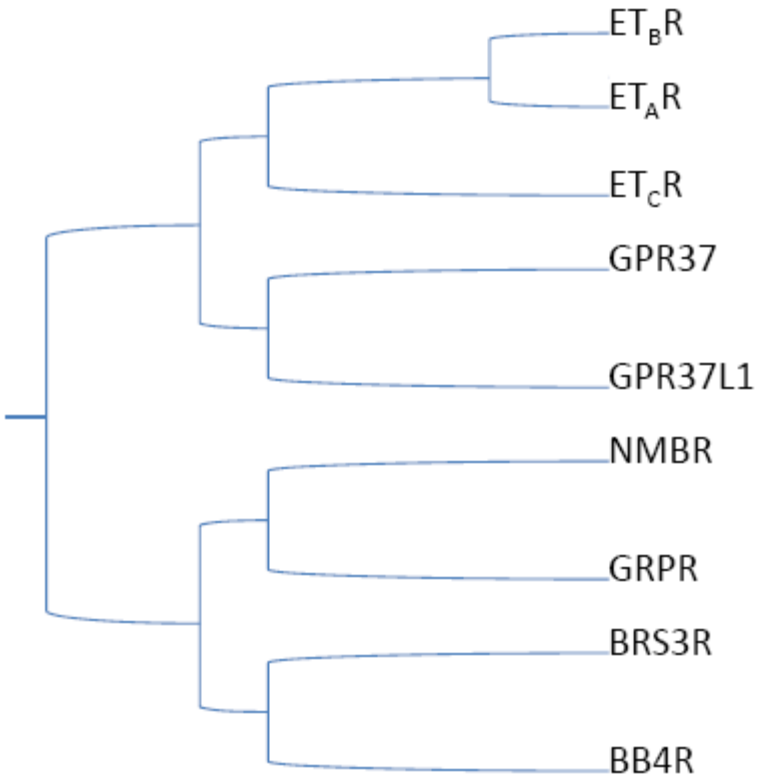
1.6 Parkin-Associated Endothelin-Like Receptor (PAEL-R)/GPR37

1.6.1 Endothelin-Like

GPR37, also known as the Parkin-associated Endothelin-Like Receptor (Pael-R), was first cloned by Zeng et al. in 1997 and termed ET_BR-LP (endothelin type B receptor-like protein) due to its significant homology, 52% similarity, to the ET_BR (Zeng et al 1997). Like ET_BR, the newly cloned receptor had a large extracellular N-terminal domain and a short third cytoplasmic domain of less than 30 amino acid residues. Northern blot analysis revealed highest expression of GPR37 in the human brain—found in all regions of the brain examined but highest in the corpus callosum and substantia nigra—but also low levels of expression in the human placenta, liver and testis (Marazziti et al 1998; Zeng et al 1997). Though expressed highest in oligodendrocytes, GPR37 localization was also well-documented in neurons of both substantia nigra and hippocampus (Imai et al 2001; Marazziti et al 2004; Marazziti et al 2007). Unfortunately, despite its homology with ET_BR and other related receptors (Figure I-2), further characterization revealed that GPR37 bound to neither endothelins nor other similar peptides like bombesin and neuropeptide Y when expressed in heterologous cells (Valdenaire et al 1998; Zeng et al 1997).

Soon after GPR37 was cloned, a receptor sharing approximately 68% overall homology with GPR37 was isolated (Valdenaire et al 1998) and later dubbed GPR37-Like 1, or GPR37L1. Similar to GPR37, GPR37L1 is also expressed widely throughout the brain, but unlike GPR37, which was reported to be expressed in neurons and oligodendrocytes (Imai et al 2001; Zeng et al 1997), high levels of transcripts of GPR37L1 were detected mainly in astrocytes (Valdenaire et al 1998). The transmembrane domains of GPR37 and GPR37L1 are highly homologous, but their sequences diverge in their N- and C- termini (Leng et al 1999; Valdenaire et al 1998). As with GPR37, the endothelins, bombesin and similar peptides were tested as possible ligands for GPR37L1,

Figure I-2. GPCR phylogenetic tree. This tree, adapted from Leng et al. (Leng et al 1999), is derived from comparison of the endothelin receptors (ET_AR, ET_BR, ET_CR), bombesin receptors (GRPR, NMBR, BRS3R, BB4R), and GPR37 and GPR37L1.


ET_BR
ET_AR
ET_CR
GPR37
GPR37L1
NMBR
GRPR
BRS3R
BB4R

but similar to GPR37, there was no detectable activation of GPR37L1 by these peptides (Valdenaire et al 1998).

1.6.2 Parkinson's disease and the Parkin-Associated Receptor GPR37

After Alzheimer's disease, Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting approximately 4 million people worldwide (Fitzgerald & Plun-Favreau 2008). Neuropathologically, PD is characterized by progressive degeneration of DA neurons in the substantia nigra pars compacta and by the presence of abnormal aggregates of protein known as Lewy bodies, which develop inside of these neurons. The etiology of PD remains unknown, but evidence indicates a connection to mitochondrial dysfunction and oxidative stress (Beal 2003; Jenner & Olanow 1996; Kosel et al 1999; Zhang et al 2000).

The loss of DA neurons and the disruption of the entire DA system results in gradual, progressive loss of movement control, producing symptoms such as resting tremor, bradykinesia, postural instability and rigidity. Other common PD-related symptoms are disturbances in the patient's gait and posture, speech and swallowing difficulties, as well as insomnia, dementia, dizziness, and akathisia, or the inability to sit still. The average onset of the most common form of PD is generally between 60 and 80 years of age, with incidence increasing from ~1% at age 65 to ~4-5% in those at least 85 years old (Giasson & Lee 2001). This form of PD is referred to as sporadic or idiopathic PD and has been relatively enigmatic due in part to the lack of a biologic marker available to use for diagnostic purposes. In fact, until about 2001, autopsy studies indicated that the final diagnosis of PD had been incorrect about one-fourth of the time.

In recent years, advances in understanding the more rare familial forms of PD have provided new insights about the disease. Familial forms of PD are linked to disease-related hereditary mutations. These account for between 5-15% of PD cases and typically have an onset below the age of 40 (Dawson & Dawson 2003; Mochizuki 2009; Schulz 2008). They can be due to autosomal dominant point mutations or gene duplications/triplications in the α-synuclein gene, and/or point mutations in the ubiquitin-C-terminal hydrolase-L1 and the leucine-rich repeat kinase genes. They also can be triggered by autosomal recessive mutations of the aforementioned gene parkin, as well as PINK1, DJ-1, and a specific ATPase gene (Schulz 2008). Parkin mutations actually account for about 50% of individuals with AR-JP and over 75% of sporadic cases in which disease onset occurs before 20 years of age (Fitzgerald & Plun-Favreau 2008).

There currently is no cure for the characteristic progressive neurodegeneration of PD, but by gaining an understanding of these identified mutations in the familial forms of the disease, and discovering yet to be identified genetically-linked mutations, further insight can be gained into treating the neuropathology of PD. One potential treatment stemming from such research is to administer an overabundance of parkin via gene therapy (Mochizuki 2009; Ulusoy & Kirik 2008).

PD has been associated with increased ER stress. Mimetics of PD specifically induce ER stress in neuronal cells (Holtz & O'Malley 2003), and expression of certain ER chaperones are up-regulated in the brain of PD patients (Conn et al 2004). In addition, two genes that are mutated in certain cases of PD, parkin and ubiquitin C-terminal esterase L1 (UCH-L1), are both involved in ER-associated degradation. UCH-L1 is a protein in neurons that can stabilize a monomeric ubiquitin to ubiquitinate unfolded proteins, targeting them for degradation (Osaka et al 2003). Parkin is an E3 ubiquitin ligase, equipped with an ubiquitin-like domain at its NT, and its main cellular role is

believed to also be ubiquitination of proteins. Interestingly, parkin expression is increased when the cell is experiencing ER stress, and healthy neuronal cells overexpressing parkin are resistant to such stress (Malhotra & Kaufman 2007). However, a variety of both homozygous and heterozygous mutations can cause rearrangements and missense mutations in the parkin gene. Such mutations are commonly observed in post-mortem brain tissue of patients with autosomal recessive juvenile Parkinson's disease (AR-JP), leading to impaired binding of parkin to putative substrates or inactive ligase activity, which results in dysfunction of the ubiquitin proteasomal pathway, reducing or eliminating the resistance to ER stress (Yang et al 2009).

GPR37 was dubbed 'parkin-associated' upon the identification of its role as an interacting partner of parkin (Imai et al 2001). When GPR37 is expressed in heterologous cells, it has difficulty folding and trafficking properly: it is found aggregated in both the cytoplasm and the ER and exists in an ubiquitinated, insoluble form due to its misfolded state (Imai et al 2001). It has been observed that an overexpression of GPR37 alone induces ER stress and dopaminergic (DA) cell death, a fate that can be alleviated via parkin overexpression (Yang et al 2003). When parkin is mutated and cannot properly target GPR37 for degradation, ER stress is induced, thereby suggesting a role for GPR37 in the pathology of AR-JP (Imai et al 2007).

Murakami et al reported the presence of GPR37 in the core of Lewy bodies (Murakami et al 2004). Parkin is often seen associated with these protein inclusions, but it is generally localized around their 'halo' and not necessarily involved in their development. This finding, then, is indicative of a possible non-parkin-related role of GPR37 in PD, perhaps linking GPR37 to the pathology of both sporadic and familial forms.

Further connections between GPR37 and PD may be derived from studies revealing that GPR37 KO mice exhibit an increased resistance to MPTP-induced toxicity, a commonly used animal model of PD (Marazziti et al 2004). Moreover, GPR37 transgenic (Tg) mice exhibit an enhanced susceptibility to such MPTP-induced toxicity (Imai et al 2007), as do *Drosophila* genetically-engineered to express mutated parkin (Pesah et al 2004) as well as rats overexpressing GPR37 (Dusonchet et al 2009). Though this may not be a direct correlation between GPR37, parkin and PD, MPTP *is* used to induce PD-like symptoms and behaviors in animals for PD research because: 1) it acts by killing certain neurons in the substantia nigra and 2) MPTP-induced pathology can be treated with the use of Levodopa, a drug used for reducing symptoms in human PD patients (Langston & Ballard 1984; Langston et al 1984a; Langston et al 1984b; Langston et al 1984c).

To get a better grasp on the relationship of GPR37 and parkin, Wang *et al*. made double-mutant mice by crossbreeding parkin KO mice with GPR37 Tg mice. They reported that these mice exhibit ER stress as well as early and progressive loss of DA neurons without the formation of Lewy body-like inclusions, similar to the pathological characteristics of AR-JP patients (Wang et al 2008). It was also reported that there was significant DA cell loss after only 12 months in the double mutant mice, as opposed to 18 months in the GPR37 Tg mice, further suggesting the potential importance of the interplay between parkin and GPR37 in PD pathology.

1.7 Overall Hypothesis

Given the utility of GPCRs as drug targets, and the possible role that GPR37 plays in a devastating disease such as PD, it is important to determine the normal physiological roles of GPR37, as well as how GPR37 may be targeted in disease states such as PD. The aim of this work is to provide more insight into the basic roles that GPR37 plays in cellular physiology by studying its cellular trafficking and signaling properties. To do this, we first sought out to develop methods to express GPR37 more efficiently at the cell surface. Since this receptor has very poor plasma membrane expression in heterologous cell lines, we wanted to determine why it was retained intracellularly and what might be done to reverse that effect. We demonstrate here the key role played by the receptor's NT in controlling GPR37 cell surface trafficking and how this information can be used to develop tools for studying GPR37 in heterologous cells.

Additionally, recent reports of heterodimers and multimers indicate the importance of interacting partners in GPCR functionality and signaling. In an effort to express full-length GPR37 at the cell surface, and also to understand more about its pharmacology, we studied the trafficking of GPR37 when co-expressed with a number of other GPCRs. We reveal here an interaction of GPR37 with D_2R and demonstrate how that interaction influences both GPR37 trafficking and D_2R ligand binding. We propose that the findings we report here have the potential to be used in developing novel pharmacological treatments targeted at the DA system. Finally, we also report here a novel interaction between GPR37 and the scaffold protein syntenin-1, which results in a dramatic increase in GPR37 surface expression in heterologous cells. As a whole, these findings provide additional methods and tools with which to study GPR37. These tools will assist in the further characterization and eventual deorphanization of GPR37, as well as in the development of therapeutics that may target GPR37 directly.

CHAPTER II: Structural Determinants Controlling Surface Trafficking of GPR37

2.1 Introduction

A major stumbling block impeding progress in understanding the ligand binding and signaling of GPR37 is its poor trafficking to the cell surface in most heterologous cell lines. Other GPCRs that exhibit trafficking defects in heterologous cells, including α_{1D}-AR (Hague et al 2004a; Hague et al 2004c; Pupo et al 2003; Uberti et al 2003), $GABA_BR1$ (Calver et al 2001; Couve et al 1998; Margeta-Mitrovic et al 2000; Margeta-Mitrovic et al 1999; Pagano et al 2001), and the cannabinoid CB1 receptor (Andersson et al 2003) share significant homology with receptors that do exhibit robust surface expression. They also have been shown to more efficiently traffic following truncation of either the receptor's NT or CT regions. GPR37L1 shares 68% homology and 48% identity with GPR37 and, similar to GPR37, is abundantly expressed in the brain (Leng et al 1999; Valdenaire et al 1998). However, like GPR37, none of the peptides tested thus far, including the endothelins, bombesin and neuropeptide Y, have produced activation of any signaling pathways in heterologous cells or *Xenopus* oocytes expressing GPR37L1 (Valdenaire et al 1998). The main identified difference between the two receptors is the diverging sequences in their CT and NT. Thus, we created truncated forms of GPR37 to shed light on the structural determinants of GPR37 that influence its plasma membrane expression. It was our hope that by creating a mutant that *does* traffic well to the cell surface, we could then express it heterologously and use it as a tool to better characterize and eventually deorphanize GPR37.

2.2 Experimental Procedures

Materials. Materials were obtained from the following sources: human embryonic kidney (HEK293) cells, ATCC (Manassas, VA); GPR37, Guthrie Research Institute (Sayre, PA); FLAG-GPR37L1, Multispan (Hayward, CA); Dulbecco's modified Eagle medium (DMEM), Lipofectamine 2000, precast 4-20% Tris-Glycine gels, AlexaFluor 488 and 800 goat-anti-mouse antibodies, Alexa Fluor 546 and 700 goat-anti-rabbit antibodies, Invitrogen (Carlsbad, CA); anti-FLAG M2 monoclonal antibody, forskolin, isoproterenol, and cAMP, Sigma (St. Louis, MO); ECL™ Anti-mouse IgG, Horseradish Peroxidase-linked whole antibody, [^{3}H]-cAMP, GE Healthcare (Buckhinghamshire, UK); anti-Na^+/K^+ ATPase antibody, Upstate/Millipore (Billerica, MA); bovine serum albumin (BSA), penicillin-streptomycin solution, Fisher (Herndon, VA); fetal bovine serum (FBS), Atlanta Biologicals (Atlanta, GA); QuikChange XL site-directed mutagenesis kit, Stratagene (Cedar Creek, TX); SuperSignal Elisa Pico ECL reagent, Pierce (Rockford, IL); nitrocellulose membranes, Bio-Rad (Hercules, CA); DAPI, AppliChem (Ottoweg, Darmstadt, Germany); head activator neuropeptide, Phoenix Pharmaceuticals (Belmont, CA) and Bachem AG (Bubendorf, Switzerland); p44/42 MAPK (ERK1/2) rabbit antibody and immobilized phsopho-p44/42 MAPK (ERK1/2) mouse antibody, Cell Signaling Technology (Danvers, MA); [^{3}H]-adenine, [^{3}H]-adenosine, American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Cell culture and transfection. HEK293 cells were maintained in DMEM supplemented with 10% FBS, and 1% penicillin-streptomycin solution at 37°C with 5% CO_2. Cells in 10-cm tissue culture dishes at a confluency of 50-60% were transfected

with 1-3 μg of cDNA mixed with 15 μl Lipofectamine 2000 in 5ml of serum-free medium. Following a 4-5 hr incubation, complete medium was added to stop the transfection. The cDNAs used were FLAG-GPR37 in pCMV2b, FLAG-GPR37L1 in pMEX, ΔCT-GPR37, $\Delta^{1\text{-}35}$, $\Delta^{1\text{-}70}$, $\Delta^{1\text{-}105}$, $\Delta^{1\text{-}140}$, $\Delta^{1\text{-}175}$, $\Delta^{1\text{-}210}$, and ΔNT-FLAG-GPR37 in pCMV2b, untagged GPR37 in pCMV2b, and empty pCMV2b vector. All cDNAs used were human.

