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Ryan H. Purcell Date

Activation and regulation of the brain-expressed adhesion G protein-coupled receptors ADGRB1/BAI1 and ADGRB2/BAI2: Implications for human disease

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Neuroscience Program

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B.A., Johns Hopkins University, 2009

Advisor: Randy A. Hall, Ph.D.

An abstract of

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James T. Laney School of Graduate Studies of Emory University

In partial fulfillment of the requirements for the degree of

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Abstract

Activation and regulation of the brain-expressed adhesion G protein-coupled receptors ADGRB1/BAI1 and ADGRB2/BAI2: Implications for human disease

By Ryan Herndon Purcell

The adhesion G protein-coupled receptors (GPCRs) are the second-largest family of GPCRs in the human genome. Most adhesion GPCRs are considered to be orphan receptors with few widely-accepted natural ligands. However, the unique architecture and common domain structures of these receptors invites the hypothesis that they may have a common activation mechanism. While the activation and signaling mechanisms of these receptors largely remain enigmatic, even less is understood in terms of how these proteins are regulated.

The first third of this dissertation focuses on progress in understanding the activation mechanisms of the adhesion GPCR BAI1/ADGRB1 (B1). We found that removing almost the entire extracellular amino terminus of the receptor has no negative effect on the signaling activity of the receptor, with the heavily truncated receptor being recognized by β-arrestins and G proteins as an active receptor conformation. Thus, contrary to the tethered cryptic agonist model that has been proposed based on studies of several other aGPCRs, activation of B1 does not appear to require a tethered cryptic agonist.

Next, we applied similar methods to study a human disease-associated mutation in the closely-related receptor BAI2/ADGRB2 (B2). This mutation was found in an adult female patient who has suffered from a degenerative neuromuscular condition since adolescence. We found that this arginine to tryptophan (R1465W) substitution in the intracellular C terminus of B2 significantly increases the receptor's signaling output, but only in the active form of the receptor that has a truncated N-terminus (B2ΔNT). This mutation also increases receptor surface expression, and our results suggest two mechanisms underlying these differences: increased flexibility of G protein coupling and disruption in the receptor's interaction with the endocytic protein endophilin A1.

In the final third of this dissertation, studies are presented on regulation of B1 signaling by C-terminal determinants. We found that deletion of the B1 proline-rich region drastically biases the receptor's signaling activity by eliminating G protein-dependent signaling to SRF-luciferase and greatly potentiating activity to NFAT. Finally, we report on the identification of three mutations in B1 that were discovered in individuals with schizophrenia. One of these mutations is in a similar location as the B2-R1465W, is also an Arg→Trp substitution, and greatly increases receptor activity.

Taken together, these studies provide a deeper understanding of two adhesion GPCRs in terms of their signaling and regulation. Moreover, the findings described here provide a basis to connect the activity of these receptors to human diseases and set the stage for the eventual targeting of these receptors by therapeutics.

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Table of Contents

List of Figures:

Chapter 1. Introduction

1.1 Cell surface receptors

Cells need to sense their extracellular environment in order to survive and communicate with other cells. Signals from outside, such as neurotransmitters, hormones, ions, and sensory stimuli, must be received and the physiological messages they convey must be transduced inside the cell. The mammalian cell membrane is teeming with transmembrane proteins, including ion channels, adhesion molecules, and receptor proteins, which allow for the decoding of extracellular signals. Among these transmembrane proteins, one class of receptors is the largest gene family in the mammalian genome with 800 individual members in humans: G protein-coupled receptors (GPCRs).

Interestingly, the concept of a specific receptor for a biologically-active molecule was once very controversial (Lefkowitz 2004, Maehle, Prull, and Halliwell 2002). Today, we take for granted that cell membrane-impermeable molecules exert their action through integral membrane receptor proteins but early work on receptors met significant resistance. It was John N. Langley who in the early 20th century (Fig. 1) first described the idea of a "receptive substance" in an effort to understand how poisons such as curare and nicotine act on muscle preparations (Langley 1905). Paul Ehrlich, who initially studied "side chains" in the context of antibodies and immunology, expanded his thinking by 1907 to rename them as "chemoreceptors" and believed that both toxic substances and drugs could interact at these sites (Maehle, Prull, and Halliwell 2002, Parascandola 1980). In fact, Ehrlich formalized his ideas about specific receptors to explain his early observation of drug-resistance in microorganisms (Parascandola 1980). Working with

three different classes of chemical dyes that are toxic to parasitic trypanosomes, Ehrlich found that the organisms that were resistant to one dye were resistant to other dyes in that class, but not to those in a separate chemical class. His explanation for this phenomenon was that the drugs only act by combining with a chemoreceptor and that a trypanosome strain could display resistance to a class of drugs if it lacked a certain chemoreceptor or if that chemoreceptor had decreased affinity for that particular drug (Parascandola 1980).

By the early 1920's, Langley had postulated that there are at least two main classes of receptive substances which can either inhibit or induce muscle contraction (Maehle, Prull, and Halliwell 2002). Another key figure, A.J. Clark, rejected prominent pseudoscientific trends of the time and pushed the field toward more quantitative analysis. In the early 1930s, Clark proposed that drugs reversibly interact with receptors and that the effect is dependent in part on the number of receptors bound (Clark 1933, Kenakin 2004, Maehle, Prull, and Halliwell 2002). However, it was not until around 1950 that receptor pharmacology truly began to mature (Parascandola 1980). Ahlquist, building on Langley's two type of receptors concept described two separate, α- and β-adrenergic (or adrenotropic) receptors (Ahlquist 1948). Ariens defined drug affinity at a receptor (Ariens 1954) and Stephenson developed a quantitative basis for receptor occupancy and drug efficacy (Stephenson 1956) among other significant milestones (Kenakin 2004).

The molecular era was heralded by a shift toward direct interrogation of receptor function, which began in the early 1970's (Lefkowitz 2004). Early work in the molecular pharmacology of GPCRs was aimed at finding the receptor that mediates an effect or is bound by a particular ligand. For example, the presence of a receptor that was specifically

bound by opiates in the nervous system was discovered by Pert and Snyder in 1973 and soon thereafter Kosterlitz and Hughes demonstrated the existence of endogenous opioids that had differential affinities for three different receptors classes (Pert and Snyder 1973, Hughes et al. 1975, Lord et al. 1977). However, the genes for the opioid receptors were not identified by molecular cloning until the early 1990's and were only then matched to previously-described functions (Evans et al. 1992, Kieffer et al. 1992, Wang et al. 1993).

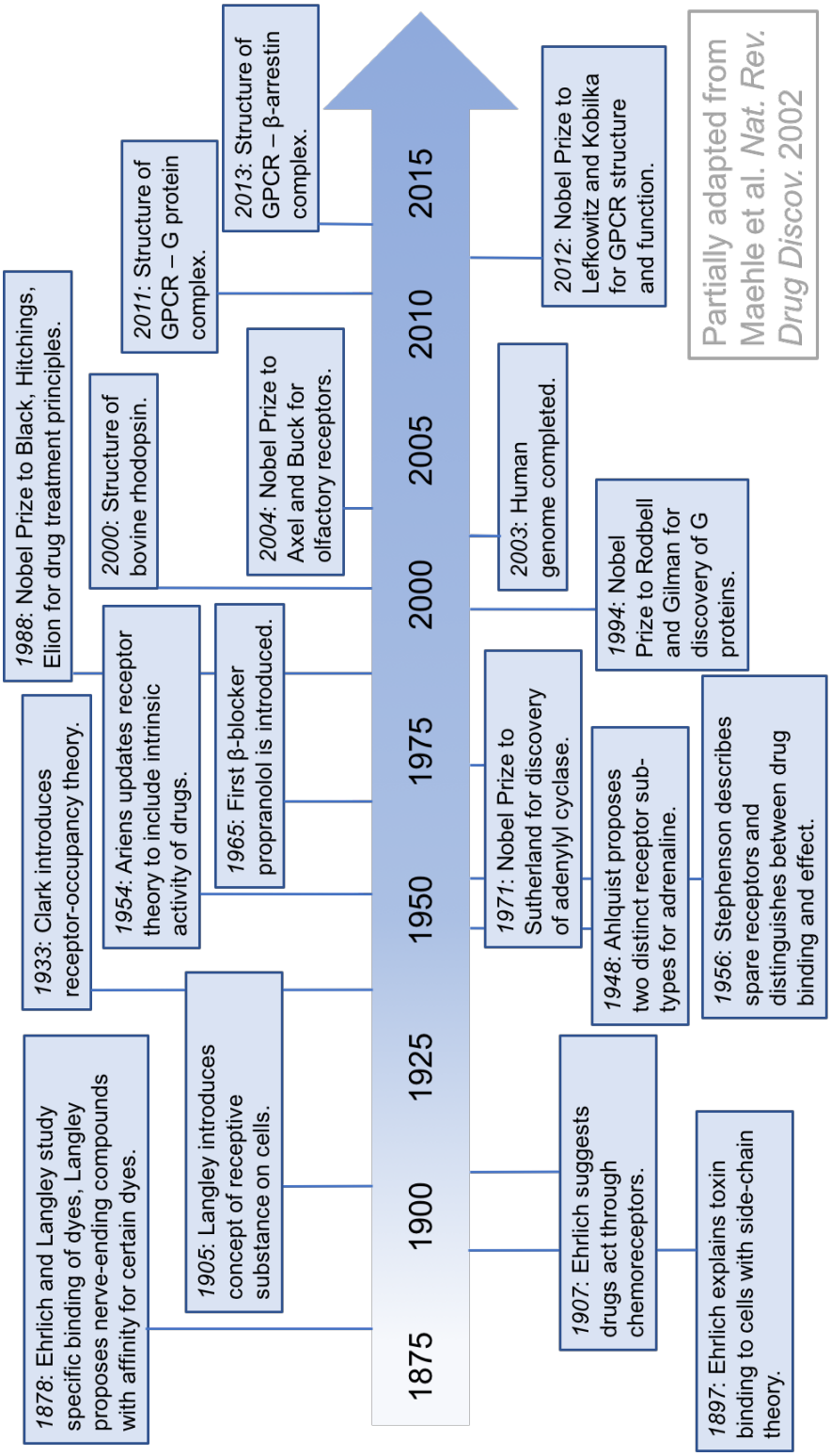
The first seven-transmembrane-spanning receptor was cloned from the retina, where rhodopsin, a GPCR activated by photons, is highly abundant (Nathans and Hogness 1983). Lefkowitz and colleagues found that the adrenergic receptors bore striking homology to rhodopsin (Dixon et al. 1986), and Buck and Axel discovered that hundreds of odorant receptors in the nose were also seven-transmembrane receptors (Buck and Axel 1991). It thus became clear to many physiologists and pharmacologists that these proteins are a prominent and extraordinarily diverse family of cell surface receptors. Indeed, it is now known that this superfamily of receptors can receive and transduce signals as diverse as large proteins (parathyroid hormone receptor), fatty acids (GPR120), sex hormones (GPR30), and neurotransmitters (adrenaline, dopamine, serotonin, glutamate, GABA, etc.) in addition to the aforementioned photons (rhodopsin) and odorant molecules at olfactory receptors (Bjarnadottir et al. 2006).

The completion of the human genome in 2003 shifted the classic GPCR research paradigm because now the sequence of the entire complement of human 7-transmembrane receptor proteins is known (Vassilatis et al. 2003). GPCRs are currently grouped into five families under the GRAFS nomenclatures: Glutamate (class C, metabotropic glutamate receptors, $GABA_B$, Ca^{2+}-sensing, several taste and pheromone

receptors), Rhodopsin-like (class A, 719 receptors including 329 non-olfactory GPCRs), Adhesion (33 human receptors, see below), Frizzled (10 Frizzled receptors and Smoothened), Secretin-like (Class B, 15 receptors) (IUPHAR 2015).

The question now, for many GPCRs, is what role they serve in the body. Of the ~400 non-olfactory human GPCRs (Fredriksson et al. 2003), roughly 1/3rd remain orphan receptors without a known endogenous ligand (Roth and Kroeze 2015). These "pharmacologically dark" receptors represent enormous potential for improving our understanding of physiology and for drug discovery.