Cell-surface luminometer-based assay. HEK293 cells transiently transfected with FLAG-tagged or untagged constructs were split into poly D-lysine-coated 35-mm dishes and grown overnight at 37°C. For internalization assays, ligand was added into incomplete media and placed on cells for a 30-60 min incubation at 37°C. The cells were washed with phosphate-buffered saline (PBS + Ca^{2+}), fixed 30 min with 2% paraformaldehyde (PFA), and washed with PBS + Ca^{2+} again. The cells were then incubated in blocking buffer (2% nonfat dry milk in PBS, pH 7.4) for 30 minutes at room temperature (RT), followed by RT incubation with horseradish peroxidase-conjugated M2-anti-FLAG antibody (1:1000) in blocking buffer for 1 h. The cells were washed twice with blocking buffer, washed once with PBS + Ca^{2+}, and incubated with SuperSignal Pico ECL reagent for 15 s. Luminescence of the entire 35-mm dish was determined using a TD20/20 luminometer (Turner Designs).

Flow cytometry. HEK293 cells that had been transiently transfected were split into poly D-lysine-coated 35-mm dishes and grown overnight at 37°C. The cells were transferred to ice, washed with PBS + Ca^{2+} once and incubated with M2 anti-FLAG antibody (1:300) in 1% BSA for 1 hour. Then the cells were washed once and incubated in the dark with Alexa Fluor 488 anti-mouse antibody (1:500) in 1% BSA for 1 hour. Again the cells were washed once, incubated for 15 minutes with 10mM Tris, 5mM

EDTA, shaken loose, and transferred to tubes containing equal volume 4% PFA. Samples were spun down, supernatant aspirated, and resuspended in 250 µl 1%BSA. Flow cytometric acquisition and analysis were performed on at least 10,000 acquired events on an LSR II flow cytometer driven by FACSDiva software (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star).

Mutagenesis. Six forward primers and one reverse primer were designed to make sequential truncations. Truncated constructs were generated via PCR using those primers and a cDNA corresponding to full-length GPR37. The PCR products were digested with BamHI and EcoRI and inserted into previously digested pCMV-2B, containing an N-terminal FLAG epitope.

New forward and reverse primers were designed to make mutations of the N-terminal of GPR37, generated from cDNA of $\Delta^{1\text{-}175}$ GPR37 containing an N-terminal FLAG epitope in the mammalian expression plasmid pCMV-2B using the QuikChange Site-Directed Mutagenesis kit. All sequences of truncated and mutated receptor were confirmed by sequence analysis (Agencourt, Beverly, MA).

Western blotting. Samples were resolved by SDS-PAGE on 4-20% Tris-Glycine gels, followed by transfer to nitrocellulose membranes. The membranes were incubated in blocking buffer (2% nonfat dry milk, 50mM NaCl, 20mM HEPES, 0.1% Tween 20) for 30 min and then incubated with primary antibody for either 1 h at RT or overnight at 4°C. Next, the membranes were washed three times in blocking buffer and incubated with either a fluorescent- or HRP-conjugated secondary antibody for 30 min, washed three times more, and finally visualized using the Odyssey imaging system (Li-Cor) or via ECL reagent followed by exposure to film.

Confocal microscopy. Cells transiently transfected with FLAG-tagged constructs were grown on poly-D-lysine coated glass slides. The cells were rinsed with PBS + Ca^{2+}, fixed with 2% PFA at RT, and washed 3 times with PBS + Ca^{2+}. Fixed cells were permeabliized and blocked by incubating 30 min at RT in saponin buffer (1% BSA, 0.08% saponin, PBS + Ca^{2+}). Next, the cells were washed 3 times and incubated with rabbit anti-FLAG antibody (1:1000) and mouse anti-Na^{+}/K^{+} ATPase (1:500) in 1% BSA at 37° C for 1 hr. After two times washing with PBS + Ca^{2+}, cells were incubated in the dark with Alexa Fluor anti-mouse 488 and anti-rabbit 546 antibodies (1:250) in 1% BSA for 1 hr at RT. Cells were washed 2 times more for 5 min with PBS + Ca^{2+}, DAPI-stained for 10 min, rinsed twice with water, and mounted with Vectashield mounting medium. Cells were examined using a Zeiss LSM 510 laser scanning confocal microscope.

ERK activation assays. HEK293 cells transiently transfected with FLAG-tagged constructs were split into poly D-lysine-coated 35-mm dishes and grown overnight at 37°C. Twenty-four hours later, they were starved in serum-free minimum essential medium overnight. To stimulate cells, 3 nM head activator peptide was added directly to the starvation medium for 2, 5, or 10 min at 37 °C. Stimulation with FBS (1%) was performed for 2 min as a positive control. At the end of the stimulation, the medium was removed, and 80 µl of sample buffer was added. The 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. Fluorescence-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).

cAMP assays. Transiently-transfected HEK293 cells were split into 24 well plates 24 h before experimentation. Because HEK293 cells do not easily take up [^{3}H]-adenine, [^{3}H]-adenosine was used to prelabel cells. Cells were prelabeled with 1 ml of incomplete media containing 1 μCi of [^{3}H]-adenosine for 2 hours. Cells were then washed with TBMX buffer [Na-Elliot's buffer (137mM NaCl, 5mM KCl, 1.2 mM $MgCl_2$, 0.44mM KH_2PO_4, 4.2mM$NaHCO_3$, 20 mM HEPES, pH 7.4], 10mM $CaCl_2$, 100mM glucose, and IBMX) and treated with 100 nM HA and/or 10 μM forskolin/isoproteronol for 10 minutes. Reactions were stopped by addition of 77% trichloroacetic acid. A 50 μl aliquot of 10 mM cAMP was added as a carrier, and plates were spun for 10 min at 2,500 rpm. The [^{3}H]-cAMP that was formed was isolated by sequential Dowex and alumina chromatography. Eluants from alumina columns were collected, 5 ml of scintillation fluid was added, and [^{3}H]-cAMP was quantified by using a liquid scintillation counter.

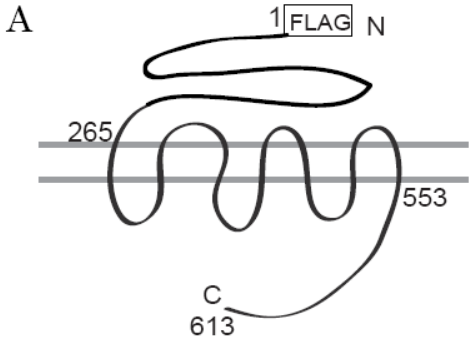
Statistical analysis. All statistical analyses were carried out using Graph Pad Prism software (GraphPad Software Inc., San Diego, CA).

2.3 Results

2.3.1 GPR37L1 exhibits robust cell surface expression but GPR37 does not.

Plasma membrane trafficking of GPR37 (Fig II-1A) and GPR37L1 (Fig II-1B) was assessed using three independent techniques: a quantitative luminometer-based surface expression assay, FACS analysis, and confocal microscopy. To be sure the surface expression of the receptors was the same across cell lines, we repeated many of these assays in CHO and Cos-7 cell lines and found similar results (data not shown). In the luminometer-based assay, we observed very little plasma membrane expression of GPR37, consistent with previous reports (Imai et al 2001; Rezgaoui et al 2006; Yang et al 2005). In contrast, GPR37L1 was robustly trafficked to the plasma membrane (Fig II-1C). FACS analysis confirmed that GPR37L1 was highly expressed on the cell surface, whereas GPR37 expression was barely detectable (Fig II-1D) despite comparable levels of Western blot staining for the two receptors (data not shown). To confirm these findings via a third independent technique, confocal microscopy studies were performed using the Na^+/K^+ ATPase as a plasma membrane marker. Mainly punctate intracellular staining was observed for GPR37 (Fig II-1E), whereas GPR37L1 was predominantly localized at the plasma membrane (Fig II-1F). MATLAB analysis of the images revealed that approximately 42% of the overall transfected GPR37L1 exhibited cell surface expression, whereas the cell surface expression for wt GPR37 was undetectable by these methods (<1%). Thus, despite the high degree of sequence similarity between the two receptors, GPR37L1 exhibited robust surface expression in our studies whereas GPR37 poorly trafficked to the plasma membrane.

Figure II-1. GPR37L1 exhibits robust cell surface expression as compared to GPR37. FLAG-tagged constructs corresponding to GPR37 (A) and GPR37L1 (B) were transiently transfected into HEK293 cells. Cell surface expression was determined using a luminometer-based assay (C) and via flow cytometry (D). Values are expressed as mean fold surface expression over empty vector ± S.E.M. Unpaired *t* tests were used to determine statistical significance (n= 23 & 3, respectively; *, $p < 0.05$; **, $p < 0.005$). Confocal imaging of cells transfected with wt GPR37 (E) or GPR37L1 (F) was done using mouse anti-Na^+/K^+ ATPase, followed by Alexa-Fluor 488, to mark the cell surface (green, left panels), rabbit anti-FLAG, followed by Alexa-Fluor 546, to detect the receptors (red, center panels), and DAPI to stain the nucleus (blue, right panels). Yellow indicates colocalization of receptor with plasma membrane (E&F, right panels).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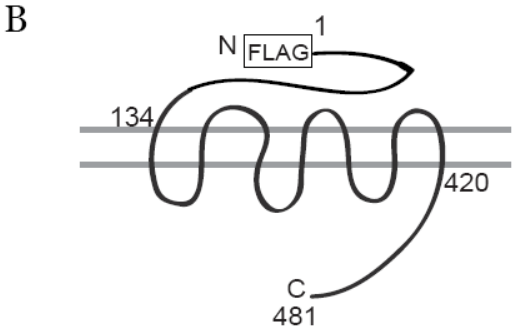
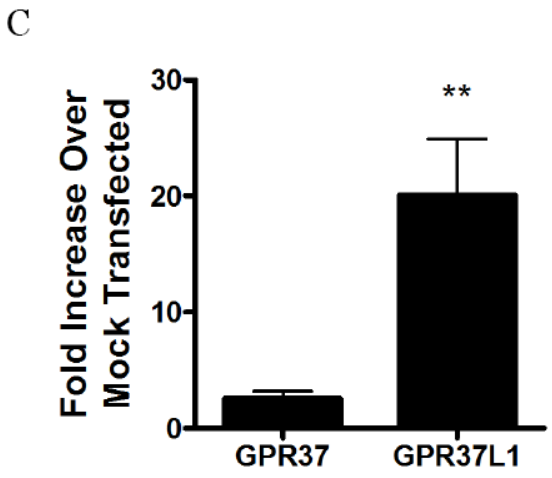
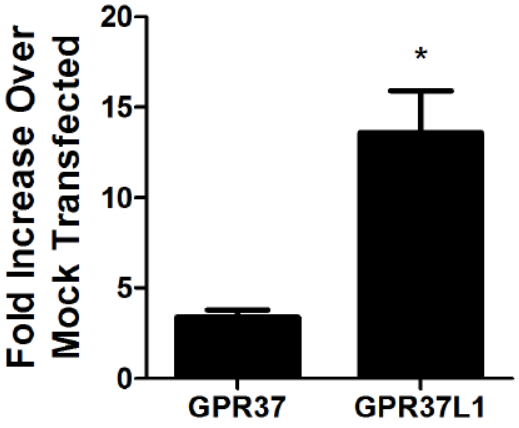
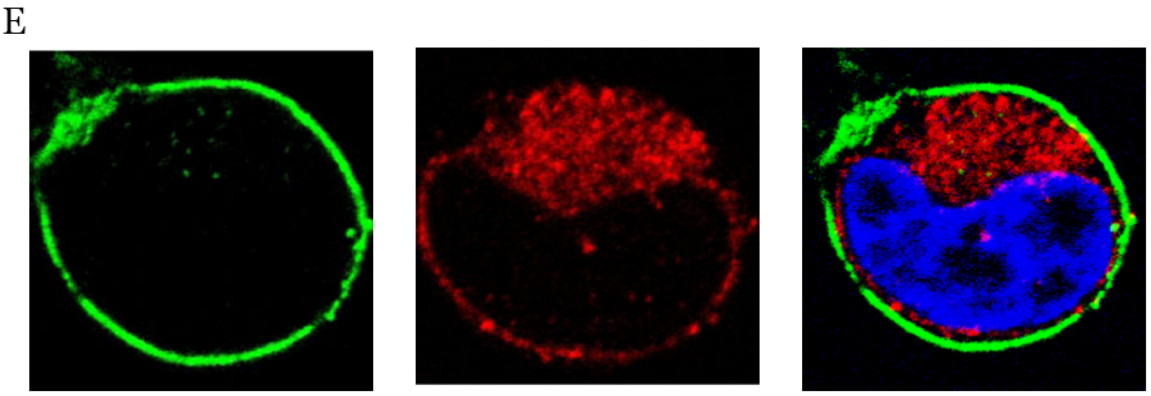
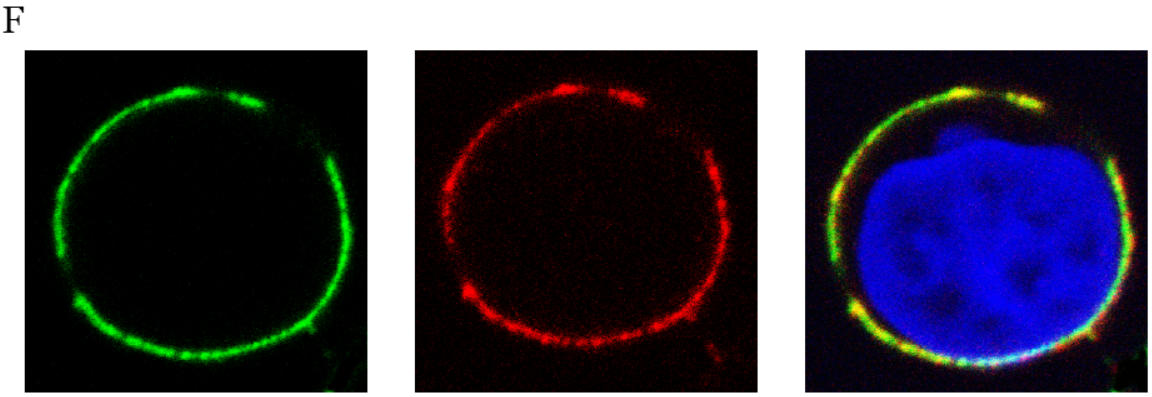
FLAG
N
265
553
C
613