Figure 1. History of receptor theory



1.2 Key features of GPCRs

The members of this family of seven-transmembrane spanning proteins are typically referred to as G protein-coupled receptors (GPCRs) because of their ability to transduce external stimuli into cellular signaling pathways mediated by guanosine nucleotide-binding regulatory proteins (G proteins) (Hepler and Gilman 1992). G proteins are heterotrimers comprised of an alpha, beta, and gamma subunit. There are at least 16 different G protein alpha subunits in the human genome (nearly 20 different proteins counting splice variants) and they are grouped into four major classes: $G\alpha_s$, $G\alpha_{i/o/z}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Milligan and Kostenis 2006). There nearly as many beta and gamma subunits, which can join their alpha counterparts in multiple combinations, adding an additional layer of complexity. The alpha-independent functions of the $G\beta\gamma$ subunits are only beginning to be understood and appreciated. Original work in this area is discussed in Chapter 3.

These components of G proteins have received far less attention but are nonetheless highly important for signaling activity as addressed in the present studies. The different subtypes of $G\alpha$ subunits were initially named for their effects on the effector protein adenylyl cyclase, an enzyme that cyclizes adenosine monophosphate (AMP) to turn it into a second messenger. $G\alpha_s$ stimulates adenylyl cyclase to increase local cAMP concentrations and $G\alpha_i$ inhibits the enzyme. In the outer segments of rod cells in the retina, the conformation of rhodopsin is shifted from inactive to active when light isomerizes the covalently linked vitamin molecule retinal from 11-*cis* to all-*trans*-retinal. This new conformation allows the receptor to interact with transducin, the G protein of the retina, which has a similar function as $G\alpha_i$ and activates a

phosphodiesterase which leads to the breakdown of a cyclical second messenger (cGMP in this case). Rhodopsin is atypical among GPCRs in that its agonist (retinal) is covalently linked to the receptor. Chapter 2 addresses a hypothesis in the field that adhesion GPCRs also have a tethered agonist.

Perhaps the most iconic feature of GPCRs is their serpentine, 7-transmembrane domain insertion in the phospholipid bilayer cell membrane with an extracellular amino (N) terminus and an intracellular carboxyl (C) terminus. Major advances in the understanding of the structure of these proteins has occurred in recent years as crystal structures have emerged for a number of GPCRs. In 2000, a 2.8 angstrom structure of bovine rhodopsin was solved by Palczewski and colleagues (Palczewski et al. 2000) but it took another 11 years for the first report of a GPCR in its active state in complex with a G protein, in this case the β2-adrenergic receptor with $G\alpha_s$ (Rasmussen et al. 2011). This structure triggered an explosion in GPCR crystallography and, as of July 2015, the structures of more than 30 GPCRs have been experimentally solved (Piscitelli et al. 2015). Unfortunately, no adhesion receptors have been crystallized to this point, and given their tremendous size and structural complexity, it seems likely that a piecemeal approach will be utilized in the coming years.

1.3 Regulation of GPCR signaling activity

The signaling activity of G protein-coupled receptors must be tightly regulated, otherwise the signaling would be difficult for the cell to decode and could be toxic. Receptors need to be activated in response to certain stimuli and cells must have mechanisms to limit or stop signaling when the message has been received or prolonged

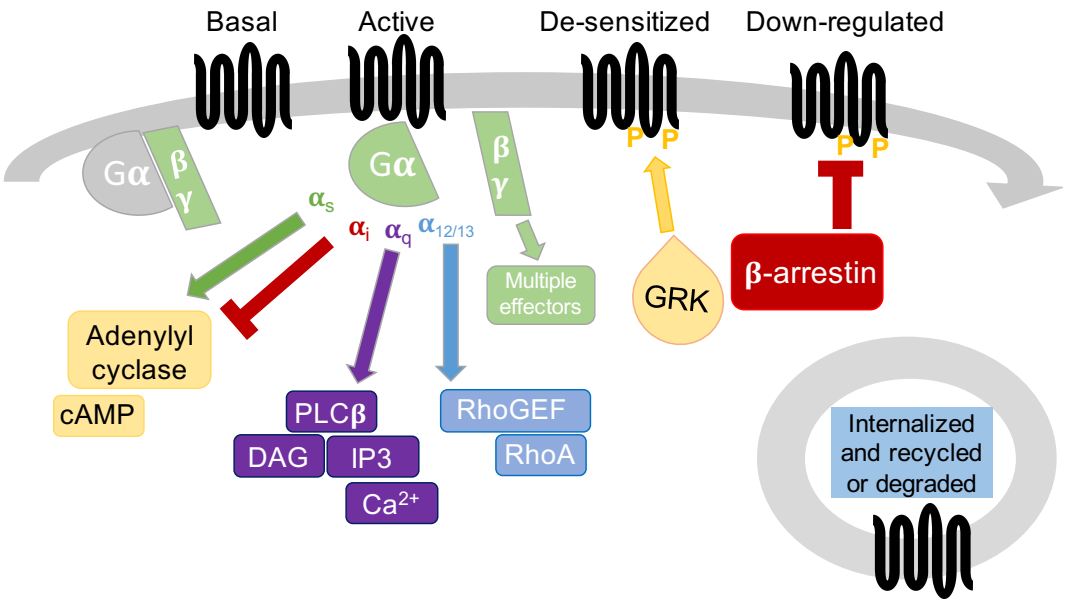
signaling threatens cellular homeostasis. In the absence of an agonist, most GPCRs exist in inactive conformations. However, most GPCRs also exhibit some level of constitutive activity in the absence of ligands, with the extent of constitutive activity varying between receptors and being dependent on receptor expression levels.

When a GPCR adopts its active conformation, it is able to interact with heterotrimeric G protein alpha subunits (Fig. 2). The receptor acts as a guanine nucleotide exchange factor (GEF) for the alpha subunit, which is a GTPase. Interaction with the receptor catalyzes the exchange of GDP for GTP, which activates the G protein. Upon activation, the G protein alpha subunit can release its βγ subunits, and the alpha and βγ subunits can exert actions on effector proteins. As mentioned above, $G\alpha_s$ stimulates adenylyl cyclase and $G\alpha_i$ inhibits the same enzyme. Other alpha subunits ($G\alpha_{q/11}$ sub-family) can stimulate calcium signaling, which is a critical signal at the presynapse for the release of neurotransmitter vesicles. The Rho pathway, activated by $G\alpha_{12/13}$ is known to initiate actin cytoskeletal remodeling, which is important in post-synaptic dendritic spines. Two other key Gα subunits not shown in Figure 2 are $G\alpha_o$ and $G\alpha_z$. These are also members of the $G\alpha_i$ sub-family and are highly expressed in the brain and nervous system but do not inhibit adenylyl cyclase. $G\alpha_z$, unlike $G\alpha_{i/o}$, is insensitive to pertussis toxin, a common inhibitor of this pathway (Ho and Wong 2001). All three of these Gα subunits are known to liberate their βγ subunits for key functions including MAP kinase signaling and the modulation of ion channels (Khan et al. 2013). See Chapter 3 for further discussion of $G\alpha_z$ signaling.

Figure 2. Regulation of GPCR signaling activity

In the basal state, G proteins (Gα/βγ) are bound to GDP and do not readily interact with GPCRs. Once a receptor adopts its active conformation, it can serve as a guanosine nucleotide exchange factor (GEF) to activate Gα subunits, which can liberate βγ subunits. Depending on the subtype of Gα, it can act on multiple effectors including adenylyl cyclase, phospholipase Cβ (PLCβ), or RhoGEFs, which can then activate or inhibit a variety of second messenger molecules (cyclic-Adenosine Monophosphate, cAMP; Diacylglycerol, DAG; Inositol 1,4,5-trisphosphate, IP3). G-protein-coupled receptor kinases (GRKs) can phosphorylate (P-) active receptors, which creates a binding site for β-arrestin. Arrestin binding to GPCRs typically occludes the G protein interaction site and therefore stops signaling activity to those pathways. Arrestins can then recruit adaptor proteins and clathrin coat proteins (not shown) to mediate the internalization of GPCRs. Receptors can then be recycled back to the cell surface for a second round of signaling or can be shuttled to the lysosome for degradation.

Figure 2. Regulation of GPCR signaling activity



Heterotrimeric G proteins are not the only cytoplasmic proteins that can recognize a GPCR in its active conformation. G-protein-coupled receptor kinases (GRKs) can phosphorylate intracellular serine and threonine residues on active GPCRs, which creates binding sites for β-arrestins (Premont, Inglese, and Lefkowitz 1995). However, there are also pathway-specific kinases such as protein kinase A or protein kinase C (not pictured), which can respond to increased concentrations of the second messengers cAMP or Ca^{2+}, respectively, and phosphorylate intracellular regions of both active and inactive GPCRs (Ferguson 2001). Thus, desensitization – defined as cellular mechanisms to safeguard against overstimulation from active receptors – can be both specific and non-specific (Ferguson 2001).

When β-arrestins bind to activated and phosphorylated receptors they sterically occlude heterotrimeric G proteins and thereby uncouple the receptor from the G protein pathways (Ferguson 2001). However, β-arrestins can also serve as scaffolds for the activation of a separate set of kinase cascades like the MAP kinase pathway (Violin and Lefkowitz 2007). In fact, it is now known that some agonists possess functional selectivity such that certain molecules can bias the receptor either more toward a conformation that activates G protein pathways or arrestin signaling pathways (Violin and Lefkowitz 2007).

The functional selectivity of molecules at GPCRs is a very active area of research because the clinical consequences can be profound. For example, morphine is a powerful analgesic but its pain relieving effects are accompanied by a high risk of dependence and side effects such as respiratory suppression and constipation (Raehal, Walker, and Bohn 2005). Interestingly, these side effects have been disentangled from the primary analgesia

in mice lacking β-arrestin-2. These animals do not develop the typical tolerance for morphine – their response to a second treatment with morphine is as robust as the first treatment – whereas wild-type animals respond only about half as well to the second dose (Bohn et al. 2000). In addition, mice lacking β-arrestin-2 are protected from two of the most clinically relevant side effects of morphine treatment, constipation and respiratory suppression (Raehal, Walker, and Bohn 2005). These data indicate that β-arrestin-2 is a key mediator of morphine tolerance and its unwanted side effects and that drugs that retain the ability to stimulate G protein signaling from the μ-opioid receptor but limit recruitment of and signaling by β-arrestin-2 could be safer analgesic agents (Raehal et al. 2011). This concept of functional selectivity or bias for a particular pathway will be discussed in Chapter 4, not based on a particular ligand but on mutations to the receptor itself. Fine tuning the functional selectivity of drugs such that they retain their primary function but are accompanied by less risk of dependence and fewer side effects is a major goal in this area.

In addition to mediating alternative signaling pathways downstream of GPCRs, β-arrestins are also involved in receptor internalization. Upon binding by β-arrestins, many GPCRs are removed from the cell surface via the canonical clathrin-coat pathway (Ferguson 2001). β-arrestins can bind to both adaptor proteins and clathrin coat proteins to facilitate the formation of clathrin-coated vesicles which are then pinched off from the intracellular side of the membrane by dynamin. Endocytosis does not necessarily mean the end of a receptor's signaling activity. An emerging area of GPCR research focuses on intracellular GPCR signaling from endosomes, though the importance of this signaling may differ for specific GPCRs (Irannejad et al. 2013).

Recently, a new pathway has been identified which also may play a key role in GPCR internalization. In the late 1990's, it was reported that the intracellular scaffold protein endophilin bound to the β_1-adrenergic receptor, but it was unclear exactly what function this interaction had (Tang et al. 1999). Endophilins are membrane-binding proteins with SH3 domains that can participate in a number of protein-protein interactions and perhaps modulate the clathrin GPCR endocytic pathway (Mousavi et al. 2004). However, in 2015, new data demonstrated that endophilins not only participate in the canonical clathrin-mediated endocytic pathway, but can mediate their own dynamin-dependent endocytic pathway without the help of clathrin (Boucrot et al. 2015). This new pathway is exciting because the kinetics appear to be faster than clathrin-mediated endocytosis, it is more dependent on the receptor's activity (clathrin endocytosis can be constitutive in some cellular compartments), and endophilin interactions were found with more than a dozen GPCRs including adrenaline, dopamine, serotonin, and histamine receptor subtypes (Boucrot et al. 2015). Much remains to be understood about this pathway including what role (if any) β-arrestins have in it. Original work presented in Chapter 3 discusses a potential role for endophilins in the regulation of ADGRB2.

Once internalized, GPCRs can either be recycled back to the cell surface or degraded by lysosomal and/or proteasomal pathways (Marchese et al. 2008). Some receptors such as protease-activated receptor 1 (PAR1) are not recycled back to the membrane (Trejo and Coughlin 1999). However, PAR1 is not a conventional GPCR. It is activated by a tethered cryptic agonist which is exposed when the protease thrombin cleaves the ectodomain of the receptor (Coughlin 2000). Clearly, due to the way this agonist induces a permanent modification of the receptor, it is unlike a reversibly-binding

ligand that can diffuse away. Therefore, receptor degradation may be the most efficient regulatory process for a receptor like PAR1. This process is relevant for adhesion GPCRs as well because their activation mechanisms may also be irreversible (see below), so it is unclear how signaling could be quenched once activation has occurred. This concept will be discussed in further detail throughout this dissertation.

Some adhesion GPCRs including the ADGRB sub-family have unusually long intracellular C termini, which allow for a number of different protein-protein interactions including with small signaling GTPases like Rac1 and Cdc42, and PDZ domain proteins at the extreme C-terminus (see Table 3). How these non-heterotrimeric G protein signaling pathways are activated, regulated, and how their signaling may intersect with heterotrimeric G protein signaling from other regions of the same protein are not well understood and are active areas of investigation.

1.4 Mutations in GPCRs can cause human diseases

The cloning of the gene for the GPCR rhodopsin was followed shortly thereafter by the identification of mutations in that gene linked to retinitis pigmentosa, a degenerative disease of the retina (Dryja et al. 1990). The identification of these mutations in the protein also led to a better understanding of the basic functions of the receptor. For example, a mutation to lysine 296, the residue that binds the light sensitive vitamin molecule retinal, makes the receptor constitutively active (Robinson et al. 1992). Another mutation in rhodopsin that imparts constitutive activity, Glycine 90 to Aspartate, causes congenital night blindness (Rao, Cohen, and Oprian 1994). Similarly, substituting

glycine 578 for aspartate in the luteinizing hormone receptor makes that protein constitutively active, which results in precocious puberty (Shenker et al. 1993). By the mid-2000's, more than 700 mutations had been found in GPCRs linked to more than 30 genes and the list continues to grow (Schoneberg et al. 2004). Adhesion GPCRs have also been linked to human disease and are discussed in detail in section 1.5. Chapter 3 will discuss a neuromuscular disease-associated human mutation in the adhesion GPCR ADGRB2/BAI2.

1.5 Adhesion GPCR family

Under the GRAFS organizational scheme, adhesion GPCRs are the second largest family of non-olfactory GPCRs. The adhesion G protein-coupled receptors (aGPCRs) are evolutionarily ancient proteins with paralogs in zebrafish (*Danio rerio*), as well as *Caenorhabditis elegans*, *Drosophila melanogaster*, and even *Dictyostelium discoideum* (Nordstrom et al. 2009). Interestingly, the much better-understood Secretin-like receptors appear to have diverged from the more ancient adhesion GPCRs (Nordstrom et al. 2009).

The aGPCR family itself – now comprised of 33 members – was only identified upon completion of the human genome.[1] Very recently, all 33 aGPCRs were renamed with the ADGR prefix, followed by a letter indicating the receptor subgroup, and number for the receptor within that group (Hamann et al. 2015). This new nomenclature has been adopted by IUPHAR and will be used as the primary identifier for receptors in this dissertation.

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[1] Portions of this chapter have been submitted for publication in *Annual Review of Pharmacology and Toxicology* for an article entitled "Adhesion G protein-coupled receptors as drug targets", co-authored by Dr. Randy Hall.

The first adhesion GPCRs (aGPCRs) were identified in the area of immunology. A homolog of the mouse macrophage marker F4/80 was determined to be a seven-transmembrane-spanning (7-TM) protein with unique structure and was named EMR1 (epidermal growth factor-like molecule containing mucin-like hormone receptor 1) as was the leukocyte activation marker CD97, both of which would later be grouped into the same subfamily and re-named ADGRE1 and ADGRE5 respectively (Baud et al. 1995, Hamann et al. 1995). Soon, the discovery of additional 7-TM proteins with long yet variable extracellular domains but with significant similarity in their transmembrane cores resulted in a grouping of the receptors to a family called LNB-TM7 for long N-terminal domain 7-TM receptors with similarity to family B (Secretin) receptors (Stacey et al. 2000).

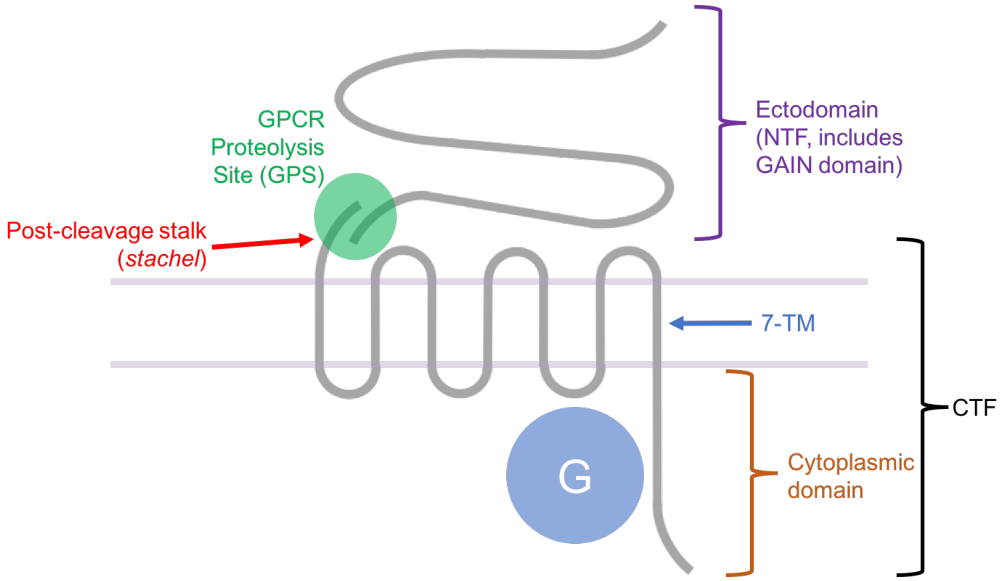
When the first aGPCRs were discovered it was thought that these were hybrid proteins. Several of the receptors including ADGRL1 (a.k.a. latrophilin, CIRL, CL-1), ADGRE5/CD97, and ADGRC1-3/CELSR1-3 had extended extracellular regions with many of the eponymous adhesion domains followed by a linker region to the seven transmembrane-spanning (7-TM) domain (Fig. 3). This apparent linker region contained the GPCR Proteolysis Site (GPS) motif, where the protein is cleaved (Krasnoperov et al. 2002). The GPS motif and 7-TM bore substantial similarity between these proteins.

Later work identified the approximately 300 amino acid region N-terminal to the GPS motif reaching almost to the start of the first transmembrane domain as a novel protein domain found in almost every aGPCR as well as several other transmembrane proteins. This novel domain was crystallized from ADGRB3/BAI3 and ADGRL1 and called the GPCR autoproteolysis inducing (GAIN) domain (Arac et al. 2012). All

aGPCRs aside from ADGRA1/GPR123 have a GAIN domain (Lagerstrom et al. 2007). Even when aGPCRs undergo GAIN domain-mediated autoproteolysis in the endoplasmic reticulum (Krasnoperov et al. 2002), the N- and C-terminal protomers are known to traffic together to the cell membrane, bound to each other in a non-covalent manner (Stephenson et al. 2013). In fact, receptor cleavage is not necessary for aGPCRs to be expressed on the cell surface (Kishore et al. 2016, Promel, Waller-Evans, et al. 2012). Moreover, the aforementioned crystal structure of the GAIN domain showed that the post-cleavage stalk would be tightly bound inside a hydrophobic groove and would not be stable in an aqueous environment, suggesting that the NT would not be easily lost (Arac et al. 2012). Most likely, interaction with a large, extracellular binding molecule would be required to shift the conformation of the NT (or remove it entirely) and relieve its inhibitory action on the 7TM region (Paavola and Hall 2012).

Figure 3. Adhesion GPCR structure

Adhesion GPCRs are characterized by long extracellular amino termini (ectodomain, NTF), which include the GAIN domain. The GAIN domain can cleave the receptors at the GPCR Proteolysis Site (GPS), which results in the post-cleavage stalk (*stachel*) becoming the new N terminus of the GPCR. The NTF and CTF can remain non-covalently associated. The CTF contains both the classic 7-transmembrane (7-TM) domain as well as the intracellular C terminus which, in aGPCRs, can be several hundred amino acids long.



1.5.1 Adhesion GPCR structure

Many adhesion GPCRs are extraordinarily long proteins (Fig. 3). In comparison to other, more commonly-studied receptors like the β_2-adrenergic receptor (413 amino acids) or the D2 dopamine receptor (443 a.a.), the BAI sub-family members are nearly four times as long at 1,522-1,585 amino acids. Among aGPCRs, the ADGRC/CELSRs (2,933-3,312 a.a.) and, of course ADGRV1 (formerly known as Very Large GPCR1 – 6,306 a.a.) are even longer. The 7TM domain typically takes up less than 300 a.a., so these receptors have very long extracellular and intracellular domains. Adhesion receptors were named as such due to the presence extracellular adhesion domains in early members of the family (McKnight and Gordon 1998, Stacey et al. 2000). However, the long amino termini of these proteins display a rich variety of protein domains with many properties beyond adhesion. For example, thirteen of the 33 human aGPCRs have a hormone-binding domain reminiscent of the Secretin family of GPCRs (Krishnan et al. 2016). It is currently unclear whether these domains might mediate ligand binding in aGPCRs.

Several of these N-terminal protein domains are restricted to single sub-families of aGPCRs (Fig. 4). For example, only the three ADGRBs (BAI1-3) have thrombospondin type-1 repeats (5 in ADGRB1 and 4 each in B2 and B3). In addition, only the ADGRCs (CELSR1-3) have cadherin or laminin-EGF domains. ADGRL1-3 each have a lectin and olfactomedin domain but ADGRL4/ELTD1 has neither. EGF-like domains are found in all of sub-family ADGRE as well as in ADGRL4 and ADGRC1-3, and ADGRF3 (Hamann et al. 2015).

The GAIN domain is now largely considered to be the archetypal feature of aGPCRs. Only ADGRA1/GPR123 lacks a GAIN domain and outside of the aGPCR family, this domain is only found in polycystic kidney disease proteins PKD1-5 in humans (Arac et al. 2012). The rich diversity of extracellular domains presents enormous opportunity for interaction with potential ligands, which may fine tune aGPCR signaling activity. Moreover, these surfaces likely represent vast potential as drug targets (see Chapter 5). To date, the extracellular domains of aGPCRs have received the most attention due to the presence of a number of known protein domains and advances in domain crystallization. Sub-family E (EMR1-4, CD97) is characterized by N-terminal EGF-like domains, sub-family C by cadherin domains, and the BAIs (ADGRB1-3) have at least four type-I thrombospondin repeats in the N terminus of each receptor. Other domains such as pentraxin and hormone-binding motifs are found across multiple sub-groups of aGPCRs.