B
N
FLAG
1
134
420
C
481


C
Fold Increase Over Mock Transfected
30
20
10
0
**
GPR37
GPR37L1


D
Fold Increase Over Mock Transfected
20
15
10
5
0
*
GPR37
GPR37L1


E


F

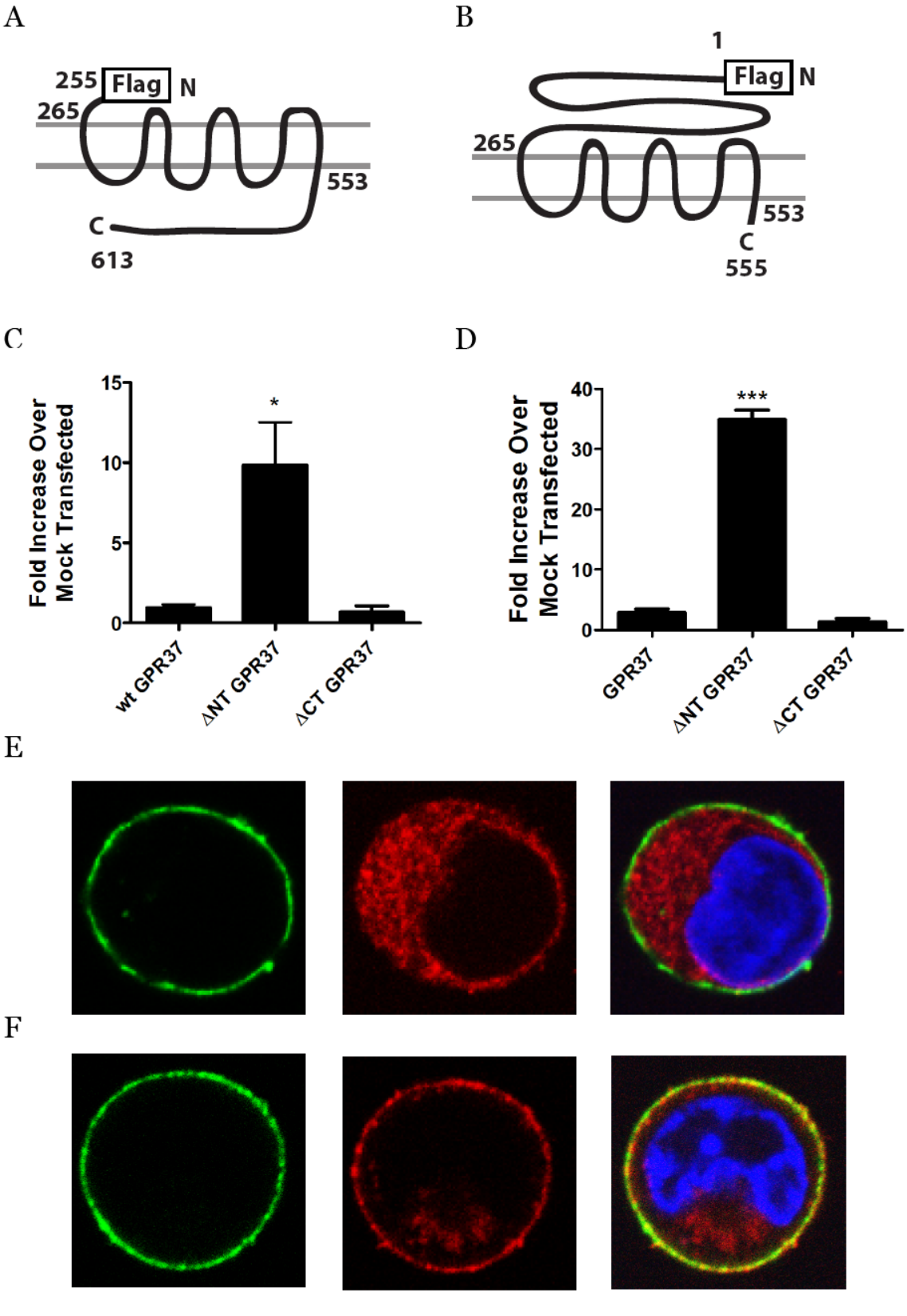
2.3.2 N-terminal truncation of GPR37 increases plasma membrane expression.

Given the striking difference in plasma membrane expression between GPR37 and GPR37L1, we next focused on determining which region of these related receptors might account for this difference. Since GPR37 and GPR37L1 are most divergent from each other at their N- and C- termini, we created two N-terminally FLAG-tagged constructs of GPR37 – one in which 255 N-terminal amino acids were deleted (ΔNT-GPR37; Fig II-2A), and a second in which 58 C-terminal amino acids were deleted (ΔCT-GPR37; Fig II-2B). In the luminometer-based assay, the surface expression of ΔCT-GPR37 was observed to be equivalent to full-length GPR37, hereafter identified as wild-type GPR37 (wt GPR37). However, the ΔNT-GPR37 mutant exhibited a striking increase in surface expression relative to either of the other two constructs (Fig II-2C). These findings were confirmed in flow cytometry experiments (Fig II-2D), as well as with confocal microscopy, in which ΔCT-GPR37 exhibited similar intracellular distribution to wt GPR37 (Fig II-2E), while ΔNT-GPR37 was predominantly associated with the plasma membrane (Fig II-F). Again, MATLAB analysis confirmed the qualitative observations, with about 38% of transfected ΔNT-GPR37 expressing at the cell surface.

2.3.3 Structural determinants on the GPR37 N-terminus control receptor surface expression.

The results with the ΔNT-GPR37 mutant suggested that a motif on the GPR37 NT is a critical determinant of the proper folding and plasma membrane localization of GPR37. To determine the location and sequence of this potential motif, we generated 6 sequentially truncated constructs of GPR37—$\Delta^{1\text{-}35}$, $\Delta^{1\text{-}70}$, $\Delta^{1\text{-}105}$, $\Delta^{1\text{-}140}$, $\Delta^{1\text{-}175}$, and $\Delta^{1\text{-}210}$—

Figure II-2. N-terminal truncation of GPR37 enhances plasma membrane expression. Constructs corresponding to N-terminal truncation *(A)* and C-terminal truncation (*B*) of GPR37 were prepared with N-terminal FLAG tags. HEK293 cells were transiently transfected with wt GPR37, ΔNT GPR37, and ΔCT GPR37. Surface expression was detected using a luminometer-based assay (C) and via flow cytometry (D). Values are expressed as mean fold over empty vector ± S.E.M. One-way ANOVA followed by Tukey's post-hoc test was used to determine statistical significance (n= 3-6; *, $p < 0.05$; ***, $p < 0.0001$). Confocal imaging of cells transfected with ΔCT GPR37 (E) or ΔNT GPR37 (F) was done using mouse anti-Na^+/K^+ ATPase, followed by Alexa-Fluor 488, to mark the cell surface (green, left panels), rabbit anti-FLAG, followed by Alexa-Fluor 546, to detect the receptors (red, center panels), and DAPI to stain the nucleus (blue, right panels). Yellow indicates colocalization of receptor with plasma membrane (E&F, right panels).


A
255
Flag
N
265
553
C
613
B
1
Flag
N
265
553
C
555
C
Fold Increase Over Mock Transfected
15
10
5
0
*
wt GPR37
ΔNT GPR37
ΔCT GPR37
D
Fold Increase Over Mock Transfected
40
30
20
10
0
***
GPR37
ΔNT GPR37
ΔCT GPR37
E
F

removing 35 more amino acids from the NT in each additional construct (Fig II-3A). Evaluation of the surface expression of these constructs was performed using the luminometer-based assay. As shown in Fig II-3B, very little surface expression was observed in the first 4 mutants, and the Δ^{1-175} mutant exhibited only a slight increase over wt GPR37. However, the Δ^{1-210} mutant exhibited a robust enhancement in surface expression, similar to that of ΔNT-GPR37. Confocal images revealing colocalization between Δ^{1-210} GPR37 and the plasma membrane confirmed the findings from the luminometer experiments (Fig II-3C); analysis of the images using MATLAB revealed that approximately 25% of the overall transfected Δ^{1-210} exhibited cell surface expression.

2.3.4 Plasma membrane expression of GPR37 does not seem to be influenced by individual N-terminal amino acids.

Based on these findings, we hypothesized that a motif was present within the 35-amino acid section between 175 and 210 of GPR37 that strongly influenced trafficking of the full-length receptor. Using site-directed mutagenesis, a panel of 12 mutants, each with unique sets of 3 consecutive amino acids mutated to alanine, was created to investigate the significance of the various residues between amino acids 175 and 210 on the GPR37 NT. However, when compared to Δ^{1-175} GPR37, we did not observe enhanced cell surface expression with any of the mutated constructs (Fig II-4).

2.3.5 Head Activator does not detectably activate wt GPR37, ΔNT GPR37, Δ^{1-210}GPR37, or GPR37L1.

The *Hydra* peptide head activator (HA) has been reported to be a ligand that activates and induces internalization of GPR37 (Rezgaoui et al 2006), so we examined whether this peptide could activate wt GPR37 or the truncated GPR37 mutants that

Figure II-3. Structural determinants on the GPR37 N-terminus control receptor surface expression. (A) Representative Western blot of N-terminal truncated constructs. HEK293 cells expressing empty pCMV2b vector, wt GPR37, Δ^{1-35}, Δ^{1-70}, Δ^{1-105}, Δ^{1-140}, Δ^{1-175}, Δ^{1-210}, and ΔNT-GPR37 were harvested, run on an SDS-PAGE gels, transferred and blotted with anti-FLAG antibody. The monomeric species of interest is indicated with a red asterisk to the left of each band. Larger molecular weight species represent aggregated receptor, as is often seen for GPR37. (B) Surface expression of these receptors was determined using a luminometer-based assay. Values are expressed as mean fold increase over wt GPR37 ± S.E.M. One-way ANOVA followed by Dunnett's post-hoc test was used to determine statistical significance. (n= 5; *, p < 0.05; **, p < 0.01) (C) Confocal imaging of HEK-293 cells Δ^{1-210} GPR37 was done using mouse anti-Na^+/K^+ ATPase, followed by Alexa-Fluor 488, to mark the cell surface (green, left panel), rabbit anti-FLAG, followed by Alexa-Fluor 546, to detect the receptors (red, center panel), and DAPI to stain the nucleus (blue, right panel). Yellow indicates colocalization of receptor with plasma membrane (C, right panel).

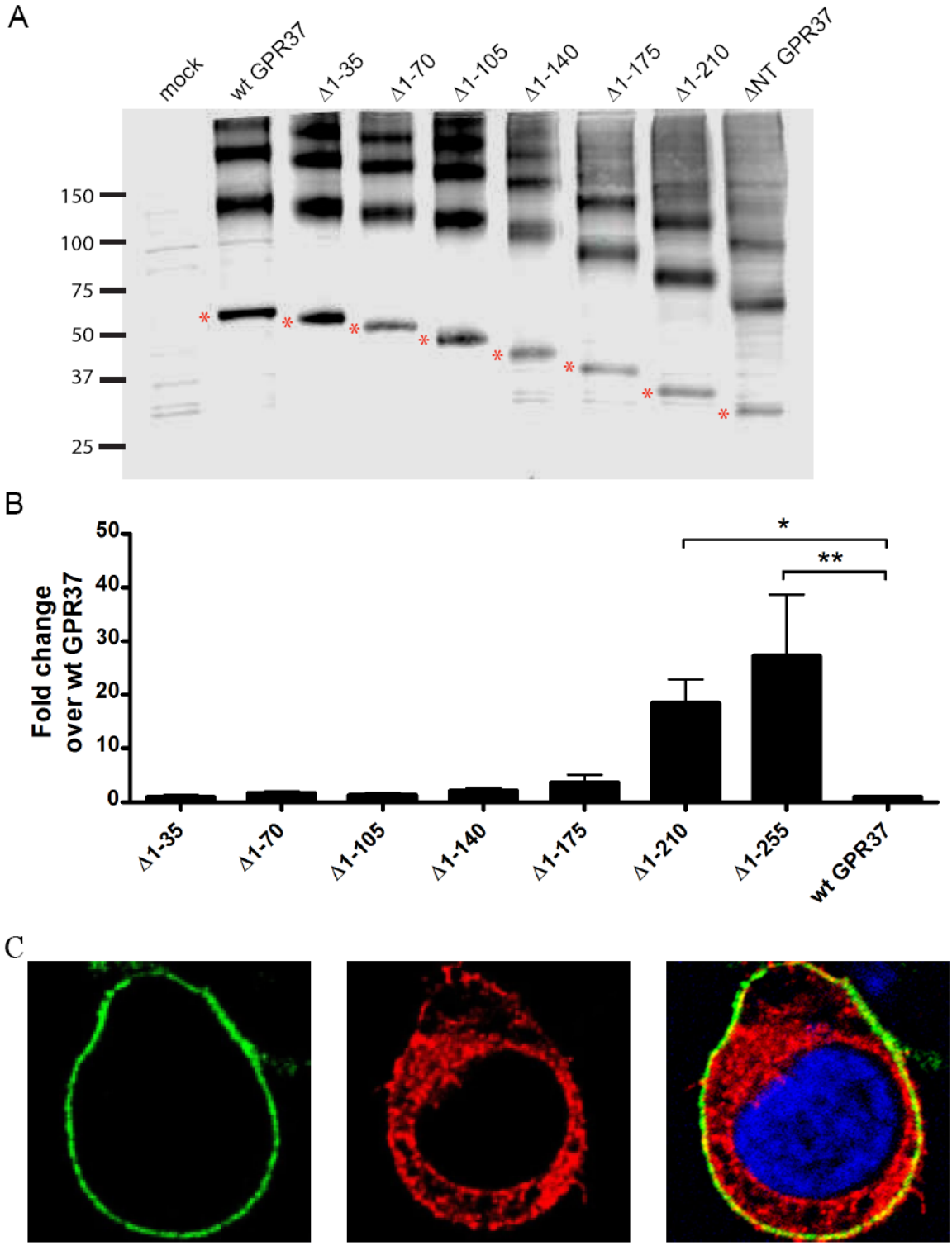

A
mock
wt GPR37
Δ1-35
Δ1-70
Δ1-105
Δ1-140
Δ1-175
Δ1-210
ΔNT GPR37
150
100
75
50
37
25
B
Fold change over wt GPR37
50
40
30
20
10
0
*
**
Δ1-35
Δ1-70
Δ1-105
Δ1-140
Δ1-175
Δ1-210
Δ1-255
wt GPR37
C

Figure II-4. Point mutations created between amino acids 175 and 210 exhibit no effect on GPR37 surface expression. HEK293 cells were transiently transfected with empty vector, wt GPR37, 12 mutant GPR37 constructs with mutations between amino acids 175-210, $\Delta^{1\text{-}175}$, and $\Delta^{1\text{-}210}$ and surface expression was measured using a luminometer-based assay. Values are expressed as mean ± SEM for fold increase over $\Delta^{1\text{-}175}$ GPR37. One-way ANOVA followed by Dunnett's post-hoc test was used to determine statistical significance. (n= 4; *, $p < 0.05$)