The GAIN domain is the most notable feature of aGPCR ectodomains. Early work on aGPCRs had described the hybrid nature of these receptors with 7-TM domains non-covalently linked to extracellular amino termini, but it was not clear that the GPCR proteolysis site was actually an integral component of a larger protein domain. In 2002, it was reported that ADGRL1 was actually a single gene product receptor that was thought of as a heterodimer of a cell adhesion protein and a GPCR, which are separated in the endoplasmic reticulum (Krasnoperov et al. 2002). In the aGPCRs ADGRL1/CL1 and ADGRB3/BAI3, crystal structures showed the GAIN domain to cut the protein near the start of the first transmembrane domain in a hydrophobic beta sheet that folds deep inside a bundle of beta strands (Arac et al. 2012). The exact mechanism of proteolysis was

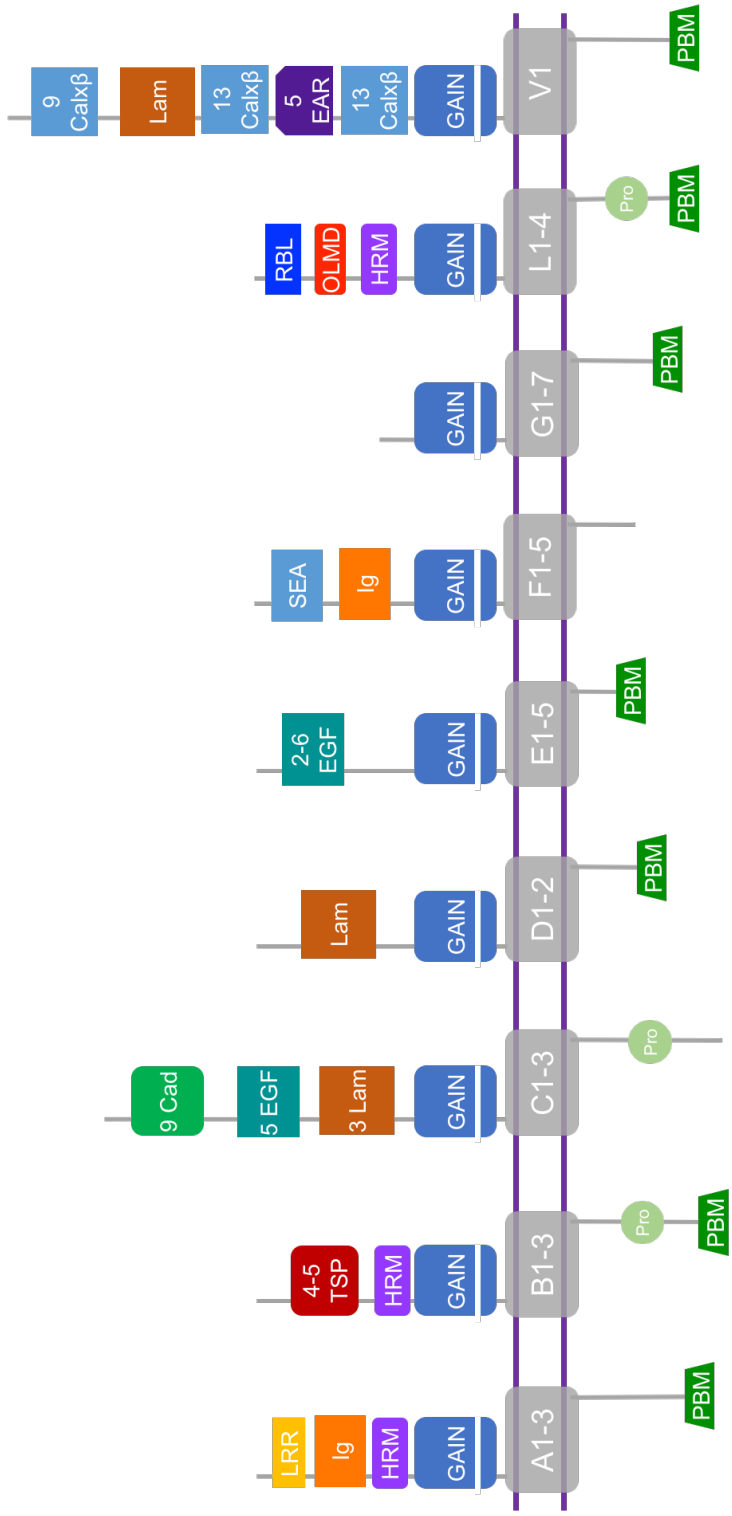
actually described many years earlier (Lin et al. 2004). Briefly, most GPCRs have the sequence (-2)Arg/His, (-1)Leu/Ile/Met, cleavage site, (+1)Ser/Thr/Cys where the hydroxyl or sulfahydral group of the amino acid in the +1 position serves as a nucleophile and can attack the -1 residue resulting in hydrolysis to cleave the protein. It is worth noting that, in brain tissue, all three BAI receptors appear to readily undergo proteolysis with a substantial fraction of the protein running at the predicted size of the CTF. However, in HEK-239T cells all three receptors are essentially uncleaved, which suggests that either these receptors may be inefficient cleavers and require an additional factor that is not present in HEK cells or, alternatively, some factor in HEK cells might inhibit GAIN proteolysis. In ADGRG1, a receptor that does in fact cleave efficiently in HEK cells, inhibiting cleavage has no effect on receptor surface expression or signaling activity suggesting that GAIN cleavage is not necessary for receptor trafficking and some functions (Kishore et al. 2016).

The 7-TM core of aGPCRs most closely resembles that of the family B, Secretin-like receptors (de Graaf et al. 2016). Many rhodopsin family GPCRs have a DRY motif in the third intracellular loop that is important in G protein coupling but adhesion receptors do not adhere to this model (Rovati, Capra, and Neubig 2007). However, most (21 of 33) aGPCRs have an E-X-X-X-X-Y motif in the third intracellular loop where the position of the Y corresponds to that of the Y in the rhodopsin-like DRY motif (de Graaf et al. 2016). In the extracellular loops, aGPCRs resemble the metabotropic glutamate receptors with short first and third extracellular loops and conserved cysteine-tryptophan residues in the second extracellular loop (de Graaf et al. 2016). The functional contributions of these structural features are largely untested for aGPCRs.

Figure 4. Adhesion GPCR sub-families

The 9 adhesion GPCR sub-families listed alphabetically with key protein domains depicted for each group. Some sub-family members have slightly different structure from what is indicated in the figure: A1-3: A1-No GAIN, HRM, IG, LRR, A2-No Ig; B1-3: Pro in B1, B1-5xTSP1, B3-CUB; C1-3: Pro in C2; D1-2: PBM only in D1; E1-5: PBM only in E5; F1-5: SEA in F1 and F5, Ig in F5; G1-7: G1-PLL, G6-CUB, Laminin, PBM only in G1; L1-4: Pro only in L1, L4-Only EGF and GAIN in NT, no PBM. Abbreviations: GAIN: GPCR autoproteolysis-inducing domain; LRR: Leucine-rich repeat; Ig: Immunoglobulin-like; HRM: Hormone receptor motif; TSP: Type-1 thrombospondin repeat; Cad: Cadherin repeat; EGF: Epidermal Growth Factor-like (includes Calcium-binding EGF-like domains); Lam: Laminin; Pro: polyproline sequence; PBM: PDZ binding motif; SEA: Sperm protein/Enterokinase/Agrin domain; RBL: rhammose-binding lectin; OLMD; olfactomedin-like; EAR: Epilepsy-associated repeat.

Figure 4. Adhesion GPCR sub-families



1.6 Adhesion GPCR activation mechanisms

Without known endogenous ligands, and with relatively few specific tools such as receptor antibodies to work with, initial progress in understanding the signaling activity of aGPCRs was slow. One early idea was that the long, extracellular N termini could include ligand binding sites, analogous to Family C GPCRs such as the metabotropic glutamate receptors (Pin, Galvez, and Prezeau 2003). This hypothesis was tested in ADGRG1 and ADGRB1, two distantly-related aGPCRs. To determine if the NT is necessary for receptor activity, the receptors were truncated near the start of the first transmembrane domain. Surprisingly, instead of resulting in inactive receptors, the truncated (ΔNT) forms of both G1 (Paavola et al. 2011) and B1 (Stephenson et al. 2013) were found to be much more active than the full-length receptors. When over-expressed in HEK-293T cells, G1ΔNT and B1ΔNT dramatically increased GTP-bound active RhoA compared to empty vector controls. Moreover, the RGS domain of p115RhoGEF, a specific $G\alpha_{12/13}$-inhibitor (Kozasa et al. 1998), blocked this activation in B1, demonstrating that it does indeed couple to G proteins – a concept that was not taken for granted with aGPCRs (Langenhan, Aust, and Hamann 2013).

Despite belonging to the same family, G1 and B1 are not closely related receptors. Therefore, these data, along with parallel studies on ADGRE5 (Ward et al. 2011) and ADGRB2 (Okajima, Kudo, and Yokota 2010), which also resulted in large increases in receptor signaling activity led to the proposal of a *disinhibition model* of signaling (Fig. 5) whereby the N terminus of aGPCRs may generally inhibit the intrinsic signaling potential of the 7-TM domain by locking the receptor in an inactive conformation (Paavola and Hall 2012). In addition to the receptors mentioned above,

ADGRG2 (Demberg et al. 2015, Peeters et al. 2015, Balenga et al. 2016), ADGRG6 (Paavola et al. 2014), ADGRD1 (Liebscher et al. 2014), ADGRF1 (Stoveken et al. 2015), and ADGRV1 (Hu et al. 2014) have also been found to highly active when the N terminus is removed via truncation.

As early as 2002, the possibility was considered that aGPCR cleavage could play a role in receptor activation as it does in the protease-activated receptors (PARs) (Krasnoperov et al. 2002). However, it was not until 2014 that it was demonstrated that peptides derived from the post-cleavage stalk sequence could activate ADGRG6 and ADGRD1 (Liebscher et al. 2014). Soon thereafter, it was independently reported that ADGRG1 and ADGRF1 could also be activated in this manner (Stoveken et al. 2015). This mechanism can be thought of as the *tethered agonist model* of aGPCR signaling where the post-cleavage stalk acts as tethered agonist to push the receptor into an active conformation. This model would likely, but not necessarily, involve removal of the NTF following GAIN cleavage.

There are technical and theoretical challenges for the tethered cryptic agonist model that are being addressed in ongoing studies. One key hurdle for these experiments is that the stalk or "*stachel"* (German for "stinger") peptides are highly hydrophobic for almost all aGPCRs. Moreover, in some cases, only certain lengths of peptide can agonize the receptor whereas even peptides 1-2 amino acids longer can strongly inhibit activity acting as inverse agonists (Stoveken et al. 2015). This remains an exciting developing area for aGPCR research. The receptors for which a tethered agonist has been identified are shown in Table 1 (Liebscher and Schoneberg 2016). Some receptors can clearly be activated by the post-cleavage stalk peptide but it is not a requisite agonist for all

adhesion receptors. For example, a truncated version of ADGRB1 shows no signaling deficits compared to B1ΔNT in gene reporter assays, and is recognized as an active conformation of the receptor by β-arrestins and $G\alpha_{13}$ (Chapter 2) (Kishore et al. 2016).

Another possibility, which has also gained experimental support, is that the stalk can act more like a lever within the cleaved but associated NTF-CTF complex to tune signaling activity based on its position. This has been termed the *tunable model* (Stoveken et al. 2015). Indeed, increasing evidence indicates that aGPCRs might generally act as metabotropic mechanosensors (Scholz et al. 2016). ADGRG5/GPR114 was found to be activated by mechanical stimulation *in vitro* in a manner that is dependent on a glutamine residue within the stachel sequence, which might be necessary for placing the stachel in the appropriate position (Wilde et al. 2016). In addition, ADGRG1 has been shown to respond to mechanical loads in mice (White et al. 2014) and the *Drosophila* homolog of the ADGRL receptors was shown to be a functional mechanoreceptor for multiple sensory modalities through the chordotonal organ (Scholz et al. 2015). The localization of ADGRV1 to auditory hair cell stereocilia suggests that this receptor might also have a mechanosensory function, but this concept has not been demonstrated to date (Scholz et al. 2016).

As a general rule, the activation state of adhesion GPCRs is governed by NTF-CTF interactions. It seems likely that most, if not all, aGPCRs express a combination of disinhibition and tethered agonist activation, which may have the potential to activate discrete downstream pathways (Kishore et al. 2016). The evidence suggesting that ADGRB1 does not seem to have tethered agonist mediated activity may be atypical among aGPCRs. Interestingly, one consequence of the high degree of sequence

homology across the aGPCR family around the GAIN cleavage site is that the agonistic portion of tethered agonist ("*stachel*") peptides from one receptor closely or exactly match that of another receptor. Indeed, this situation has recently been reported – activating peptides derived from ADGRF1/GPR110 can activate F1 and also the closely-related receptor ADGRF5/GPR116 as well as ADGRG2/GPR64, which resides in a separate sub-group of aGPCRs (Demberg et al. 2017). Interestingly, it was also observed that there is some degree of pathway specificity when using peptides from one receptor to activate another. An obvious consequence of these findings is that if drugs were designed to mimic the agonistic portion of these peptides, then they may lack specificity but may, on the other hand, have broader use (Demberg et al. 2017).

Figure 5. Models of adhesion GPCR activation

Adhesion GPCR activation follows the general rule that activity state is governed by NTF-CTF interactions. The tethered agonist (stachel) can be unmasked when the NTF is completely removed. Alternatively, the stachel can act as a lever where, as its position is modulated by NTF movements, it tunes activity levels. Finally, disinhibition is a general model where the intrinsic activity of the 7-TM protomer (CTF) is unveiled when the NTF is pulled away from the CTF or shed completely from the protein complex.