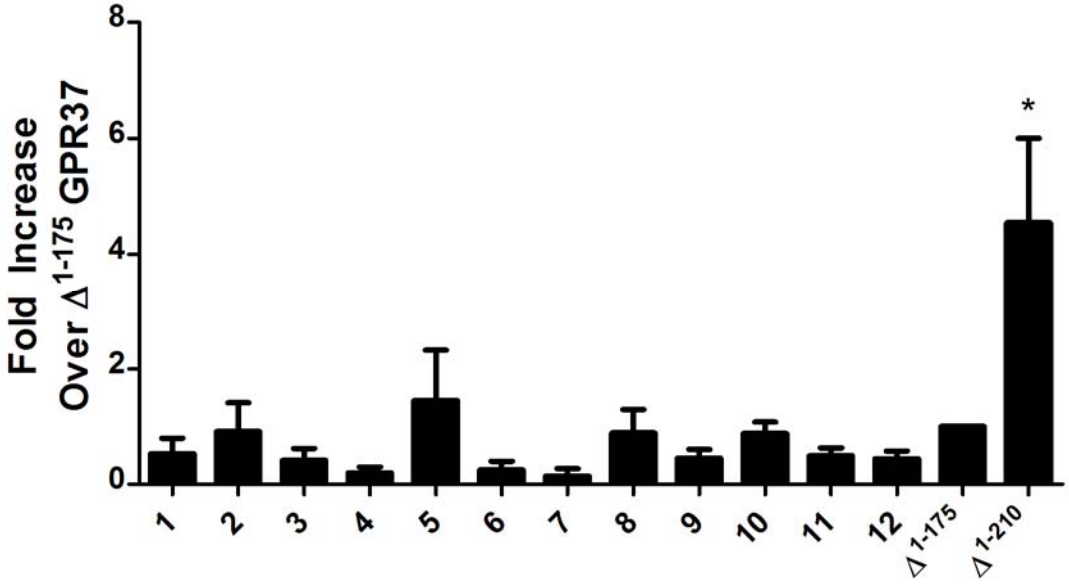
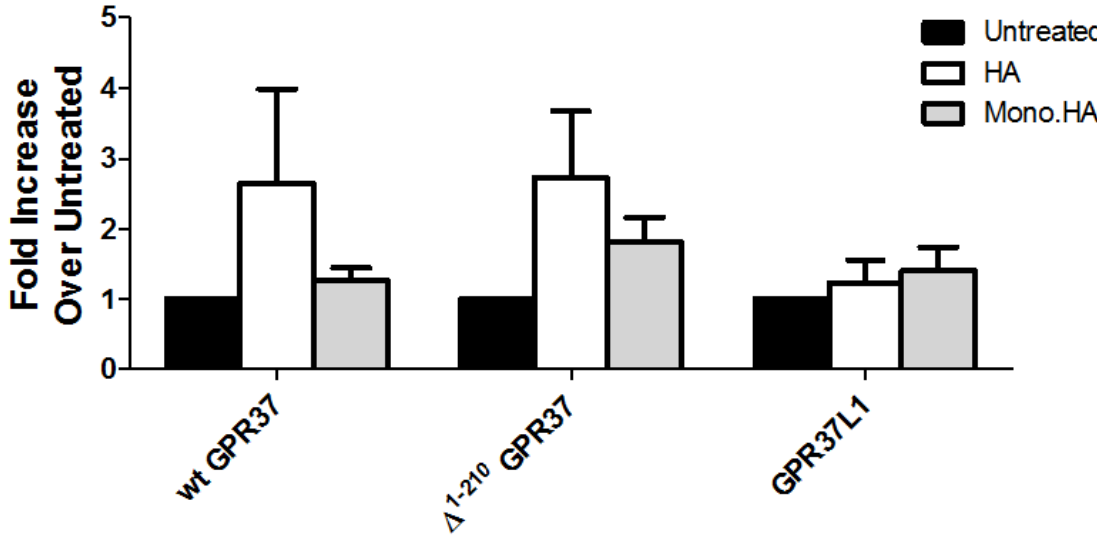

Fold Increase
Over Δ1-175 GPR37
8
6
4
2
0
*
1
2
3
4
5
6
7
8
9
10
11
12
Δ 1-175
Δ 1-210

exhibit superior surface expression. HEK293 cells transfected with wt GPR37, ΔNT, or $\Delta^{1\text{-}210}$ were treated with 3 nM HA for 20 minutes, and luminometer-based surface assays were preformed to measure agonist-induced receptor internalization. Neither wt GPR37 nor the well-expressed ΔNT mutant exhibited any significant change in surface expression upon HA stimulation (data not shown). In further studies, we also increased the concentration of HA, used HA from two different sources, and used the potentially more active monomeric form of HA, but despite the changes in parameters, we again saw no evidence of agonist-induced internalization upon stimulation with HA (Fig II-5*A*).

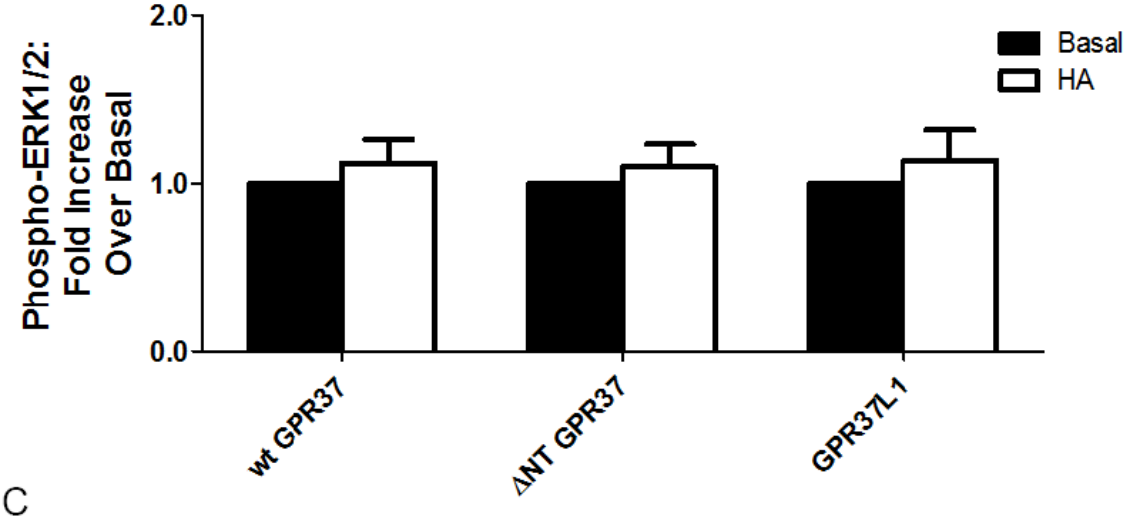
HA stimulation of GPR37 reportedly induces receptor coupling to a pertussis-toxin sensitive G protein, indicating that HA may promote GPR37 coupling to $G\alpha_i$ or $G\alpha_o$ (Rezgaoui et al 2006). To assess this possibility, we measured stimulation of ERK1/2 phosphorylation in GPR37-transfected HEK293 cells. Activation of $G\alpha_i/G\alpha_o$-coupled GPCRs in HEK293 cells typically induces robust phosphorylation of ERK (Luttrell 2005). However, in comparison to cells transfected with empty vector, HA stimulation did not produce any effect on ERK1/2 phosphorylation in cells expressing wt GPR37 or ΔNT GPR37 (Fig II-5B). We also studied inhibition of adenylyl cyclase (AC) activity by stimulating transfected cells with forskolin and/or isoproteronol, in the presence or absence of HA. Again we were unable to discern an inhibition of cAMP following HA treatment (Fig II-5C). Likewise, Ca^{2+} mobilization assays revealed no evidence of GPR37-mediated calcium transients induced by HA (data not shown).

Figure II-5. Head activator does not stimulate GPR37 internalization or signaling. HEK293 cells were transiently transfected with wt GPR37, ΔNT GPR37, Δ^{1-210} GPR37 or GPR37L1. Prior to fixing, cells were treated with 10 μM HA for 20 minutes. Surface expression with and without either form of HA treatment was determined using a luminometer-based assay (A). Values are expressed as mean fold over untreated ± S.E.M. (*n*=3) ("*Mono.*" indicates the monomerized form of HA) ERK activation was measured in cells treated with 3nM HA for 2-10 minutes (B). Values are expressed as mean fold over basal (untreated) ± S.E.M. (*n*=3). Inhibition of cAMP production was measured in cells treated with 1 μM monomerized HA, 100 nM HA and/or 10 μM forskolin/isoproteronol (C). Values are expressed as mean ± S.E.M. for fold over basal cAMP (*n*=4).

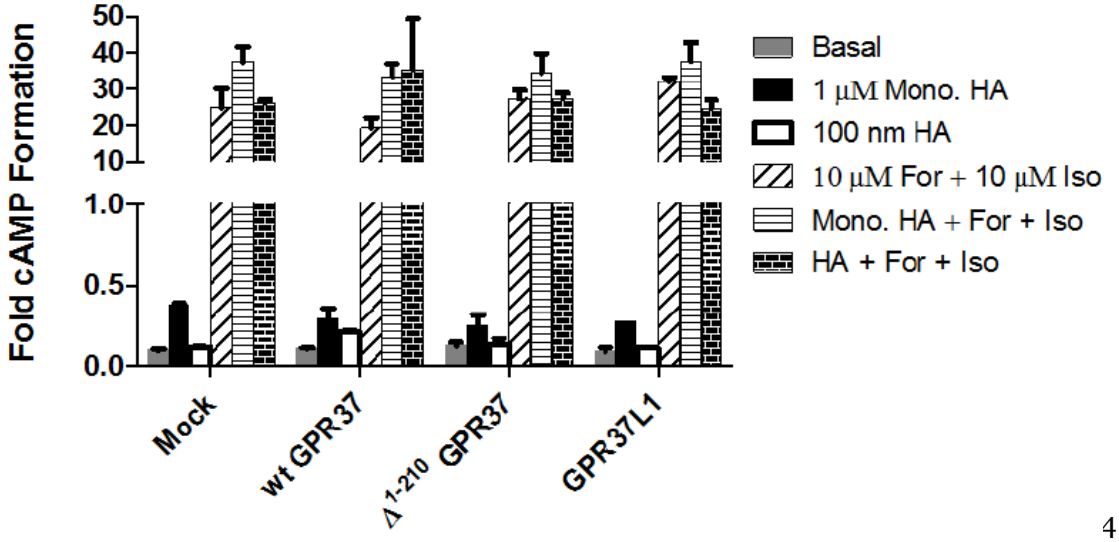
A


Fold Increase Over Untreated
0
1
2
3
4
5
Untreated
HA
Mono.HA
wt GPR37
Δ1-210 GPR37
GPR37L1

B


Phospho-ERK1/2: Fold Increase Over Basal
0.0
1.0
2.0
Basal
HA
wt GPR37
ΔNT GPR37
GPR37L1

C


Fold cAMP Formation
10
20
30
40
50
0.0
0.5
1.0
Basal
1 μM Mono. HA
100 nm HA
10 μM For + 10 μM Iso
Mono. HA + For + Iso
HA + For + Iso
Mock
wt GPR37
Δ1-210 GPR37
GPR37L1

2.4 Discussion

Most GPCRs must reach the plasma membrane in order to achieve proper functional activity. Thus, the identification of ligands for orphan GPCRs can be greatly impeded if the receptors exhibit trafficking defects when expressed in heterologous cells. For this reason, we sought to find ways to enhance surface trafficking of GPR37, an orphan receptor that is well-known to suffer from trafficking defects upon heterologous expression (Imai et al 2001; Rezgaoui et al 2006; Yang et al 2003). In the studies shown in this chapter, we have identified one approach by which GPR37 trafficking to the plasma membrane can be enhanced: truncation of the receptor's N-terminus.

The effects of truncating the GPR37 N-terminus are similar to the effects of N-terminal truncations on the surface trafficking of the α_{1D}-adrenergic receptor (Hague et al 2004a) and CB1 cannabinoid receptor (Andersson et al 2003; Nordstrom & Andersson 2006). For both of these receptors, N-terminal truncations greatly improve receptor surface expression, although it is not certain if the receptors' N-termini possess specific ER retention motifs that are removed by the truncations or if instead there are global difficulties in folding that get resolved through the removal of hard-to-fold regions. There is evidence that the CB1 N-terminus undergoes proteolysis as part of the receptor's post-translational processing in some native cell types (Nordstrom & Andersson 2006), but at present there is no comparable evidence that the α_{1D}-adrenergic receptor or GPR37 undergo proteolysis of their N-termini as part of their processing in native cells.

After observing that N-terminal truncations could greatly enhance GPR37 surface trafficking in heterologous cells, we sought to determine if the surface-expressed truncated mutant versions of GPR37 were functionally active. Since the *Hydra* peptide head activator (HA) has been reported to be an agonist for GPR37 (Rezgaoui et al 2006),

we explored potential HA-mediated stimulation of wt GPR37 and the truncated versions of GPR37. However, in a variety of assays under a variety of different conditions, we were unable to detect any evidence for HA-induced activation of GPR37, GPR37L1 or any of the mutant versions of GPR37 that we had created. It is possible, of course, that truncations to the GPR37 N-terminus might destroy the binding site for HA, but comparable N-terminal truncations to the ET_BR, the most closely-related receptor to GPR37 and GPR37L1, do not disrupt ligand binding (Doi et al 1997; Klammt et al 2007).

In addition, studies of α_{1D}-AR have revealed NT truncation dramatically *increases* the receptor binding site density, as detected by radioligand binding. The ability of truncated α_{1D}-AR to release of intracellular calcium also increased, indicating proper functioning of the receptor as well (Pupo et al 2003). Thus, there is reason to believe that the truncated GPR37 mutants still may be functionally active. As for our studies on full-length GPR37, the discrepancy between the positive findings of Rezgaoui et al. (Rezgaoui et al 2006) and our negative findings for HA stimulation of wt GPR37 might be explained by differences in the cell lines used or other technical factors. Regardless of the explanation, it seems that GPR37 and GPR37L1 should still be considered orphan receptors at the present time, especially since it is not clear that a peptide similar to HA exists in vertebrates. Though there were a handful of papers several decades ago reporting HA-like immunoreactivity in sections of mammalian brains (Bodenmuller & Schaller 1981; Bodenmuller et al 1980; Ekman et al 1990), there have not been any positive follow-up studies in the past twenty years to confirm these early observations.

In conclusion, we have shown that GPR37L1, a close relative of GPR37, exhibits robust surface expression in heterologous cells. We have also shown truncation of the NT (255 amino acids) of GPR37 significantly enhances surface expression of the

receptor, and removing only 210 amino acids has similar effects with possibly less change to the structure and function of the receptor. Thus, if it is assumed that GPR37 and GPR37L1 are activated by the same ligand, or at least related ligands, it seems that GPR37L1 may prove the superior choice for screens attempting to identify the ligand(s) for this orphan receptor pair. However, screens focused solely on GPR37 may benefit from application of N-terminal truncation in order to achieve improved surface trafficking and enhanced functionality of GPR37.

CHAPTER III: Regulation of GPR37 Surface Expression by Protein-Protein Interactions

3.1 Introduction

Though it is acknowledged that not all GPCRs require the presence of their NT to bind ligand or to function properly (Andersson et al 2003; Frielle et al 1989; Sakamoto et al 1993), it is typically preferable to use the wild-type version of a receptor in characterization studies, to have the utmost confidence in translating the results *in vitro* to *in vivo*. For instance, though the orphan receptor GPRC6A is believed to be a promiscuous L-α-amino acid receptor, the work done to come to that conclusion utilized a chimeric receptor, exposing the GPRC6A binding domain on the cell surface while attached to the seven-transmembrane region of the homologous goldfish receptor 5.24 due to an inability of the full-length GPRC6A to properly traffic (Wellendorph et al 2005). Therefore, although activation of the chimeric receptor was clearly observed upon stimulation with basic amino acids, further studies are required to confirm the endogenous ligand for GPRC6A.

In the studies described in the preceding chapter, we made sequential truncations of GPR37 to try to create a well-trafficked mutant of GPR37 that would have robust surface expression, but would not diverge too extensively in its sequence from the full-length receptor. Initially, removal of 255 amino acids, all but 10 amino acids from the NT, revealed an enormous boost in plasma membrane expression. In our follow-up studies, we determined that removal of just 210 amino acids would still provide robust plasma membrane expression, while not creating as severe a change as the entire NT truncation. Unfortunately, no specific amino acid mutations could be identified in the section between amino acids 175 and 210 to allow for creation and use of a mutated full-length GPR37. We still believe that the $\Delta^{1\text{-}210}$ mutant is likely to bind GPR37 ligands and

is therefore a potentially useful tool for receptor characterization; however, to provide more accurate pharmacological characterization of GPR37, we also sought out other ways of enhancing proper trafficking of the full-length receptor to the cell surface.

Some GPCRs are found to have genetic mutations in specific disease states, which makes it difficult for them to properly fold and be processed, and they consequently wind up retained in the ER, leading to disease-related symptoms (Janovick et al 2002; Leanos-Miranda et al 2002; Morello & Bichet 2001; Ulloa-Aguirre et al 2003). It has been shown that certain antagonists for these mutated receptors can be used pharmacologically, allowing for release of the receptors from the ER, thereby significantly improving the patient's condition (Bernier et al 2004a; Bernier et al 2004b; Janovick et al 2003; Janovick et al 2002; Leanos-Miranda et al 2002; Morello et al 2000; Ulloa-Aguirre et al 2003). Presuming this a feasible method for receptors with difficulty trafficking in normal state, we attempted to increase surface expression of GPR37 using an ET_BR-specific antagonist which theoretically is structurally similar to true GPR37 antagonists.

Interactions between GPCRs have also been shown in some cases to strongly influence receptor surface expression, as well as receptor functional activity and pharmacological properties (Ferre et al 2008; Hague et al 2006; Prinster et al 2005). In some cases, it is believed that dimerization could perhaps mask an ER retention motif on a poorly trafficked receptor, thereby allowing for release and plasma membrane trafficking (Prinster et al 2005). In other cases, it seems that the dimer pair alters functionality as well as pharmacology of the individual partners, such as in the case for the TRs, whereby T1R2 + T1R3 heterodimerization is required to form functional sweet taste receptors, but the heterodimerization of T1R1 + T1R3 forms functional umami taste receptors (Nelson et al 2001). In addition, PDZ (postsynaptic density protein, disc large

and zonula occuludens) scaffold proteins also have the capacity to affect surface expression and functional activity of certain receptors (Weinman et al 2006). For instance, the NHERF scaffolds mediate recycling and subcellular localization of the β_2 adrenergic receptor (Cao et al 1999) and also regulates Ca^{2+} signaling of mGluR5 (Paquet et al 2006). Therefore we examined the capacity of GPR37 to associate with PDZ scaffold proteins and other GPCRs. Our studies have revealed that co-expression with certain other receptors enhances GPR37 trafficking to the plasma membrane and that interactions with the PDZ scaffold protein syntenin-1 also strongly promote GPR37 surface expression.

3.2 Experimental Procedures

Materials. Materials were obtained from the following sources: human embryonic kidney 293 (HEK293) cells, ATCC (Manassas, VA); HA-A_{2A}R, HA-A_{2B}R, EE-$G\alpha_{i1}$, GPR37, HA-NPY_1R, HA-NPY_2R, HA-D_2R, HA-D_1R, Missouri S&T cDNA Resource Center (Rolla, MO); Dulbecco's modified Eagle medium (DMEM), Lipofectamine 2000, precast 4-20% Tris-Glycine gels, AlexaFluor 800 goat-anti-mouse antibodies, Alexa Fluor 700 goat-anti-rabbit antibodies, Invitrogen (Carlsbad, CA); anti-FLAG M2 monoclonal antibody, haloperidol, (-)-quinpirole hydrochloride, GDP, GTP, anti-FLAG M2-agarose, butaclamol, dopamine, Sigma (St. Louis, MO); anti-HA 3F10 polyclonal antibody, anti-HA 12CA5 monoclonal antibody, complete protease inhibitors, Roche (Indianapolis, IN); ECL™ anti-mouse IgG, horseradish peroxidase-linked whole antibody, [^{3}H]-spiperone, GE Healthcare (Buckhinghamshire, UK); anti-Na^+/K^+ ATPase antibody, Upstate/Millipore (Billerica, MA); penicillin-streptomycin solution, ScintiSafe™ scintillation fluid, Fisher (Herndon, VA); FBS, Atlanta Biologicals (Atlanta, GA); SuperSignal Elisa Pico ECL reagent, Pierce (Rockford, IL); nitrocellulose membranes, Bio-Rad (Hercules, CA); [^{3}H]-YM-019151-2, [^{35}S]-GTPγS, Perkin Elmer (Waltham, MA); Brandel filters, Brandel Inc. (Gaithersburg, MD); IRL-2500, Tocris (Ellisville, MO).

Cell culture and transfection. HEK293 cells were maintained in DMEM supplemented with 10% FBS, and 1% penicillin-streptomycin solution at 37°C with 5% CO_2. Cells in 10-cm tissue culture dishes at a confluency of 50-60% were transfected with 1-3 μg of cDNA mixed with 15 μl Lipofectamine 2000 in 5ml of serum-free medium. Following a 4-5 hr incubation, complete medium was added to stop the transfection.

The cDNAs used were FLAG-GPR37 in pCMV2b, $\Delta^{1\text{-}210}$FLAG-GPR37 in pCMV2b, 3xHA D_2R, 3xHA-$A_{2A}R$ and $A_{2B}R$, HA-NPY_1R and NPY_2R, and EE-tagged $G\alpha_o$ in pCDNA3.1, untagged DAT in pcDNA3.1 (-)/Neo, and empty pCMV2b vector. All cDNAs used were human.

Surface luminometer assay. HEK293 cells transiently transfected with FLAG-tagged or HA-tagged constructs were split into poly D-lysine-coated 35-mm dishes and grown overnight at 37°C. For internalization assays, ligand was added into incomplete media and placed on cells for 30-60 min at 37°C. The cells were washed with phosphate-buffered saline (PBS + Ca^{2+}), fixed 30 min with 2% paraformaldehyde (PFA), and washed with PBS + Ca^{2+}again. The cells were then incubated in blocking buffer (2% nonfat dry milk in PBS + Ca^{2+}, ph 7.4) for 30 min at room temperature (RT), followed by RT incubation with horseradish peroxidase-conjugated M2-anti-FLAG antibody (1:1000) or 12CA5-anti-HA antibody (1:1000) in blocking buffer for 1 h. For tracking HA-tagged receptors, cells were washed and incubated with anti-mouse IgG, horseradish peroxidase-linked whole antibody (1:4000) 30 min at RT. The cells were washed twice with blocking buffer, washed once with PBS + Ca^{2+}, and incubated with SuperSignal Pico ECL reagent for 15 s. Luminescence of the entire 35-mm dish was determined using a TD20/20 luminometer (Turner Designs).

Western blotting. Samples were resolved by SDS-PAGE on 4-20% Tris-Glycine gels, followed by transfer to nitrocellulose membranes. The membranes were incubated in blocking buffer (2% nonfat dry milk, 50mM NaCl, 20mM HEPES, 0.1% Tween 20) for 30 min and then incubated with primary antibody for either 1 h at RT or overnight at 4°C. Next, the membranes were washed three times in blocking buffer and incubated with

either a fluorescent- or HRP-conjugated secondary antibody for 30 min, washed three times more, and finally visualized using the Odyssey imaging system (Li-Cor) or via ECL reagent followed by exposure to film.

Immunoprecipitation studies. HEK293 cells expressing HA-D_2R with either FLAG-GPR37 or FLAG-Δ^{1-210} GPR37 were harvested by washing once in ice-cold PBS and scraping in harvest buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1 mM benzamidine, protease inhibitor tablet, 1% Triton X-100; pH 7.4). Cell lysates were then solubilized, immunoprecipitated with anti-FLAG M2 affinity resin, and washed by repeated centrifugation and homogenization. Samples were heated, then probed via Western blotting, using anti-FLAG M2 or anti-HA 3F10 antibodies.

Ligand binding studies. For preparation of cell lysates to be used in ligand binding assays, transfected cells grown on 100-mm dishes were rinsed with 2 ml PBS + Ca^{2+}, then starved for 1 hour in 5ml PBS + Ca^{2+}. The cells were scraped into 1 ml of ice-cold binding buffer (20 mM HEPES, 100mM NaCl, 5mM MgCl, 1.5 mM CaCl, 5mM KCl, 0.5mM EDTA, protease inhibitor tablet, pH 7.4). Cells were frozen at -20°C until use. On the day of the assay, cells were thawed and centrifuged at 13,500 rpm for 15 min to separate membranes. Membranes were then resuspended in 1 ml binding buffer, triturated, and incubated with increasing concentrations of unlabeled ligands in the presence of 0.5 nM [^{3}H]-spiperone to generate competition curves. The samples were incubated for one hour at 25°C. Nonspecific binding was defined as [^{3}H]-spiperone binding in the presence of 50μM (+)-butaclamol, and represented less than 10% of total binding in all experiments. Incubations were terminated via filtration through GF/C filter paper, previously soaked in a 0.05% polyethylenemine solution, using a Brandel

cell harvester. On the harvester, filters were rapidly washed three times with ice-cold wash buffer (10 mM HEPES, 50mM NaCl), and radioactive ligand retained by the filters was quantified via liquid scintillation spectrometry. The fitting of curves for one site versus two sites was performed, and goodness of fit was quantified using F tests, comparing sum-of-squares values for the one-site versus two-site fits.

[^{35}S] GTPγS binding assays. Cell membranes (60 μg) derived from cells that had been transfected with EE-tagged G proteins, D_2R ± wt GPR37 were incubated in 200 μl assay buffer (20mM HEPES, 100mM NaCl, 10mM $MgCl_2$, pH 7.4) containing 10 μM GDP, 0.001% saponin, and various concentrations of quinpirole. All experiments were performed in triplicate. The reaction was initiated by the addition of cell membranes and incubated for 30 minutes. A 20 μl volume of [^{35}S] GTPγS (0.1 nM final concentration) was added, and the incubation continued for another 30 minutes. At the end of the incubation, samples were spun down 20 minutes, washed twice with assay buffer, and resuspended in 200 μl H_2O. Each sample was added to 5 ml scintillation fluid and radioactivity was determined by liquid scintillation spectrometry. Data was normalized to maximal response induced by quinpirole.

Purification of Fusion Proteins. Wild-type and mutant (removal of final cysteine) GST-tagged constructs (termed GPR37-CT and GPR37-Mut-CT, respectively) were created by PCR followed by insertion into the pGEX-4T1 vector. Overnight cultures of BL-21 DE Gold cells transformed with pGEX-4T1, pGEX4T-1CT or pGEX 4T-1Mut were diluted 1:143 into 1L of LB broth supplemented with appropriate antibiotics and grown to an optical density (A600) of 0.6-0.7 at 37°C. IPTG was then added to the culture and allowed to incubate for 2 h. Bacteria were pelleted by centrifugation, and the GST,

GPR37-CT-GST, or GPR37-Mut-GST fusion proteins were purified using Sigma GSH agarose. The fusion proteins remained attached to GST agarose for the syntenin-1 pull-down or were eluted for use in the PDZ array. Eluted proteins were concentrated using the Amicon Ultra protein concentration system via centrifugation at 4°C to remove excess glutathione, and the concentration of the purified proteins were determined by QuickStart™ Bradford protein assay according to the manufacturer's protocols (Bio-Rad).

Screening of the PDZ domain proteomic array. To identify novel PDZ domains interacting with GPR37, we screened the PDZ domain array as previously described (Fam et al 2005; He et al 2006). Briefly, 96 different purified PDZ domains were spotted at 1µg per bin onto Nytran SuperCharge 96-grid nylon membranes (Schleicher and Schuell BioScience, Keene, NH). The membranes were allowed to dry overnight and then blocked in blocking buffer for 30 min at room temperature. The arrays were then overlaid with either control GST, GPR37-CT-GST, or GPR37-Mut-GST (100 nm in blocking buffer) overnight at 4°C. The overlaid arrays were washed three times for 5 min each with 20 ml blocking buffer, incubated with anti-GST horseradish peroxidase-conjugated antibody (Amersham, 1:4000) for 1 h at room temperature, washed again three times for 4 min each with 20 ml blocking buffer, and ultimately visualized via chemiluminescence.

Fusion Protein Pull-down Assays. HEK-293 cells transiently transfected with HA-syntenin-1 (kindly provided by Paul Coffer, UMC Utrecht) were harvested in a detergent-free harvest buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, protease inhibitor tablet). The cells were spun down to isolate the membranes, which were then incubated

in harvest buffer containing 1% Triton X-100 for 1h at 4°C with end-over-end agitation. The samples were spun down again to separate the soluble lysates from insoluble material. A sample of the solubilized protein was retained to ensure syntenin-1 expression and solubilization. The remaining lysates were divided evenly and incubated with glutathione agarose beads loaded with GST, GPR37-CT-GST, or GPR37-Mut-GST fusion proteins end-over-end for 1h at 4°C. The beads were washed 5 times with harvest buffer containing 1% Triton X-100 and then heated in sample buffer to strip proteins from the beads. The samples were then analyzed via Western blot for pull-down of syntenin-1 using an anti-HA antibody.

Statistical analysis. All statistical analyses were carried out using Graph Pad Prism software (GraphPad Software Inc., San Diego, CA).

3.3 Results

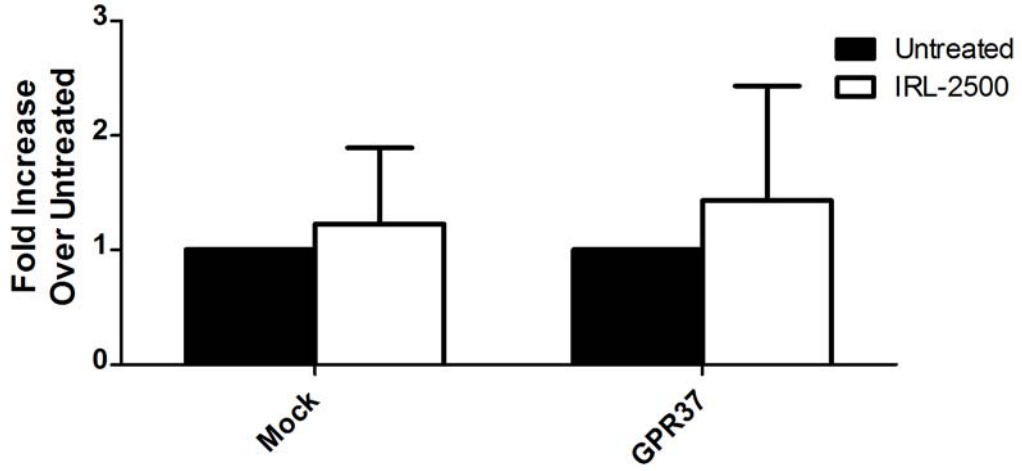
3.3.1 Antagonists of ET_BR do not influence the cellular trafficking of GPR37.

Knowing that specific antagonists are able to improve the surface trafficking of certain ER-retained GPCRs (Bernier et al 2004a; Bernier et al 2004b; Janovick et al 2003; Janovick et al 2002; Leanos-Miranda et al 2002; Morello et al 2000; Ulloa-Aguirre et al 2003), we explored the same idea for GPR37 using a known endothelin receptor antagonist. In HEK293 cells transfected with GPR37, we observed surface expression of the receptor using the luminometer-based surface expression assay with or without treatment of the selective ET_BR antagonist IRL-2500, which we hypothesized might have some ability to bind GPR37. However, we did not observe any shift in surface expression of GPR37 upon ET_BR antagonist stimulation (Fig III-1).

3.3.2 Dopamine receptor D_2R interacts with GPR37.

Studies of other poorly trafficked GPCRs, such as the aforementioned $GABA_BR1$ and α_{1D}-AR have revealed that receptor surface expression can sometimes be greatly enhanced upon co-expression with interacting GPCRs (Prinster et al 2005). GPR37 has been reported to associate with the dopamine transporter (DAT) (Marazziti et al 2007), but there have not yet been any studies of possible GPR37 interactions with other GPCRs. Thus, we co-expressed GPR37 in HEK293 cells with DAT, as well as a handful of GPCRs that possess similar regional expression patterns and quantified GPR37 surface expression. In comparison with GPR37 expressed alone, the luminometer-based surface assay revealed an increase in GPR37 surface expression when co-expressed with the

Figure III-1. ET_BR antagonists do not enhance cell surface trafficking of GPR37. HEK293 cells were transiently transfected with empty pCMV2B or FLAG-tagged GPR37. Cells were treated with IRL-2500 16 hr prior to fixing. Cell surface expression was then determined using a luminometer-based assay. Values are expressed as mean fold over untreated ± S.E.M. (n=3)


Fold Increase
Over Untreated
3
2
1
0
Untreated
IRL-2500
Mock
GPR37

adenosine A_{2A} receptor ($A_{2A}R$), as well as a much larger increase when co-expressed with the dopamine D_2 receptor (D_2R) (Fig III-2A). Neither DAT nor any of the other receptors ($A_{2B}R$ and neuropeptide receptors NPY_1 and NPY_2) had any effect on surface expression of GPR37. To examine whether D_2R might form complexes with GPR37 in cells, we performed co-immunoprecipitation studies, which revealed a robust interaction between GPR37 and D_2R (Fig III-2B-E). Both wt GPR37 (Fig III-2B & 2D) and the Δ^{1-210} mutant (Fig III-2C & 2E) co-immunoprecipitated with both the immature (unprocessed) and the mature (glycosylated) forms of D_2R (lower and upper bands, respectively), indicating the interaction between these two receptors likely occurs in the ER and is maintained after glycosylation. The Δ^{1-210} mutant exhibited a consistently stronger interaction with D_2R than did wt GPR37, probably due to the enhanced plasma membrane expression of the mutant receptor. Therefore, we used the Δ^{1-210} mutant for further studies on the effects of GPR37 on D_2R properties.