Figure 5. Models of adhesion GPCR activation

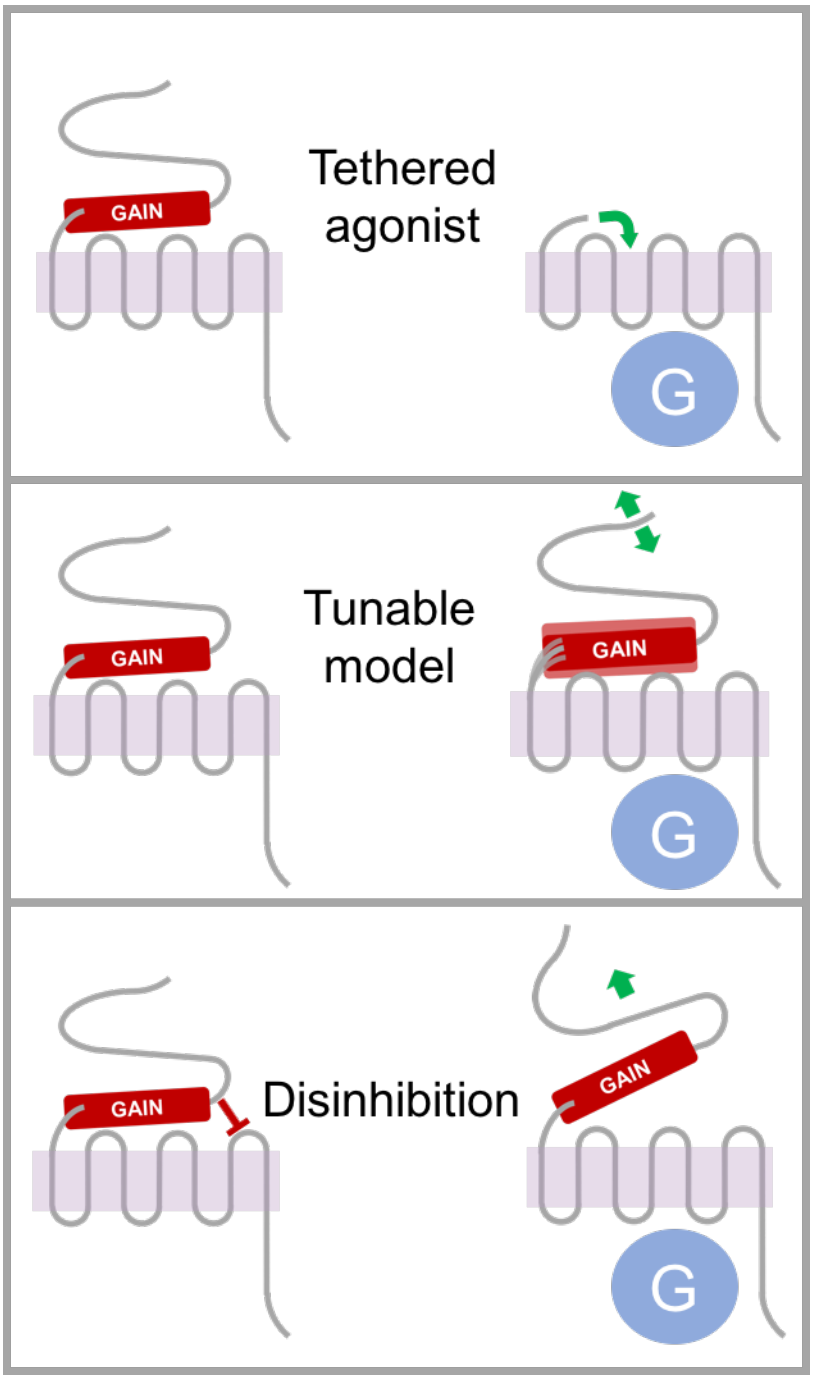


Table 1. Tethered agonist-activated adhesion GPCRs

Receptor	Evidence	Reference
ADGRB1 (BAI1)	Putative tethered agonist removal does not disrupt signaling activity.	(Kishore et al. 2016)
ADGRD1 (GPR133)	Cell-based assays, P12-15 best agonists.	(Liebscher et al. 2014)
ADGRF1 (GPR110)	*In vitro* G protein coupling data only. P9-15, 17-18 activated, P16 and 21-23 inhibited.	(Stoveken et al. 2015)
	Cell-based assays found P10-19 generally activated both cAMP and NFAT.	(Demberg et al. 2017)
ADGRF5 (GPR116)	P13 best activator of NFAT reporter.	(Demberg et al. 2017)
ADGRG1 (GPR56)	*In vitro* G protein coupling and cell-based reporter assays. P7 peptide activated by P10-15 inhibited.	(Stoveken et al. 2015)
	Tethered agonist is not required for all signaling pathway outputs.	(Kishore et al. 2016)
ADGRG2 (GPR64)	P15 best peptide among 11-20. Cell-based cAMP assay.	(Demberg et al. 2015, Balenga et al. 2016)
ADGRG5 (GPR114)	P18-20 best activators in cAMP assay. Activity also dependent on Q230 in agonist sequence.	(Wilde et al. 2016)
ADGRG6 (GPR126)	Development was disrupted in zebrafish with mutated tethered agonist sequence, peptide treatment rescued phenotype.	(Liebscher et al. 2014)
ADGRL1 (LPHN1)	Functional effects of peptide on *C. elegans* homolog LAT-1 *in vivo*.	(Muller et al. 2015)

1.6.1 Adhesion GPCR Signaling Pathways and Ligands

Understanding the signaling pathways downstream of aGPCRs is critical for drug discovery as well as a fundamental understanding of receptor function. In addition, as the activation mechanisms of aGPCRs have received increasing scrutiny in recent years, detecting the intracellular transduction of the signal has become increasingly important. The ability to couple to heterotrimeric G proteins was not always presumed for the adhesion family of GPCRs. However, seminal signaling studies on ADGRL1 (Lelianova et al. 1997, Rahman et al. 1999) demonstrated clear G protein coupling and now many aGPCRs have been found to couple to G proteins. The currently-known G protein pathways activated by each aGPCR, both of the heterotrimeric and small GTPase variety, are listed in Table 2 and summarized here.

ADGRG1 is one of the most intensively-studied aGPCRs due to its involvement in human disease (discussed below) and several studies have investigated its G protein coupling. A polyclonal antibody directed at the ectodomain of G1 was found to activate $G\alpha_{12/13}$ signaling downstream of the receptor as evidenced by inhibition with the RGS domain of p115RhoGEF, C3 exoenzyme, and dominant negative RhoA (Iguchi et al. 2008). Later work established that a version of G1 truncated to the GPS strongly activates the Rho pathway (Paavola et al. 2011), can be co-immunoprecipitated with $G\alpha_{13}$ (Kishore et al. 2016), and can directly stimulate $G\alpha_{13}$ in reconstitution assays (Stoveken et al. 2015). Others have found that G1 can associate with $G\alpha_q$ (Little, Hemler, and Stipp 2004) although G1-mediated activation of $G\alpha_q$ has not been observed (Stoveken et al. 2015).

Another ADGRG sub-family member, GPR126/ADGRG6 has been shown to couple to both $G\alpha_s$ and $G\alpha_i$ (Liebscher et al. 2014, Mogha et al. 2013, Paavola et al. 2014). It is possible that G6 may couple differentially to these distinct G proteins in a manner that is dependent on the mechanism of receptor of activation and/or cellular context. Other aGPCRs have also exhibited the capacity to couple to multiple G proteins, as for example studies on ADGRG2 have provided evidence of coupling to $G\alpha_s$, $G\alpha_i$, $G\alpha_{12/13}$ and $G\alpha_q$ (Demberg et al. 2015, Peeters et al. 2015). Many traditional GPCRs can couple to multiple proteins, with coupling often strongly regulated by factors such as receptor phosphorylation (Daaka, Luttrell, and Lefkowitz 1997) or association with scaffold proteins (Mahon et al. 2002), and in future studies it will be interesting to explore whether such regulation also occurs for aGPCRs.

Two BAI subfamily members were have been demonstrated to couple to G proteins. ADGRB1 was found to activate the Rho pathway in a manner that was sensitive to the RGS domain of p115RhoGEF (Stephenson et al. 2013). Furthermore, B1 can be co-immunoprecipitated in complex with $G\alpha_{13}$ (Kishore et al. 2016). Despite a high degree of similarity with the 7-TM structure of B1, ADGRB2/BAI2 has a unique signaling profile and its activity to the NFAT luciferase reporter was found to be potentiated by the addition of $G\alpha_{16}$, a promiscuous G protein (Okajima, Kudo, and Yokota 2010). This result demonstrates the receptor's capacity for G protein coupling but not necessarily its specificity. Chapter 3 discusses the identification of a G protein that couples to B2. The cognate G protein(s) for ADGRB3 have not yet been identified.

The pharmacology of the ADGRA1-3 sub-family is poorly understood without any description of G protein coupling for any of these three receptors. Nonetheless,

ADGRA2/GPR124 can be co-activated (with Frizzled) by WNT7A to stimulate β-catenin signaling and, unlike G protein signaling via many other aGPCRs, this activation is dependent on the presence of an intact N terminus, presumably to participate in WNT or Frizzled binding (Posokhova et al. 2015). ADGRC2 and 3 (CELSR2 and 3) have been found to activate Ca^{2+} signaling in a phospholipase C and ER-calcium store dependent manner but it is unclear whether a heterotrimeric G protein mediates this signaling activity (Shima et al. 2007). The largest aGPCR, ADGRV1, has been found to constitutively couple to $G\alpha_i$ (Hu et al. 2014), and also to signal to protein kinase A and C by way of $G\alpha_s$ and $G\alpha_q$ (Shin et al. 2013).

As displayed by Table 2, there are still many aGPCRs for which G protein coupling has not been established. This lack of fundamental understanding for so many adhesion receptors is indicative of the enormous potential for basic discovery that these receptors represent.

Table 2. aGPCR signaling pathways

Receptor	Pathway(s)	References
ADGRA1 (GPR123)	unknown	
ADGRA2 (GPR124)	β-catenin, Cdc42	(Posokhova et al. 2015, Kuhnert et al. 2010)
ADGRA3 (GPR125)	unknown	
ADGRB1 (BAI1)	$G\alpha_{12/13}$, Rac1	(Duman et al. 2013, Park et al. 2007, Stephenson et al. 2013)
ADGRB2 (BAI2)	$G\alpha_{16}$	(Okajima, Kudo, and Yokota 2010)
ADGRB3 (BAI3)	unknown	
ADGRC1 (CELSR1)	Rho kinase	(Nishimura, Honda, and Takeichi 2012)
ADGRC2 (CELSR2)	Ca^{2+}	(Shima et al. 2007)
ADGRC3 (CELSR3)	Ca^{2+}	(Shima et al. 2007)
ADGRD1 (GPR133)	$G\alpha_s$	(Bohnekamp and Schoneberg 2011, Gupte et al. 2012)
ADGRD2 (GPR144)	unknown	
ADGRE1 (EMR1)	unknown	
ADGRE2 (EMR2)	$G\alpha_{15}$	(Gupte et al. 2012)
ADGRE3 (EMR3)	unknown	
ADGRE4 (EMR4)	unknown	
ADGRE5 (CD97)	$G\alpha_{12/13}$	(Ward et al. 2011)
ADGRF1 (GPR110)	$G\alpha_q$	(Gupte et al. 2012, Stoveken et al. 2015)
ADGRF2 (GPR111)	unknown	
ADGRF3 (GPR113)	unknown	
ADGRF4 (GPR115)	$G\alpha_{15}$	(Gupte et al. 2012)
ADGRF5 (GPR116)	$G\alpha_q$	(Tang et al. 2013)
ADGRG1 (GPR56)	$G\alpha_{12/13}$, $G\alpha_q$	(Little, Hemler, and Stipp 2004, Paavola et al. 2011, Stoveken et al. 2015)
ADGRG2 (GPR64)	$G\alpha_s$, $G\alpha_i$, $G\alpha_{12/13}$,$G\alpha_q$	(Demberg et al. 2015, Peeters et al. 2015)
ADGRG3 (GPR97)	$G\alpha_o$	(Gupte et al. 2012)
ADGRG4 (GPR112)	unknown	
ADGRG5 (GPR114)	$G\alpha_s$	(Gupte et al. 2012)
ADGRG6 (GPR126)	$G\alpha_s$, $G\alpha_i$	(Liebscher et al. 2014, Mogha et al. 2013, Paavola et al. 2014)
ADGRG7 (GPR128)	unknown	
ADGRL1 (LPHN1)	$G\alpha_o$, $G\alpha_q$	(Lelianova et al. 1997, Rahman et al. 1999)
ADGRL2 (LPHN2)	unknown	
ADGRL3 (LPHN3)	unknown	
ADGRL4 (ELTD1)	unknown	
ADGRV1 (VLGR1)	$G\alpha_i$	(Hu et al. 2014)