Receptor-receptor interactions often modulate the endocytic trafficking of GPCRs (Bouvier 2001; Prinster et al 2005). In some cases, interaction with a partner receptor can inhibit normal agonist-induced internalization of a given GPCR, whereas in other cases, stimulation by the ligand of one receptor can induce an interacting receptor to be co-internalized (Lavoie et al 2002; Uberti et al 2005). To observe the effects of Δ^{1-210} co-expression on the agonist-induced internalization rate of D_2R, and also to assess the possibility of Δ^{1-210} co-internalization upon D_2R agonist stimulation, luminometer-based surface expression assays were carried out. Upon treatment with the D_2R agonist quinpirole for 30 minutes, the individually expressed D_2R internalized by 32 ± 9%. Similarly, when co-expressed with Δ^{1-210} GPR37, quinpirole-stimulated D_2R internalized by 33 ± 2%. Co-internalization of Δ^{1-210} GPR37 was not observed upon co-expression with D_2R and quinpirole stimulation (Fig III-3), and co-immunoprecipitation

Figure III-2. Physical association between D_2R and GPR37. HEK293 cells were transiently transfected with FLAG-tagged GPR37 ± untagged DAT, HA-$A_{2A}R$, HA-$A_{2B}R$, HA-D_2R, HA-NPY_1R, or HA-NPY_2R. (A) Surface expression of GPR37 was determined using a luminometer-based assay. Values are expressed as mean fold over wt GPR37 ± S.E.M. One-way ANOVA followed by Dunnett's post-hoc test was used to determine statistical significance. (n=3-8; *, p <0.05, **, p <0.005) (B-E) Cells were harvested, lysed, and immunoprecipitated (IP) with anti-FLAG antibody. Western blot detection of membrane (M), soluble lysate (L), and IP fractions with anti-FLAG (B&C) or anti-HA antibody (D&E) revealed robust co-immunoprecipitation of D_2R with both GPR37 (B&D) and mutant $\Delta^{1\text{-}210}$ GPR37 (C&E).

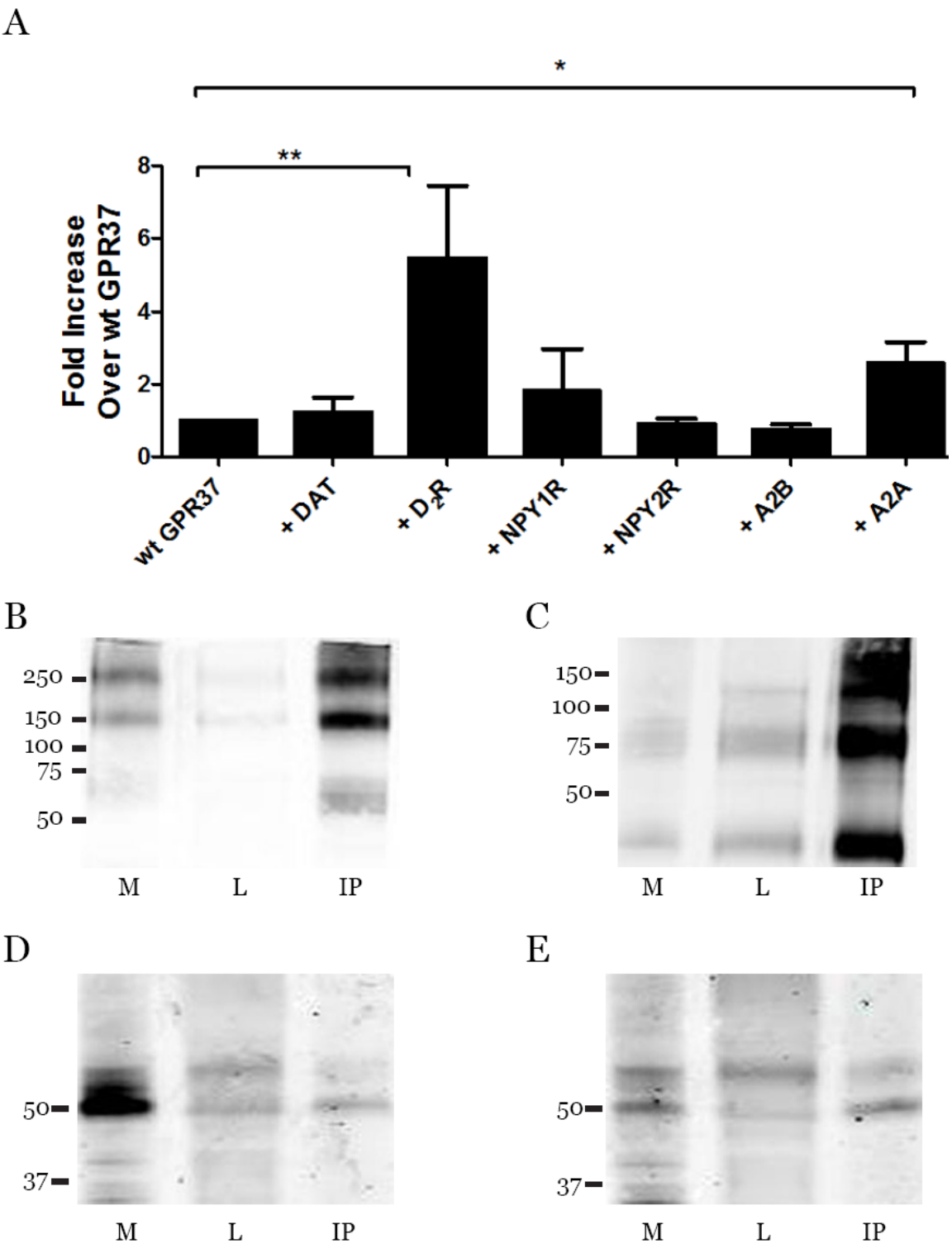
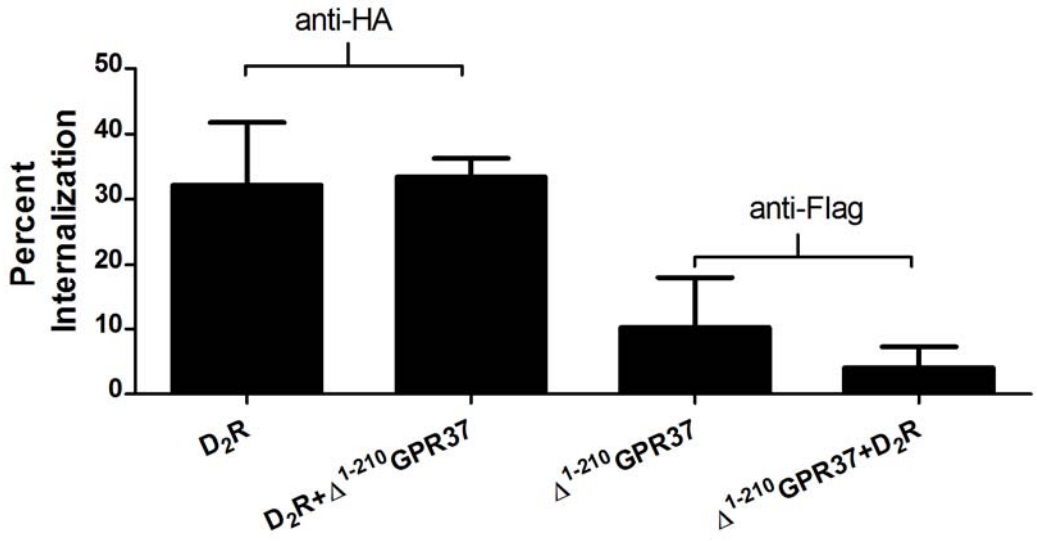

A
*
**
Fold Increase Over wt GPR37
8
6
4
2
0
wt GPR37
+ DAT
+ D2R
+ NPY1R
+ NPY2R
+ A2B
+ A2A
B
250
150
100
75
50
M
L
IP
C
150
100
75
50
M
L
IP
D
50
37
M
L
IP
E
50
37
M
L
IP

Figure III-3. Quinpirole stimulation induces no effect on surface expression of $\Delta^{1\text{-}210}$ or its interaction with D_2R. Surface expression of FLAG-$\Delta^{1\text{-}210}$ GPR37 was measured in the prese nce or absence of HA-D_2R with or without quinpirole treatment (100 μM, 1 hr, 37°C) using a luminometer-based assay (anti-FLAG). In the same way, surface expression of HA-D_2R was detected in the presence or absence of FLAG-$\Delta^{1\text{-}210}$ GPR37 with or without quinpirole treatment (anti-HA). Values are expressed as mean percent internalization ± S.E.M. (*n*=5).


anti-HA
anti-Flag
Percent Internalization
50
40
30
20
10
0
D2R
D2R+Δ1-210GPR37
Δ1-210GPR37
Δ1-210GPR37+D2R

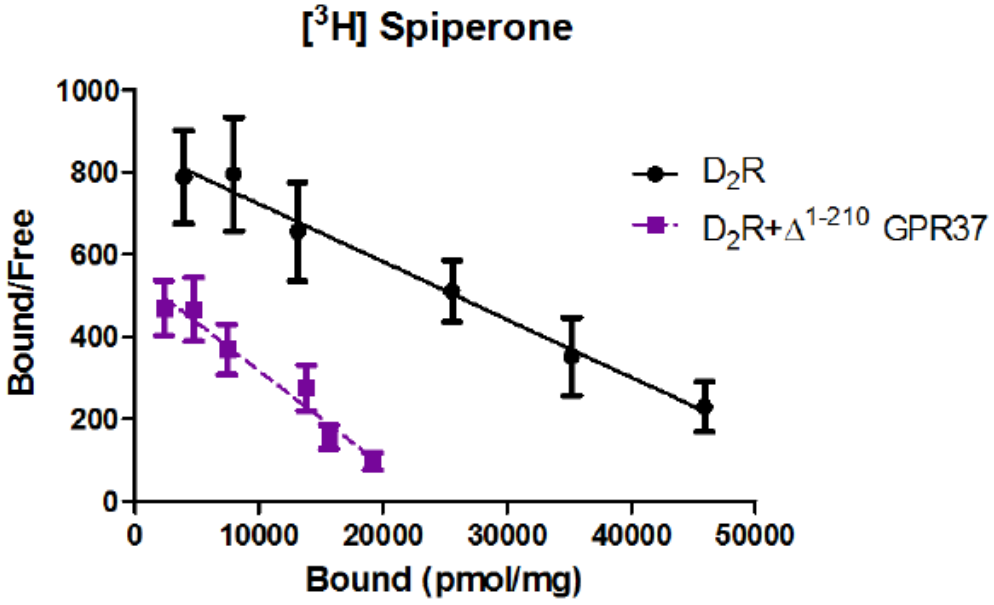
experiments performed in the absence and presence of quinpirole treatment revealed that the interaction between D_2R and GPR37 was unchanged upon agonist stimulation (data not shown). Thus, we found no evidence for agonist regulation of the interaction, and also no evidence that the interaction altered agonist-promoted internalization of D_2R.

3.3.3 Co-expression with Δ1-210 GPR37 alters D_2R ligand-binding properties.

To determine if the physical interaction between GPR37 and D_2R might have effects on D_2R functionality, ligand binding studies were performed using radiolabeled versions of the D_2R antagonists spiperone and YM-09151. Both ligands exhibited modest but significant increases in affinity for D_2R when D_2R was co-expressed with $\Delta^{1\text{-}210}$ GPR37 (Table 1; Fig.III-4A&B). Competition curves were also performed for displacement of [^{3}H]-spiperone binding by unlabeled versions of the D_2R agonists, dopamine and quinpirole, and the D_2R antagonist haloperidol. The affinities of these ligands for D_2R were also somewhat altered when D_2R was co-expressed with $\Delta^{1\text{-}210}$ GPR37, with the magnitude of the change being ligand-specific (Table 1). To investigate whether these differences in ligand binding might correspond to changes in functional activity, [^{35}S]-GTPγS binding studies were performed on membranes derived from cells over-expressing $G\alpha_o$ protein and D_2R in the absence and presence of $\Delta^{1\text{-}210}$ GPR37. However, no significant shift in EC_{50} was observed when $\Delta^{1\text{-}210}$ GPR37 was co-expressed with D_2R, compared to D_2R alone (data not shown).

Figure III-4. D_2R antagonists exhibit enhanced affinity for D_2R in the presence of Δ^{1-210} GPR37. HEK293 cells were transiently transfected with D_2R in the presence or absence of Δ^{1-210} GPR37. (A&B) Scatchard plots for the ligand-binding studies were performed using radiolabeled versions of the D_2R antagonists spiperone (A) and YM-09151 (B). (*n*=3)

A


[3H] Spiperone
Bound/Free
1000
800
600
400
200
0
0
10000
20000
30000
40000
50000
Bound (pmol/mg)
D2R
D2R+Δ1-210 GPR37

B

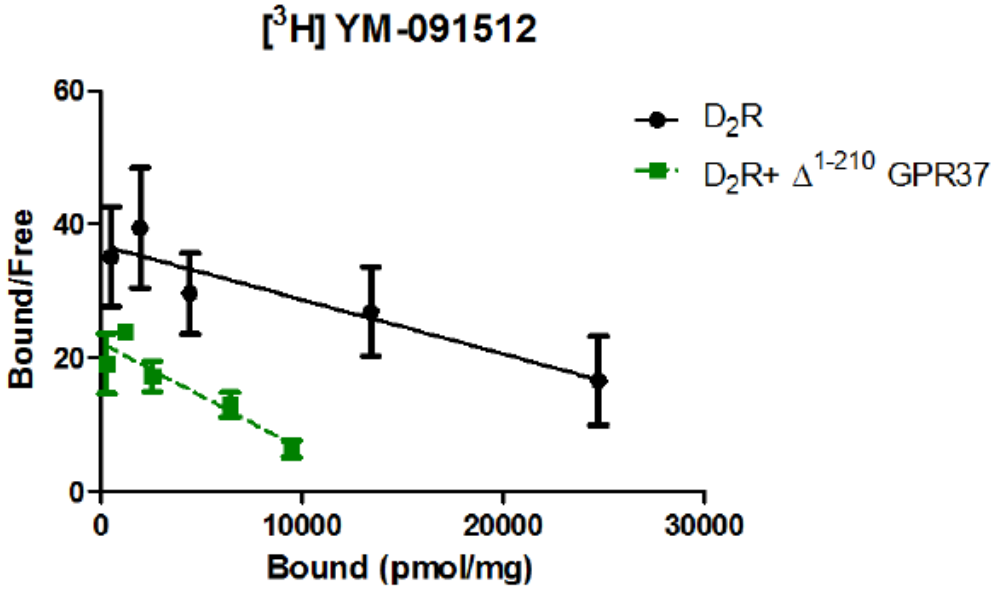

[3H] YM-091512
Bound/Free
60
40
20
0
0
10000
20000
30000
Bound (pmol/mg)
D2R
D2R+ Δ1-210 GPR37

Table III-1. Co-expression with $\Delta^{1\text{-}210}$GPR37 modulates D_2R ligand binding affinity.