1.6.2 Adhesion GPCR Ligands

While most aGPCRs are still considered to be orphan receptors, a number of extracellular interacting proteins have been identified, and some of these interacting partners may represent authentic endogenous agonists for the receptors. Early on, ADGRL1 was found to be the calcium-independent receptor for the black widow spider venom neurotoxin α-latrotoxin (Krasnoperov et al. 1997, Lelianova et al. 1997, Sugita et al. 1998) but the ADGRLs have been also associated with endogenous ligands. Pre-synaptic neurexins (Boucard, Ko, and Sudhof 2012), post-synaptic FLRT proteins (O'Sullivan et al. 2012), and teneurins (Boucard, Maxeiner, and Sudhof 2014, Silva et al. 2011) have all been found to bind with high affinity to ADGRL extracellular regions and are thought to promote intercellular and perhaps transsynaptic adhesion. Furthermore, ADGRL3 was recently found to engage in a ternary complex with the transmembrane cell guidance protein Unc5 and FLRT2 (Jackson et al. 2016). Binding of Lasso/teneurin-2 was found to stimulate Ca^{2+} signaling in hippocampal neurons expressing ADGRL1 (Silva et al. 2011) but α-latrotoxin also induces Ca^{2+} influx in a heterotrimeric G protein-independent manner (Sugita et al. 1998) and it is largely unknown how these extracellular ligands modulate receptor signaling activity.

Similarly, a number of interacting partners have been identified for the ADGRB/BAI sub-family of aGPCRs, but their effects on signaling still remain elusive. The N-terminal type-1 thrombospondin repeats of ADGRB1 bind to phosphatidylserine, thereby allowing the receptor to recognize and mediate the internalization of apoptotic cells (Park et al. 2007). Moreover, this function has been linked to intracellular signaling via the RacGEF ELMO/DOCK180. The role of B1 in macrophages has been further

extended to include the recognition of Gram-negative bacteria, also via surface lipopolysaccharide (LPS) interaction with the thrombospondin repeats (Billings et al. 2016, Das et al. 2011). Like the ADGRL1-3 receptors, which share similar ectodomain structures, all three ADGRB proteins have at least 4 N-terminal type-1 thrombospondin repeats but it is unclear if ADGRB2 and B3 also bind phosphatidylserine and LPS. In the brain, ADGRB3 is a target of the C1ql proteins, which are secreted proteins bearing the complement pathway-like C1q globular domain. B3 was first reported to bind to C1qL3 (Bolliger, Martinelli, and Sudhof 2011) and subsequently was shown to bind to C1ql1 in the cerebellum in a manner that regulates synaptogenesis (Sigoillot et al. 2015).

Another complement cascade protein, CD55/decay accelerating factor was found to be a ligand for ADGRE5/CD97 (Hamann et al. 1996), though with relatively low affinity (Lin et al. 2001). The EGF-like domains of ADGRE2 and the longest form of ADGRE5 are nearly identical and both bind the glycosaminoglycan chondroitin sulfate (Stacey et al. 2003). However, despite this high degree of ectodomain similarity, ADGRE2 only weakly interacts with CD55 (Lin et al. 2001) suggesting that even aGPCRs with very similar ectodomains may nonetheless have highly specific interactomes (Stacey et al. 2003).

The members of ADGRG sub-family of receptors have been found to bind to multiple extracellular proteins. G1 was shown to inhibit melanoma growth and metastasis by interacting with a component of the extracellular matrix (ECM), tissue transglutaminase (TG2), which binds to the extracellular N terminus of G1 (Xu et al. 2006). Further work on this interaction revealed a unique paradigm where G1 prevents excess TG2-mediated ECM crosslinking by internalizing TG2 and degrading it, thereby

retarding melanoma growth and progression (Yang et al. 2014). Additionally, G1 has been found to bind to collagen III in a manner that stimulates $G\alpha_{12/13}$-mediated signaling to the Rho pathway (Luo et al. 2011).

The N terminus of ADGRG6 has been found to bind to collagen IV (Paavola et al. 2014) and laminin-211 (Petersen et al. 2015), both of which may be key developmental signals that can stimulate receptor activation and increased cAMP levels. Interestingly, ADGRG6 expressed in Schwann cells was also found to be a target of the prion protein (Kuffer et al. 2016). The flexible tail of the prion protein contains a domain that is very similar in amino acid sequence to the G6-interacting motif of collagen IV, and indeed the prion protein was also found to stimulate cAMP signaling through interaction with G6 (Kuffer et al. 2016).

To date, ADGRF1/GPR110 and ADGRF5/GPR116 are the only receptors in the ADGRF sub-group for which extracellular interacting molecules have been identified. ADGRF1 was reported to be activated by synaptamide, a metabolite of the omega-3 fatty acid docosahexaenoic acid in brain tissue (Lee et al. 2016). Mice lacking ADGRF5 and surfactant protein D phenocopy each other in lung tissue and it has been found that surfactant protein D can interact the with ectodomain of F5 (Ludwig, Seuwen, and Bridges 2016). However, as for many of the interactions described in this section, it is not yet clear what effect this interactions may have on G protein signaling or other signaling downstream of F5 (Fukuzawa et al. 2013).

1.7 Adhesion GPCRs in disease

Adhesion GPCRs have been found to be fundamentally important in normal physiology and have also been linked to a growing list of diseases. The following section is divided into three of the most consistent disease categories that aGPCRs have been implicated in: neuropsychiatric disorders, cancer, and immune/cardiac/pulmonary disorders. The aim of this section is to highlight emerging evidence for these receptors as potential therapeutic targets.

1.7.1 Neuropsychiatric disorders

The most intensively-studied adhesion GPCR in neurological dysfunction has been ADGRG1, mutations of which cause a brain developmental disorder known as bilateral frontoparietal polymicrogyria (BFPP, mentioned above) (Piao et al. 2004).[2] G1 is highly expressed in neural progenitor cells (NPCs) and plays a key role in inducing NPCs to stop migrating at their appropriate position in the brain (Iguchi et al. 2008). Disease-associated mutations of G1 typically interfere with receptor folding, trafficking and/or signaling, and a number of distinct disease-causing mutations have been described (Chiang et al. 2011, Jin et al. 2007). Patients with BFPP also exhibit myelination deficits (Piao et al. 2005, Piao et al. 2004), and indeed more recent work in mice has revealed that G1 is expressed in oligodendrocytes during development and loss of G1 function results in deficient myelination (Ackerman et al. 2015, Giera et al. 2015).

Another prominent disease-associated aGPCR is ADGRV1. Mutations to this receptor cause Usher syndrome type 2C, which is characterized by deafness and

[2] Portions of this chapter have been submitted for publication in *Annual Review of Pharmacology and Toxicology* for an article entitled "Adhesion G protein-coupled receptors as drug targets", co-authored by Dr. Randy Hall.

blindness (McMillan and White 2010). V1 is expressed at high levels in the stereocilia in the cochlea as well as the ciliary membrane of photoreceptors (McGee et al. 2006, van Wijk et al. 2006). This receptor appears to be important for key aspects of ciliary function. Many distinct disease-causing mutations have been identified on the receptor's massive (>5,000 amino acid) N-terminus (Weston et al. 2004). Some of these mutations introduce stop codons, meaning that the expressed receptor would be devoid of the seven-transmembrane region that is necessary for signaling (Weston et al. 2004). However, at least one disease-associated mutation is found on the receptor's cytoplasmic C-terminus and has been shown to modulate V1 coupling to G proteins (Hu et al. 2014).

Mouse, zebrafish, and human studies have established that ADGRC1 is required for neural tube closure (Curtin et al. 2003, Robinson et al. 2012). Similarly, the other two CELSR/ADGRC receptors are critical in neuronal migration and axon guidance (Tissir and Goffinet 2013). Other aGPCRs that are important in distinct aspects of nervous system development include ADGRG6/GPR126, which is required for peripheral nervous system myelination (Mogha et al. 2013, Monk et al. 2009, Monk et al. 2011), and ADGRA2/GPR124, which regulates CNS angiogenesis. Knockout of A2 has been shown to result in embryonic death due to disruption of angiogenesis in the CNS and resultant hemorrhaging (Anderson et al. 2011, Cullen et al. 2011, Kuhnert et al. 2010). A2 is widely expressed in the vasculature (Kuhnert et al. 2010), where it can promote Wnt signaling to regulate angiogenesis (Posokhova et al. 2015, Vanhollebeke et al. 2015, Zhou and Nathans 2014).

The latrophilins/ADGRL1-3 and BAIs/ADGRB1-3 appear to be critical for synapse formation and strengthening. ADGRL1-3 receptors are found in both pre- and

post-synaptic compartments (Meza-Aguilar and Boucard 2014). Members of the ADGRL sub-family have been linked to both autism spectrum disorder (ASD) and schizophrenia (SZ), though mostly due to their extra- and intracellular interactions. As mentioned above, ADGRL1 binds with high affinity to neurexins (Boucard, Ko, and Sudhof 2012), which have been genetically linked to both SZ and ASD (Reichelt, Rodgers, and Clapcote 2012). Interestingly, L1 can compete for neurexin binding with neuroligins (Boucard, Ko, and Sudhof 2012), which have also been linked to ASD (Jamain et al. 2003). Across the cell membrane, the cytoplasmic domain of ADGRL receptors can interact with the SHANK scaffold proteins (Kreienkamp et al. 2000, Tobaben, Sudhof, and Stahl 2000), which have been associated with ASD (Guilmatre et al. 2014). Human studies have consistently linked ADGRL3 to attention deficit hyperactivity disorder (ADHD) (Arcos-Burgos et al. 2010, Ribases et al. 2011) and animal studies have supported this finding. Studies in mice have revealed that targeted deletion of L3 results in hyperactivity and disrupted dopamine and serotonin transport (Wallis et al. 2012), as well as significant changes in the relative strengths of connections between different layers of the neocortex (O'Sullivan et al. 2014). Moreover, deletion of L3 in zebrafish also results in hyperlocomotor behavior and other changes consistent with changes in synaptic connections (Lange et al. 2012).

Meanwhile, ADGRB1 and B3 have been shown to be critical for dendritic maturation and stability. Knockdown of B1 in cultured neurons results in drastically altered dendritic spine morphology (Duman et al. 2013), and genetic deletion of B1 induces perturbations to the post-synaptic density (PSD) regions of excitatory synapses in vivo as well as impairments in synaptic plasticity and spatial memory (Zhu et al. 2015).

While less is known about the signaling properties of ADGRB3 relative to B1, it has been genetically linked to schizophrenia (DeRosse et al. 2008) and several studies have identified B3 as an important component of hippocampal and cerebellar synapses. In cultured neurons, C1ql3 was found to decrease spine density through actions at B3 (Bolliger, Martinelli, and Sudhof 2011). Additional experiments have shown that B3 is critical for dendritic development *in vitro* (Lanoue et al. 2013) and through interactions with C1ql proteins (discussed above) B3 is a necessary component of excitatory synapses on cerebellar Purkinje neurons (Sigoillot et al. 2015). Interestingly, both B1 and B3 have two RacGEF interaction sites in their C termini. The ELMO/DOCK-binding RKR motif was found to mediate the dendritic spine effects of B3 (Lanoue et al. 2013) but was not important for these functions dependent on B1 (Duman et al. 2013). Instead, B1 was shown to activate Rac1 through its C-terminal PDZ-binding motif where it interacts with the RacGEF Tiam1 (Duman et al. 2013). While ADGRB2 is also enriched in the nervous system (Kee et al. 2004, Shiratsuchi et al. 1997), and also contains these same RacGEF-binding motifs, it does not appear to be required for spine maturation and synaptic function. Mice lacking B2 were found to have no gross behavioral or anatomical defects but, surprisingly, were found to increased hippocampal neurogenesis and improved resilience compared to wild-type animals in mood disorder related behavioral tests (Okajima, Kudo, and Yokota 2011).

1.7.2 Cancer

Adhesion receptors are increasingly associated with cancer (Aust et al. 2016). Whole genome analysis found that *ADGRB3* and *ADGRL3* were among the most

extensively mutated genes in tumors (Kan et al. 2010). In the late 1990s, ADGRE5 and ADGRG1 were found to be differentially expressed in tumor cells: G1 was down-regulated in the most highly metastatic melanoma cell line in comparison to lines with less metastatic potential (Zendman et al. 1999) and E5 was found to be undetectable in normal thyroid tissue but to be expressed in thyroid carcinomas with its expression level highest in aggressive tumors (Aust et al. 1997). ADGRG1 was later shown to constrain melanoma growth and metastasis (Xu et al. 2006) by inhibiting angiogenesis through VEGF suppression (Yang et al. 2011) and internalization of the tumor-promoting extracellular matrix enzyme tissue transglutaminase TG2 (Yang et al. 2014).

Angiogenesis is critical for tumor growth and, in addition to ADGRG1 mentioned above, several other aGPCRs may regulate angiogenesis in multiple ways. Among these, ADGRB1 may be the most important as it is known to be lost in glioblastoma due to epigenetic silencing by methyl-CpG-binding domain protein 2 (MBD2) (Zhu et al. 2011). ADGRB1 inhibits angiogenesis through the release of N-terminal type-1 thrombospondin repeat fragments termed vasculostatins (Cork et al. 2012). The most N-terminal fragment is liberated by matrix metalloproteinase-14 cleavage to result in a 40 kDa protein (vasculostatin-40) whereas the entire N terminus (containing four additional thrombospondin repeat domains) can be released due to GAIN domain proteolysis.

By contrast to ADGRB1, ADGRE5 is upregulated in glioblastoma (GBM) and increases the invasiveness of GBM cells (Safaee et al. 2013). Moreover, rather than inhibit angiogenesis, the NT of ADGRE5 has been found to promote angiogenesis through chemotactic recruitment of endothelial cells, which is initiated by binding to integrins (Wang et al. 2005). In fact, ADGRE5 expression is induced in a wide range of

cancer cell lines and correlates with metastatic aggressiveness (Aust et al. 2016). One possible mechanism linking ADGRE5 to invasiveness in prostate and thyroid cancer involves heterodimerization with the LPA receptor and signaling via $G\alpha_{12/13}$ and the Rho pathway (Ward et al. 2013, Ward et al. 2011). ADGRF5 has been found to drive breast cancer metastasis through Rho pathway signaling but via $G\alpha_q$/p63RhoGEF rather than $G\alpha_{12/13}$ (Tang et al. 2013). Interestingly, it was hypothesized that ADGRF5 may be involved in metastasis due to its N terminal adhesion domains but, similar to ADGRE5, rather it appears to be G protein signaling that promotes tumor metastasis. However, this effect could be difficult to disentangle as ectodomain shedding may disrupt cell adhesion but it also activates ADGRE5 and very likely activates ADGRF5 as well.

ADGRL4/ELTD1 has also been found to be a pro-angiogenesis adhesion GPCR that is upregulated in GBM tumors and endothelial cells (Dieterich et al. 2012, Masiero et al. 2013, Towner et al. 2013). Very little is known about this receptor's signaling activity but it was reported that ADGRL4 expression was regulated by multiple pro-angiogenic factors including the Notch ligand DLL4, VEGF, and bFGF (basic Fibroblast Growth Factor) (Masiero et al. 2013). Importantly, siRNA knockdown of ADGRL4 attenuated vascular endothelial cell sprouting *in vitro* and inhibited tumor growth *in vivo* indicating that this receptor may also be a therapeutic target for multiple human cancers.

Recently, ADGRD1/GPR133 was implicated in GBM as well (Bayin et al. 2016). ADGRD1 was found to be expressed in hypoxic GBM cells and its expression levels in patients were inversely correlated with survival. In addition, it was reported that knockdown of D1 in mouse brain limited tumor growth and improved survival. These data suggest that ADGRD1 inhibitors could provide a new therapeutic avenue for GBM

by limiting the ability of GBM cells to survive in a hypoxic environment (Bayin et al. 2016).

1.7.3 Immune, Cardiac, and Pulmonary

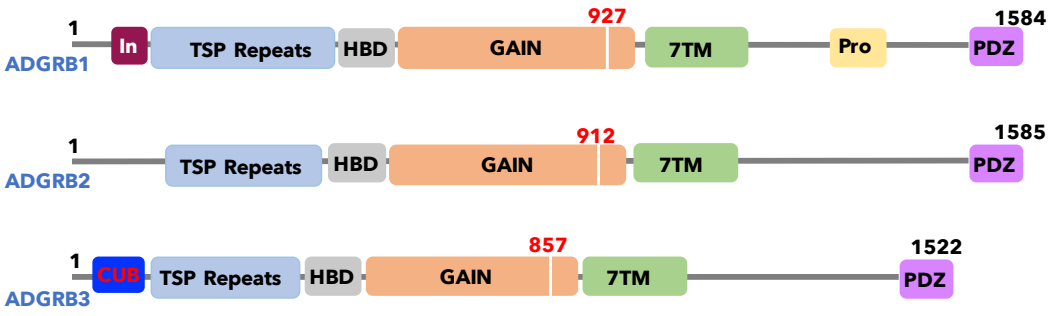
Adhesion GPCR research essentially began in immunology and aGPCRs remain highly relevant to the field. Expression in immune tissue is a common feature for all five members of the ADGRE family (EMR1-4, CD97), two ADGRG receptors (GPR56, GPR97), and ADGRB1 (Hamann et al. 2016). ADGRE2/EMR2 is widely expressed in myeloid tissue including macrophages, monocytes, and mast cells. A recent study found that a missense mutation that switches a cysteine to tyrosine upstream of the cleavage site in ADGRE2 cosegregated with vibratory urticaria, a condition where hives develop on the skin from typically innocuous stimuli, in two large families (Boyden et al. 2016). This study concluded that the loss of this cysteine residue destabilized the interactions between the extracellular NTF and the 7-TM domain, which lowered the threshold of mechanical stimulation that was necessary to induce mast cell degranulation. As mentioned above, ADGRE5 can bind to CD55 on T-cells (Capasso et al. 2006), and ADGRB1 can recognize phosphatidylserine (Park et al. 2007) and lipopolysaccharide (Das et al. 2011). In the CNS, ADGRB1 was shown to play a key role in the microglial clearance of dying neurons in collaboration with TIM-4 (Mazaheri et al. 2014) and may also have a role in astrocytic phagocytosis (Sokolowski et al. 2011). In general, while aGPCRs have been found to be involved in myriad immune functions, in most situations it is not yet clear how receptor adhesion and/or G protein signaling contribute.

The hybrid protein conceptualization of aGPCRs is epitomized by ADGRG6. Complete loss of G6 in mice results in embryonic lethality due to cardiovascular failure (Waller-Evans et al. 2010) and G6 is also required for myelination (Monk et al. 2011). However, in zebrafish, reintroduction of the G6 ectodomain up to the GPS rescued the cardiac defect but not myelination, indicating that the NT fragment alone is sufficient for cardiac functions but that the 7-TM GPCR domain is required for myelination (Patra et al. 2013). Mice lacking ADGRL4/ELTD1 displayed exaggerated cardiac hypertrophy following pressure overload (Xiao et al. 2012), which suggests that this receptor could be targeted in hypertrophic cardiomyopathy (Musa, Engel, and Niaudet 2016). Another ADGRL receptor, ADGRL2 may be involved in the epithelial-mesenchymal transition in heart valve development (Doyle et al. 2006).

Several aGPCRs are expressed in the lung (Ludwig, Seuwen, and Bridges 2016) but only ADGRF5/GPR116 has been studied in detail. Loss of functional ADGRF5 dramatically disrupts lung surfactant homeostasis in multiple mouse models (Bridges et al. 2013, Yang et al. 2013). In mice lacking F5, pulmonary surfactant, which is required for air breathing, accumulates in lung tissue (Bridges et al. 2013, Ludwig, Seuwen, and Bridges 2016). Mice lacking surfactant protein D have a similar phenotype to F5-null mice, and these proteins were found to interact in a co-immunoprecipitation, but it is unclear if surfactant protein D is an endogenous ligand for ADGRF5 (Fukuzawa et al. 2013).

Figure 6. The BAI sub-family of adhesion GPCRs

The domain and motif structure layout of ADGRB1-3. All three receptors contain N-terminal type-1 thrombospondin repeats ("TSP Repeats" B1: a.a. 264-575, B2: a.a. 314-528, B3: a.a. 206-508) a hormone-binding domain ("HBD" B1: a.a. 577-643, B2: a.a. 531-595, B3: a.a. 510-576) and GPCR autoproteolysis inducing domains ("GAIN" B1: a.a. 662-938, B2: a.a. 615-919, B3: a.a. 595-867) with the putative cleavage site marked by a break and first amino acid after cleavage in red. 7TM domains (B1: a.a. 948-1188, B2: a.a. 937-1174, B3: a.a. 881-1146) are followed by long intracellular C terminal tails ending in identical PDZ-binding motifs ("PDZ" B1: a.a. 1581-1584, B2: a.a. 1582-1585, B3: a.a. 1519-1522). B1 is characterized by a unique integrin-binding motif ("In" a.a. 231-233) and a C-terminal proline-rich region ("Pro" a.a. 1393-1437). B3 is unique among the three receptors in having an N-terminal CUB domain (a.a. 30-159).



1.8 The BAI sub-family of adhesion GPCRs

Brain-specific angiogenesis inhibitor 1 (BAI1/ADGRB1) (Nishimori et al. 1997) and shortly thereafter BAI2/ADGRB2 and BAI3/ADGRB3 were identified by molecular cloning (Shiratsuchi et al. 1997). These receptors are 45% identical at the amino acid level and are very similar in domain structure: all three contain at least four type-1 thrombospondin repeats in their distal N-termini, a juxtamembrane GAIN domain, similar 7-TM structures, and an identical C-terminal PDZ binding motif (Stephenson, Purcell, and Hall 2014). However, there are subtle differences in key domains that may underlie some of their unique functions. For example, only B1 contains an N-terminal integrin-binding motif and an extended proline-rich region in its C-terminus whereas B3 contains an N-terminal CUB domain.

The BAI receptors are enriched in the central nervous system but not limited to it. In fact, two seminal early reports on the function of B1 focused on its role in macrophages (Park et al. 2007) and skeletal muscle (Hochreiter-Hufford et al. 2013). Using a yeast two-hybrid screen in search for a receptor protein upstream of the Rac1 guanine-nucleotide exchange factor (GEF) complex ELMO1/DOCK180, B1 was identified (Park et al. 2007). A C-terminal RKR (arginine-lysine-arginine a.a. 1489-1491) motif was found to be the site of this interaction. This result suggested that the long C-terminus of B1 can function in signaling pathways beyond canonical heterotrimeric G protein signaling. This study also found that the N-terminal thrombospondin repeats of B1 can bind to phosphatidylserine, a marker of cellular apoptosis. Moreover, it was demonstrated that expression of B1 improved phagocytosis and together the results

suggested that B1 may function in the recognition and initiation of phagocytosis against apoptotic cellular targets. Interestingly, apoptosis is also an important part of the myoblast fusion process in skeletal muscle and mice lacking the receptor were reported to have smaller myofibers and impaired muscle regeneration following injury (Hochreiter-Hufford et al. 2013).

Even in the nervous system, B1 expression is not restricted to neuronal cell types. Studies have demonstrated its role in phagocytosis both in the astrocytes (Sokolowski et al. 2011) and microglia (Mazaheri et al. 2014) indicating that the receptor likely has a complex role in neural tissue. However, the most salient functions of B1 identified thus far may be at the synapse (Stephenson, Purcell, and Hall 2014). In the brain, mRNA transcripts of B1, B2, and B3 all peak within the first 10 postnatal days, a period of robust synaptic growth (Kee et al. 2004). Moreover, data from genetic knockdown and knockout studies have revealed the importance of B1 at the synapse.