	Agonists	K_i ± S.E.M(nM)	Antagonists	K_D/K_i ± S.E.M.(pM)
D_2R alone	**Dopamine**	97 ± 27	**Haloperidol**	1114 ± 86
$D_2R + \Delta^{1\text{-}210}$GPR37		41 ± 10		480 ± 76
D_2R alone	**Quinpirole**	82 ± 38	**Spiperone**	72 ± 20
$D_2R + \Delta^{1\text{-}210}$GPR37		36 ± 9		43 ± 10
D_2R alone			**YM-09151**	1228 ± 567
$D_2R + \Delta^{1\text{-}210}$GPR37				621 ± 193

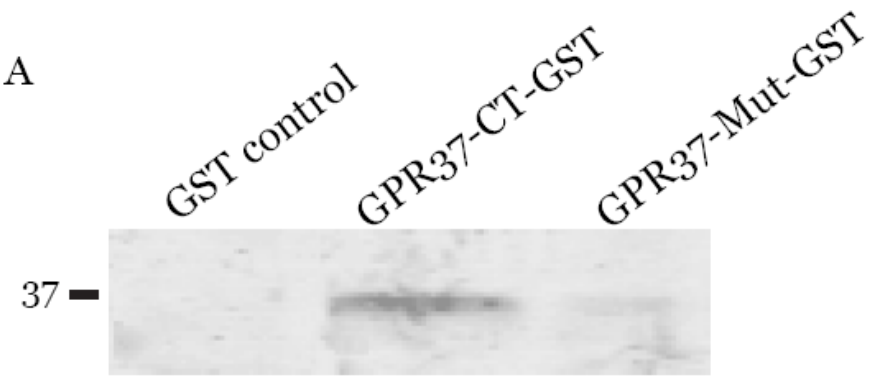
Ligand binding studies were performed, as described in *Materials and Methods*, on membranes derived from HEK293 cells that had been transfected to transiently express HA-D_2R in the absence or presence of FLAG-$\Delta^{1\text{-}210}$GPR37. Estimates of K_i or K_D (± S.E.M.) for each ligand are provided. For the curves of dopamine and quinpirole displacement of [^{3}H]-spiperone binding, two-site fits were not significantly better than one-site fits, and therefore one-site fit values are shown.

3.3.4 Co-expression of GPR37 with syntenin-1 enhances GPR37 surface expression.

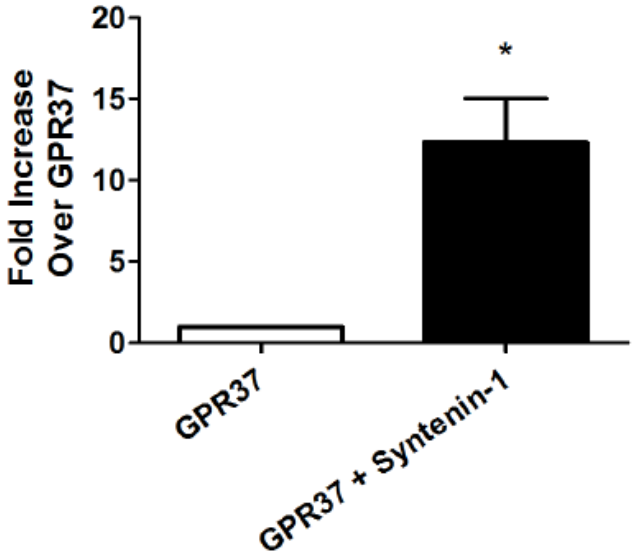
The GPR37 CT possesses a consensus Class 1 PDZ domain-binding motif (G-T-H-C). To examine whether GPR37 might interact via this motif with PDZ domain-containing scaffold proteins, we prepared the GPR37 CT as a GST fusion protein and screened it against a proteomic array of 96 purified PDZ domains (Fam et al 2005; He et al 2006). However, the GPR37-CT-GST fusion protein did not exhibit detectable binding to any of the PDZ domains on the array (data not shown). In addition to this proteomic approach toward searching for GPR37-interacting partners, we also took a bioinformatics approach and noted that GPR37 terminates in precisely the same C-terminal motif as the glycine transporter GlyT2 (G-T-x-C). Since GlyT2 has been shown to interact via this motif with the atypical PDZ scaffold syntenin-1 (Armsen et al 2007; Ohno et al 2004), we specifically examined whether GPR37 and syntenin-1 might interact. As shown in Figure III-5A, pull-down analyses revealed a robust interaction between the GPR37-CT-GST and syntenin-1. This interaction was not seen using a mutated version of the GPR37-CT-GST protein (GPR37-Mut-GST), in which the final cysteine residue had been removed to disrupt the PDZ-binding motif. Moreover, when syntenin-1 was co-expressed with full-length GPR37 in HEK-293 cells, the result was a striking 10-fold increase in the amount of GPR37 that could be detected in the plasma membrane (Fig III-5B). Interestingly, co-expression of syntenin-1 with $\Delta^{1\text{-}210}$ GPR37 still resulted in a three-fold increase in the surface expression of the truncated mutant receptor, revealing that a combination of approaches (truncation of the receptor's NT and co-expression of the receptor with a CT-binding partner) can work synergistically to maximize GPR37 trafficking to the plasma membrane in heterologous cells (Fig III-5C).

Figure III-5. Physical association between GPR37 and syntenin-1. (A) Pull-down studies were performed examining syntenin-1 interactions with control GST, GPR37-CT-GST, and a mutant version of GPR37-CT-GST (GPR37-Mut-GST) with the PDZ-binding motif removed. Robust binding of syntenin-1 was observed only with wild-type GPR37-CT-GST. (B&C) HEK-293 cells were transiently transfected with wt GPR37 (B) or $\Delta^{1\text{-}210}$ GPR37 (C) in the absence and presence of co-expressed HA-syntenin-1. Surface expression of the receptors was determined using a luminometer-based assay. Values are expressed as mean fold increase over receptor alone ± SEM. (n=3-4; *, $p < 0.05$)

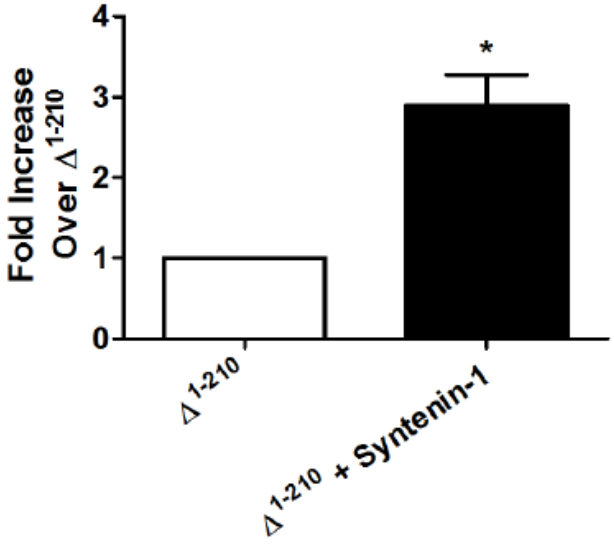
A


GST control
GPR37-CT-GST
GPR37-Mut-GST
37

B


Fold Increase Over GPR37
20
15
10
5
0
*
GPR37
GPR37 + Syntenin-1

C


Fold Increase Over Δ1-210
4
3
2
1
0
*
Δ1-210
Δ1-210 + Syntenin-1

3.4 Discussion

Most seven transmembrane receptors exhibit characteristic coupling to G-proteins to elicit intracellular responses upon activation and have been important pharmacological targets in the discovery of many important drugs. The traditional dogma in regards to this family of receptors is that they all function individually, as monomers, and their functions are individually regulated. Part of this mindset stemmed from the one-track minded goal of initial GPCR research—identify individual genes coding for different individual members of the GPCR superfamily and characterize the individual receptors (Tallman 2000). However, in recent years this idea has been evolving and it is now commonly accepted that many GPCRs function as dimers or even multimers, with subsequently altered expression levels, function, and pharmacological properties depending on receptor-receptor interactions. The gateway to this new school of thought was unlocked when the discovery was made that non-functional $GABA_BR$ subunits required dimerization to properly traffic and function (White et al 1998). This was the first widely accepted example of functional consequence from dimerization of receptors. Since then, though, further evidence has emerged, strongly supporting the case for GPCRs functioning in other ways beyond the assumed monomeric form, with no limitations to specific subfamilies, regional locations, or functionality (Gines et al 2000; Hague et al 2006; Jordan et al 2000; Lavoie et al 2002).

In our studies, we identified two distinct approaches beyond N-terminal truncation by which GPR37 trafficking to the plasma membrane can be enhanced: co-expression with certain other GPCRs and co-expression with the PDZ scaffold syntenin-1. The dopamine receptor D_2R and adenosine $A_{2A}R$ both had notable effects on the plasma membrane expression of GPR37 when co-expressed in HEK293 cells. Interestingly, D_2R and $A_{2A}R$ are known to be capable of functional interactions with each

other and are also known to be found abundantly in the striatum (Fuxe et al 2007), a brain region where GPR37 is highly expressed (Lein et al 2007; Zeng et al 1997). Many studies on cross-talk between D_2R and $A_{2A}R$ have focused on how stimulation of one of the receptors can directly influence the properties of the other partner (Fuxe et al 2007). However, in the absence of a functional ligand for GPR37, such cross-talk studies on the putative heterodimers consisting of GPR37/D_2R and GPR37/$A_{2A}R$ are not possible at the present time. Thus, we focused on determining whether co-expression with GPR37 might alter D_2R properties.

In ligand binding studies, we found that co-expression with GPR37 induced modest shifts in the affinity of D_2R for various ligands. Although these shifts were only in the range of 1.5- to 2.5-fold, the true magnitude of the changes may be underestimated in our studies, since our transfection efficiency was not 100%. Moreover, it is unlikely that there would be 100% efficient co-assembly of the receptors even in cells that were doubly-transfected with D_2R and GPR37. Thus, any observed changes in the properties of the D_2R/GPR37 heterodimer relative to D_2R alone would likely be underestimated in co-expression studies of this type. As for the potential *in vivo* relevance of the GPR37 effects on D_2R properties, it is interesting to note that the affinity of D_2R for [^{3}H]-YM-09151-2 in GPR37 knockout mice is decreased by approximately two-fold (Marazziti et al 2007), which is strikingly consistent with the approximate two-fold increase in D_2R affinity for [^{3}H]-YM-09151-2 that we observed upon co-transfection of D_2R with GPR37. If it is true that associations between GPR37 and D_2R can subtly influence D_2R antagonist binding properties *in vivo*, this is a point of significant clinical interest given the widespread use of D_2R antagonists in treating schizophrenia (Strange 2008). Since GPR37 is co-expressed *in vivo* with D_2R in some neuronal populations but not others (Lein et al 2007), it may be possible to develop D_2R antagonists with enhanced regional

selectivity by developing compounds that preferentially target the D_2R/GPR37 complex relative to D_2R alone (or vice versa).

The second approach that we found to result in enhanced surface expression of GPR37 was co-expression with the PDZ scaffold syntenin-1. The interaction of GPR37 with syntenin-1 was quite specific, as screens of a proteomic array consisting of 96 other PDZ domains with the GPR37-CT did not reveal detectable interactions with any other PDZ domains. GPR37 terminates in a motif, G-T-x-C, that is identical to the motif found at the C-terminus of the syntenin-1 binding partner GlyT2 (Armsen et al 2007; Ohno et al 2004). For GlyT2, the primary functional consequence of interaction with syntenin-1 is enhanced trafficking to synapses (Armsen et al 2007). For several other syntenin-1-interacting proteins, including pro-transforming growth factor α (Fernandez-Larrea et al 1999), CD63 (Latysheva et al 2006) and the Notch ligand Delta1 (Estrach et al 2007), co-expression with syntenin-1 has been shown to markedly enhance trafficking to the plasma membrane, similar to the effects on GPR37 that we observed in our studies. Interestingly, syntenin-1 is known to be highly expressed in oligodendrocytes (Chatterjee et al 2008), the cell type in which GPR37 is most abundantly expressed (Imai et al 2001). Since GPR37 is an orphan receptor, it is not possible at the present time to test whether the GPR37/syntenin-1 association has effects on the receptor's functional properties, but the dramatic effects of syntenin-1 on GPR37 surface expression my prove useful in screens for potential GPR37 ligands that would allow for deorphanization of the receptor.

In summary, we have elucidated two distinct approaches by which the trafficking of GPR37 to the plasma membrane can be enhanced. Screens focused on GPR37 may benefit from application of one or both of the approaches described here – co-expression with partner receptors and/or co-expression with syntenin-1 – in order to achieve

improved surface trafficking and enhanced functionality of GPR37. Moreover, the observed synergistic effect of co-expression with syntenin-1 with the truncated form of GPR37 demonstrates that combinations of these approaches can prove useful in studies of GPR37. Furthermore, when more is known about the ligand binding and signaling capabilities of GPR37, the interactions described here between GPR37 and other receptors and GPR37/syntenin-1 may eventually help to shed light on the regulation of GPR37 functional activity *in vivo*.

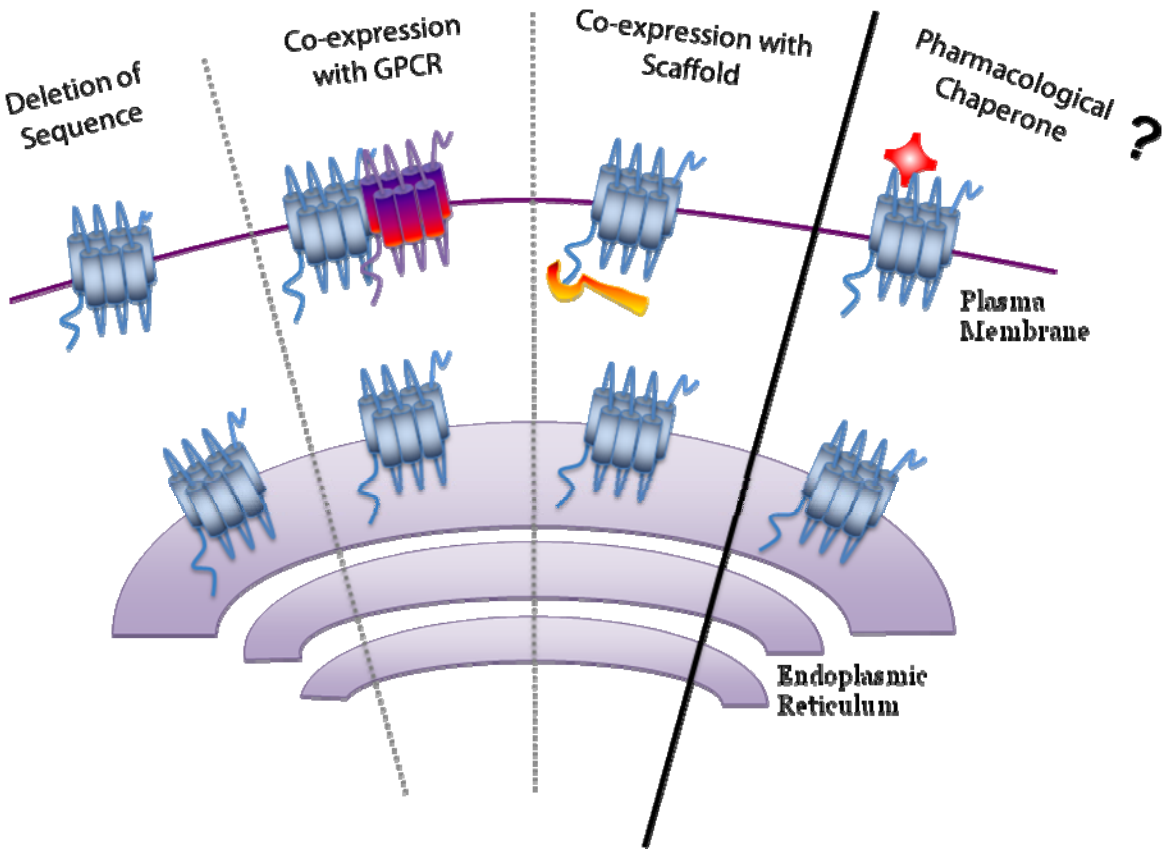
Chapter IV: Conclusion

The studies described in the preceding chapters elucidate three different approaches that can aid in GPR37 trafficking to the plasma membrane: truncation of the receptor's N-terminus, co-expression with other GPCRs (specifically D_2R and $A_{2A}R$), and co-expression with the PDZ scaffold syntenin-1 (Fig IV-1). In addition, comparisons between GPR37 and its closest relative GPR37L1 revealed robust surface expression of GPR37L1 at the plasma membrane in heterologous cells, but not of GPR37. Little has been published in regards to GPR37L1, but its strong surface expression in the studies shown here indicates it should be easier to study in heterologous systems than GPR37. Given the striking similarity of the two receptors, it seems likely that achieving a better understanding of the signaling or ligand binding of GPR37L1 would lead to insights about GPR37 function.

We determined that the first 210 amino acids of the GPR37 NT are responsible for the intracellular retention observed in heterologous cells, but we were unable to tease out any specific amino acids between amino acids 175 and 210 that may be involved in its anterograde trafficking. Interestingly, a number of GPCRs, including ET_BR, which is the orphan pair's closest relative, do not require their entire NT for proper agonist binding or function (Doi et al 1997; Frielle et al 1989; Klammt et al 2007; Meunier et al 2000). Therefore, the $\Delta^{1\text{-}210}$ mutant may still be functionally active and, thus, another useful tool for further analysis and characterization of GPR37. The neuropeptide head activator has been reported to activate GPR37 in a variety of cell lines and oocytes (Rezgaoui et al 2006). However, in our hands, we were unable to detect any evidence for HA-induced activation of GPR37, GPR37L1, or any of the mutant versions of GPR37. Therefore, we cannot say for certain whether or not the $\Delta^{1\text{-}210}$ mutant is active, but based on the analogy with endothelin receptors, the $\Delta^{1\text{-}210}$ mutant is likely to be a useful tool in future studies on GPR37 in heterologous cells.

Figure IV-1. Experimental methods for enhancement of GPR37 surface expression.

When expressed in heterologous cells, GPR37 exhibits poor plasma membrane trafficking, which we determined to be enhanced via three distinct mechanisms: truncation of a portion of the N-terminus (deletion of sequence), co-expression with other GPCRs (notably D_2R and $A_{2A}R$), and co-expression with scaffolding proteins (notably syntenin-1). Though no ligand is currently known, the far right panel indicates the possibility that further research can lead to the discovery of one or more pharmacological chaperones that may be used to release GPR37 from the endoplasmic reticulum and allow for enhanced trafficking of GPR37 to the plasma membrane.

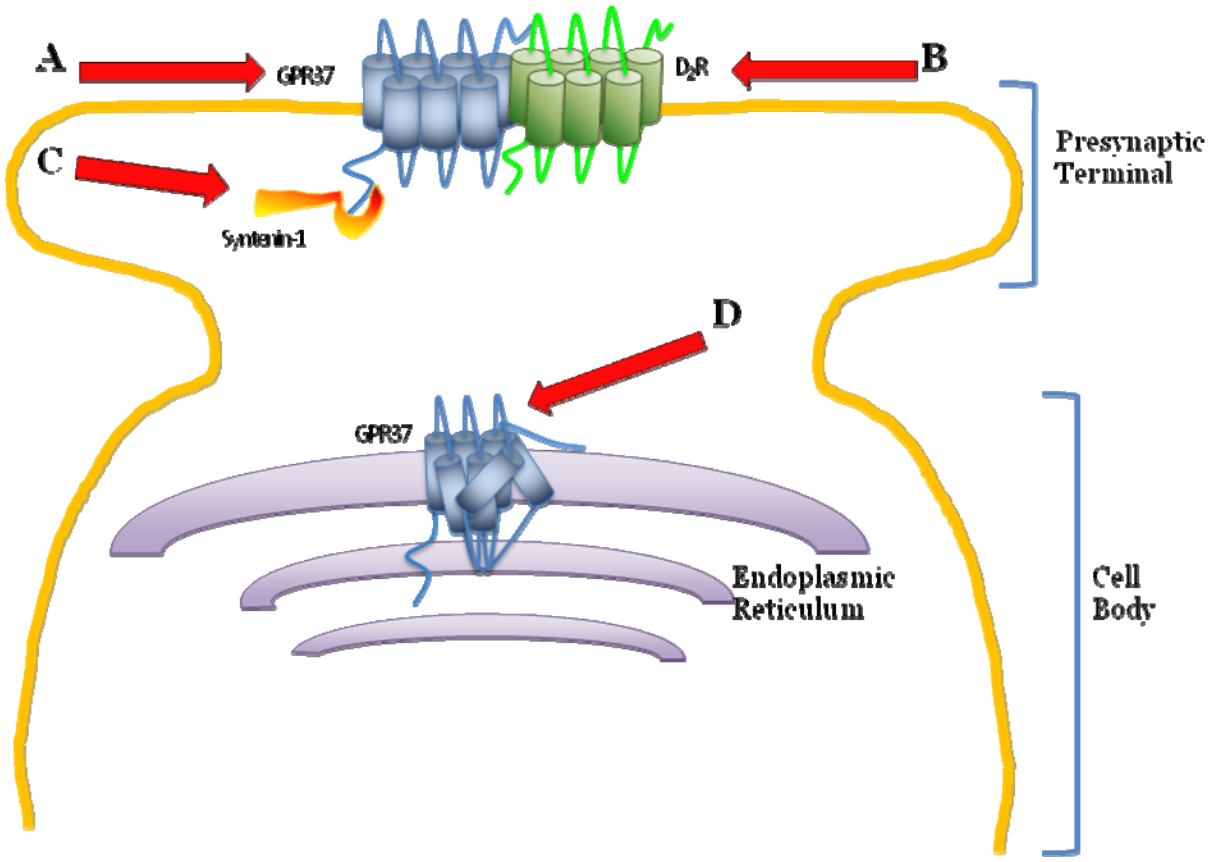

Deletion of Sequence
Co-expression with GPCR
Co-expression with Scaffold
Pharmacological Chaperone
?
Plasma Membrane
Endoplasmic Reticulum

We found a novel interaction between GPR37 and D_2R when the two receptors are heterologously co-expressed. This interaction positively influences the forward trafficking of GPR37, such that the surface expression of GPR37 is significantly enhanced when co-expressed with D_2R. In ligand binding experiments, we observed a modest increase in affinity of D_2R for both agonists and antagonists in the presence of full-length or truncated GPR37 as compared to D_2R expressed alone. Interestingly, D_2-like receptors are pre-synaptic and found mostly in the striatum (Lein et al 2007; Mizuno et al 2007). GPR37 has been shown to be located presynaptically, and is distributed widely throughout the brain, including significant expression in the striatum (Lein et al 2007; Marazziti et al 2007). Therefore, there is a reasonable possibility that these receptors could also interact *in vivo*. In fact, when measuring the B_{max} and K_D of the specific D_2R antagonist [^{3}H]- YM-09151-2 in striatal tissue of GPR37 KO mice, there was an observed two-fold decrease in affinity in comparison to tissue from wt animals (Marazziti et al 2007), which is remarkably in accordance with our observations *in vitro*.

Receptors that can subtly modulate the properties of other receptors, such as the ability of GPR37 to increase the D_2R affinity for certain agonist and antagonist may be viewed as a novel class of pharmacological targets. The dopaminergic system is one of the most highly targeted systems for a number of different diseases and disorders, including depression, schizophrenia, Parkinson's disease, hyperactivity, acute delirium, and alcohol withdrawal (Fuxe et al 2008; Lan et al 2009; Lee et al 2007). One of the major targets in this system is D_2R, but due to the receptor's widespread expression, D_2R-related treatments often cause many severe adverse side-effects, such as drug-induced parkinsonism and akathisia, which are a hindrance to patient compliance (Buckley 1999; Jackson et al 2008). These effects are most highly associated with the use of haloperidol, a conventional neuroleptic or first-generation antipsychotic. Such

potent drugs have mostly been replaced by the use of second-generation antipsychotics, due to their characteristic lack of extrapyramidal side effects at clinically effective doses (Bonham & Abbott 2008). However, these so-called atypical antipsychotics are not all more efficacious than haloperidol—some are less so—and they all come with their own host of adverse side-effects, including sedation, hyperglycemia, and significant weight gain, resulting in an increased risk for diabetes and cardiovascular difficulties (Ananth et al 2004; Leucht et al 2009), which also result in patient non-compliance (Leucht et al 2008). Thus, if various interacting partners of D_2R, such as GPR37, could be targeted to alter the affinity of haloperidol or other D_2R antagonists in only specific regions of the brain, the prevalence of debilitating side effects may be reduced and the quality of the treatment increased (Fig IV-2A). This concept is already beginning to be applied elsewhere, as in the case for treatment of PD or schizophrenia. Stimulation of $A_{2A}R$ and/or mGluR5 simultaneously with D_2R stimulation alters the affinity of D_2R for its ligands (Fuxe et al 2008). Thus, a drug cocktail targeting one or both of these receptors concomitantly with D_2R drugs could lower the necessary dose of L-Dopa, reducing the extrapyramidal side effects and possibly providing neuroprotective effects as well. In theory, agonists of those adenosine and glutamate receptors also may assist in treatment of schizophrenia by reducing or eliminating the need for D_2R antagonists (Fuxe et al 2008). Given that GPR37 already plays a role in the pathology of PD and, based on our trafficking studies, appears also to have a relationship with $A_{2A}R$, it is possible that GPR37 could be another beneficial target in the treatment of these diseases (Fig IV-2A). Ideally, one might specifically target the GPR37/D_2R heterodimer with ligands for both D_2R and GPR37 (Fig IV-2A&B), but currently the pharmacological properties of the pair upon co-stimulation are impossible to study due to the lack of known ligands for GPR37.

Figure IV-2. Possible therapeutic targets involving GPR37 and its interacting partners. Many orphan GPCRs have the potential to be therapeutic targets, and our studies on GPR37 have suggested several different ways in which this receptor may be targeted in the future for therapeutic benefit. (A) By targeting GPR37 with a ligand, dopaminergic activity could be altered in certain brain regions, resulting in lower doses of dopaminergic treatments necessary for diseases such as Parkinson's disease or schizophrenia. (B) Since our studies have revealed that D_2R can have altered pharmacological properties when co-expressed with GPR37, drugs acting preferentially at the D_2R/GPR37 heterodimer may have improved regional specificity and/or alternate downstream effects relative to drugs preferentially targeting D_2R alone, which could make such compounds therapeutically desirable. (C) Syntenin-1 or the PDZ motif on the CT of GPR37 could be a target to modulate anterograde trafficking of GPR37, thereby controlling GPR37 in a manner that might be clinically useful. Additionally, the PDZ motif of GPR37 could possibly be a target to help prevent self-aggregation of GPR37, analogous to current strategies targeting aggregates of α-synuclein as novel approaches to PD treatment (Bodles et al 2004). (D) Pharmacological chaperones for GPR37 may be used to target the misfolded GPR37 stuck in the ER, allowing for release of the receptor from the ER, reduced neuronal death, and a slowed progression of PD.


A
GPR37
D2R
B
C
Synteinin-1
Presynaptic Terminal
D
GPR37
Endoplasmic Reticulum
Cell Body

Our studies have also revealed a novel, specific interaction between the PDZ scaffold syntenin-1 and GPR37. GPR37 terminates in the amino acids G-T-x-C, a motif identical to that found on the C-terminus of the glycine transporter GlyT2, which also interacts with syntenin-1 (Armsen et al 2007; Ohno et al 2004). For GlyT2 (Armsen et al 2007), as well as other interacting proteins pro-TGFα (Fernandez-Larrea et al 1999), CD63 (Latysheva et al 2006), and the Notch ligand Delta1 (Estrach et al 2007), syntenin-1 co-expression markedly enhances trafficking to the plasma membrane. We observed the same effect on GPR37, with about a ten-fold increase of GPR37 plasma membrane expression when co-expressed with syntenin-1. Interestingly, both syntenin-1 (Chatterjee et al 2008) and GPR37 (Imai et al 2001) are known to be expressed in oligodendrocytes; therefore this is another interaction that potentially exists *in vivo*. Similar to the aforementioned GPCR heterodimers, many receptor-scaffold interactions seem to be enhanced via agonist stimulation (Hall & Lefkowitz 2002). Unfortunately, with no known GPR37 ligand, the functional properties of GPR37 cannot be studied in regards to this association, but syntenin-1 may be yet another useful tool for the future characterization and deorphanization of GPR37. Additionally, the GPR37/syntenin-1 interface may prove itself to be a valuable drug target (Fig IV-2C), as targeting domains that mediate protein-protein interactions is an increasingly popular idea in drug development (Dev 2004).

PD is widely believed to be caused by a combination of toxin exposure and genetic factors (Lang & Lozano 1998). Environmental toxins can cause oxidative stress in dopaminergic neurons, which leads to ER stress and the misfolding of certain proteins that are prone to aggregation. The buildup of the toxic aggregates can induce further ER stress and cellular damage in a feed-forward loop, which may be why brain tissue from PD patients shows clear signs of ER stress (Takahashi & Imai 2003) and why genetic

mutations to proteins that control ER-associated degradation induce early-onset forms of PD (Schulz 2008). Long-term treatment of PD with Levodopa, the most common treatment for PD patients, comes with complications and adverse side effects, including its ability to auto-oxidize, leading to ROS production and exacerbating the disease progression (Zhou et al 2008). It also only alleviates the symptoms of PD, rather than treating or stopping the progressive loss of neurons. Thus, a great need exists for new treatments that are able to slow or stop the progression of disease. One might envision a cocktail of therapeutics, to both limit oxidative stress and alleviate ER stress induced by misfolded, aggregated proteins.

GPR37 is not the only substrate of parkin, but it was one of the first to be discovered and one of only two parkin substrates along with the programmed cell death-2 isoform 1 (PDCD2-1) (Fukae et al 2009) that has been shown to accumulate in brains of AR-JP patients (Imai et al 2001; Murakami et al 2004). Furthermore, overexpression of GPR37 in rats (Dusonchet et al 2009), mice (Imai et al 2007; Wang et al 2008) and flies (Yang et al 2003) greatly potentiates the death of dopaminergic neurons, and GPR37 KO mice are resistant to MPTP-induced killing of dopaminergic neurons (Marazziti et al 2004). The weight of this evidence suggests that GPR37 is somehow involved in PD pathology. Therefore, pharmacological chaperones that reduce misfolding of GPR37 are logical candidates for novel therapeutics in treating PD (Fig IV-D). Pharmacological chaperones, which are oftentimes antagonists or allosteric modulators of receptors, have proven to be useful in treatments for disease states caused by misfolded receptors (Bernier et al 2004b; Bernier et al 2006; Janovick et al 2002; Leanos-Miranda et al 2002; Morello et al 2000; Noorwez et al 2003; Ulloa-Aguirre et al 2003). The mechanisms by which such chaperones can promote forward trafficking of misfolded receptors are not entirely understood, but it seems that in many cases, the ER-

retained receptors either achieve enhanced folding upon chaperone binding or are masked from the ER quality control, enabling the receptors to leave the ER and eventually reach the cell surface. In our efforts with an ET_BR antagonist and 4-PBA, a fatty acid seen to act as a chaperone (Jin et al 2007) and reported to reduce GPR37-induced stress (Kubota et al 2006), we were unable to promote the forward trafficking of GPR37. However, when more is understood about the ligand binding and functionality of GPR37, it is possible that targeting the receptor pharmacologically to reduce its misfolding, perhaps in combination with additional pharmacological chaperones to target other misfolded proteins that contribute to PD pathology, could represent a potential novel approach to treatment of PD (Fig IV-1).

In conclusion, we have demonstrated that GPR37 trafficking is controlled by an N-terminal domain that, when removed, allows for ER release and robust cell surface expression of the receptor. We also identified a novel interaction between GPR37 and the dopamine D_2R, in which D_2R ligand affinity is altered upon co-expression of GPR37. This receptor pair may prove to be a valuable target for antipsychotic agents, used in diseases and disorders such as schizophrenia, alcohol withdrawal, and Parkinson's disease. Finally, we have elucidated a novel interaction between GPR37 and the scaffold protein syntenin-1, which, together with truncation of the receptor, can synergistically enhance the surface expression of GPR37. The findings reported here may ultimately lead to enhanced understanding of GPR37 functional activity *in vivo* and insights into the potential role of GPR37 in the etiology of PD, which can lead to potential new therapies for PD treatment.

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