In 2013, B1 was identified in another yeast two-hybrid screen, this time using the PDZ domain of the Rac1GEF Tiam1 as bait (Duman et al. 2013). B1 was found to recruit Tiam1 and the neuronal polarity protein Par3 to the post-synaptic density in dendritic spines. Knockdown of B1 in cultured neurons resulted in mislocalization of the Tiam1 complex, less activated Rac1 in spines, and aberrant spine morphology. Importantly, these defects could be rescued by re-introducing B1, suggesting that the receptor is necessary for these processes. It is also worth noting that these effects were independent of the C-terminal RKR ELMO1 interacting motif, further evidence that there are multiple non-redundant signaling pathways downstream of the receptor. The relative importance of these pathways may differ in different cellular contexts depending on the complement

of signaling proteins expressed in the cell and the type of extracellular signal the receptor receives.

Concurrent with these findings were biochemical data, which buttressed the case for B1 as an important synaptic protein. In a screen against an array containing PDZ domains of multiple proteins, B1 mostly interacted with those that are known to be found at the synapse (Stephenson et al. 2013). Indeed, Percoll fractionation of mouse brain lysates demonstrated that B1 is highly enriched in the post-synaptic density (PSD) and genetic deletion of *adgrb1* resulted in a significant reduction in the key PSD protein PSD-95 (Zhu et al. 2015).

Further study of mice completely lacking B1 revealed a significant cognitive deficit and dramatically altered synaptic physiology. *Adgrb1*$^{-/-}$ mice performed significantly worse in the Morris water maze, indicating a deficit in spatial learning and memory (Zhu et al. 2015). Based on this finding, it was hypothesized that the knockout animals may have a deficit in hippocampal synaptic plasticity. A common technique for measuring synaptic plasticity is to induce long-term potentiation (LTP) with a short burst of high frequency stimulation, or alternatively, long-term depression (LTD) with low frequency stimulation. Following LTP protocols, synapses are typically strengthened for a short period of time so that further stimulation of any kind will result in a more robust post-synaptic response. LTD leads to the opposite situation – it subsequently becomes more difficult to achieve the same response in the post-synaptic cell after the synapse is weakened. Intriguingly, in hippocampal slice preparations from *adgrb1*$^{-/-}$ mice, LTP was intact but synapses apparently lacked the capacity for LTD and, in fact, demonstrated a

paradoxical synaptic potentiation in response to LTD stimulation protocols (Zhu et al. 2015).

In light of the dramatic effects of reducing or removing ADGRB1 from synapses, it is somewhat surprising that the gene has not been implicated in neurological or psychiatric disorders to date. However, the B1-interacting PDZ protein DLG1/SAP97 is one of 19 genes lost in 3q29 microdeletion syndrome, which is among the highest known genetic risk factors for schizophrenia (Mulle et al. 2010). Based on the hypothesis that disruption of a receptor upstream of DLG1 might mediate some of the risk for schizophrenia (SZ), *ADGRB1* was sequenced in multiplex SZ families and several potential mutations were identified. One of these mutations is in a very similar region to the disease-associated R1465W ADGRB2 mutation (Chapter 3) and appears to also lead to a gain of function in terms of receptor signaling. These data will be presented and discussed in Chapter 4.

1.9 Research aims

Non-olfactory G protein-coupled receptors are exquisite therapeutic targets yet the adhesion family of GPCRs remains the largest family of orphan, "pharmacologically dark" receptors in the human genome. The members of the BAI sub-family (ADGRB1-3) possess enormous potential as brain-enriched receptors that can modulate dendritic morphology and synaptic function. In the absence of an identified endogenous ligand, it is therefore important that progress is made in understanding the activation, regulation, and function of these enigmatic receptors. Given the shared domain structure of many

aGPCRs, it is likely that insights from one member of the family will inform studies of at least several others.

The findings included in this dissertation are the result of three aims:

I. Determine if ADGRB1 is activated by a tethered agonist.

II. Investigate the effects of a disease-linked C-terminal mutation in ADGRB2.

III. Examine the contribution of the ADGRB1 cytoplasmic proline-rich region for protein-protein interactions and receptor signaling activity.

The initial aim of my dissertation research was to determine what role if any the post-cleavage stalk of ADGRB1 has in receptor activation. Previous work in our laboratory had demonstrated that B1 is in fact a G protein-coupled receptor and activates the Rho pathway (Stephenson et al. 2013). The BAI sub-family, however, is in the minority of adhesion receptors in that they are not readily cleaved in heterologous cells (Stephenson, Purcell, and Hall 2014). We sought to take advantage of this trait, which allowed us to study the signaling activity of the full-length, cleavage-mimetic, and further truncated forms in isolation. These data are discussed in Chapter 2.

The second aim of my project was to extend our investigation to ADGRB2, which was catalyzed by the discovery of a disease-associated mutation in the receptor in a human patient. The initial results of these studies led to more questions about how the signaling pathways downstream of B2 differ from B1, and how the receptors are regulated. The C-terminal tails of both B1 and B2 extend almost 400 amino acids into the cell from the end of the 7^{th} transmembrane domain but very little is understood about the structure and function of this region. Chapter 3 will focus on B2.

Finally, we investigated the role of the extended proline-rich region (PRR) in B1. This compositionally biased region is unique to B1 among the ADGRB receptors and we hypothesized that it is a key site of protein-protein interaction. We found that loss of the PRR dramatically biases the signaling output of B1.

In addition, we examined other cytoplasmic features of B1. The R1465W mutation in B2 led us to investigate three new mutations in B1 that have been linked to schizophrenia. These studies have directed our attention to new regions of the proteins that were not previously identified as critical loci of signaling and regulatory activity. Chapter 4 is devoted to the presentation of these data and a broader discussion of the C-terminal determinants of ADGRB1 signaling activity.

Ultimately, our aim has been to gain a deeper understanding of the structure and function of these receptors in ways that may be generalizable to additional aGPCR family members and may have clinical relevance for understanding and treating human disease.

Chapter 2. ADGRB1 activation – is there a tethered agonist?

2.1 Introduction

Adhesion G protein-coupled receptors (aGPCRs) are an unusual group of 33 seven-transmembrane-spanning (7TM) proteins that form the second largest family of GPCRs in humans (Langenhan, Aust, and Hamann 2013).[3] As described in Chapter 1, aGPCRs are widely distributed and critical for many physiological processes, including cell adhesion, neural development, angiogenesis and immune system function (Hamann et al. 2015, Promel, Langenhan, and Arac 2013). Despite their essential roles, the activation and signaling mechanisms aGPCRs are poorly understood, with most members still considered orphan receptors with no known ligands.

Nearly all aGPCRs have an N-terminal juxtamembrane GPCR Autoproteolysis-Inducing (GAIN) domain, which can cleave the receptor into two non-covalently associated protomers (Arac et al. 2012). As reviewed in Chapter 1, N-terminal cleavage is thought to be a critical activation step because a number of groups have reported that aGPCR mutants that mimic post-cleavage receptors exhibit enhanced constitutive activity; these include BAI1/ADGRB1 (Stephenson et al. 2013), BAI2/ADGRB2 (Okajima, Kudo, and Yokota 2010), GPR56/ADGRG1 (Paavola et al. 2011, Stoveken et al. 2015), GPR126/ADGRG6 (Paavola et al. 2014) CD97/ADGRE5 (Ward et al. 2011), GPR110/ADGRF1 (Stoveken et al. 2015) and VLGR1/ADGRV1 (Hu et al. 2014). These data prompted the proposal of a *disinhibition model* of aGPCR activation. In this model, the N-terminal fragment (NTF) inhibits the constitutive signaling ability of the 7TM protomer until the NTF is engaged by a large extracellular ligand, which results in a

[3] Portions of the text of this chapter, and much of the data presented in it, were published in the *Journal of Biological Chemistry* in 2016 (Kishore et al. 2016).

conformational change and/or removal of the NTF to relieve inhibition and unleash maximal receptor activity (Paavola and Hall 2012).

The disinhibition model is a general model that leaves open the mechanistic question of precisely *how* aGPCR NTF regions inhibit receptor signaling. At least two more mechanistically specific models have been discussed, one in which the NTF acts as a tethered allosteric antagonist to suppress signaling by the 7TM region and another model in which the NTF lacks antagonist activity *per se* but instead masks a cryptic agonist that becomes unveiled upon cleavage and removal of the NTF (Hamann et al. 2015). Multiple recent reports have provided evidence in support of the cryptic tethered agonist model (Table 1). Peptides mimicking the remaining post-cleavage NT stalk (also known as the "stachel") can activate GPR126/ADGRG6 and GPR133/ADGRD1 (Liebscher et al. 2014). Similarly, GPR110/ADGRF1 and GPR56/ADGRG1 can also be activated by stalk-mimetic peptides (Stoveken et al. 2015). These findings have raised the question of whether signaling by all aGPCRs is dependent upon agonistic sequences in the receptors' N-terminal stalk regions.

In the studies described here, we performed a series of tests of the cryptic agonist model for BAI1/ADGRB1, hereafter referred to as "B1". B1 is a receptor of great physiological interest because it has been shown to play a critical role in regulating macrophage phagocytosis (Park et al. 2007), muscle development (Hochreiter-Hufford et al. 2013) and synaptic plasticity in the brain (Duman et al. 2013, Zhu et al. 2015, Stephenson, Purcell, and Hall 2014).

To explore the importance of the stalk region of this aGPCR for receptor signaling, we created a mutant form of B1 that lack almost the entire NT, including the

stalk region. According to the cryptic agonist model, these deletions should render the receptor completely inactive due to a lack of the tethered agonist that is necessary for receptor activation. Since most if not all GPCRs can couple to multiple downstream pathways that may be differentially activated by distinct receptor active conformations (Kenakin 2011), the signaling activities of the B1 stalkless mutant was assessed in a battery of different assays to provide a panoramic view of the importance of the stalk region for receptor signaling.

Our aim in these studies was to determine whether the juxtamembrane stalk region of the ADGRB1 – that which would be left over after GAIN domain autoproteolytic cleavage – could serve as a tethered agonist as suggested by two early reports on other aGPCRs (Liebscher et al. 2014, Stoveken et al. 2015). Our 2016 publication addressed the activity of the potential tethered agonist in both ADGRG1 and ADGRB1 (Kishore et al. 2016). For ADGRG1, the findings were somewhat mixed and, in agreement with Stoveken and colleagues, the stalk peptide is necessary for some signaling pathways downstream of the receptor (Stoveken et al. 2015). The findings related to B1 are presented below.

2.2 Experimental procedures

2.2.1 Cell culture

HEK293T/17 cells (ATCC, Manassas, VA) were maintained in a humid, 5% CO_2, 37°C incubator with standard growth medium (DMEM (Gibco), 10% FBS (Atlanta Biologicals), 1% penicillin/streptomycin (Sigma)). Transfections utilized Mirus TransIT-LT1 (Madison, WI).

2.2.2 DNA Constructs

Full-length BAI1/ADGRB1 was provided by Dr. Erwin Van Meir (Emory University) and has been described previously (Kaur et al. 2003). B1ΔNT (927-1584) and B1-SL (944-1584) were sub-cloned into pcDNA3.1+ between 5' *EcoRI* (B1ΔNT: AGA CCA GAA TTC ATG TCC ACC TTC GCC ATC TTA GCC CAG CTC) or *HindIII* (B1-SL: AGA CCA AAG CTT ATG GCG ACT CTG CCG TCG GTG ACG CTC) and 3' *XbaI* (AGA CCA TCT AGA TCA GAC CTC GGT CTG GAG GTC GAT GAT GTC).

HA-ubiquitin was a gift from Keqiang Ye (Emory University), and HA-βarrestin2 (Luttrell et al. 1999) was a gift from Robert Lefkowitz (Addgene plasmid # 14692). EE-Gα_{13} was purchased from the cDNA Resource Center (cdna.org).

2.2.3 Western Blot

Protein samples were reduced in 1x Laemmli buffer, electrophoresed in 4-20% Tris-glycine gels and transferred to nitrocellulose membranes (Bio-rad, Hercules, CA).

Non-specific binding was blocked with 5% milk (in 50mM NaCl, 10mM HEPES pH 7.3, 0.1% Tween-20 (Sigma)) and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. The B1 C-terminal antibody was generously provided by Dr. Erwin Van Meir (Emory) and has been described previously (Stephenson et al. 2013), mouse monoclonal EE antibody from Abcam (Cambridge, MA), and Flag-HRP from Sigma. Blots were then washed, incubated with HRP-conjugated secondary antibodies (GE Healthcare) and visualized with Thermo Scientific SuperSignal West solutions on a Li-Cor Odyssey Imager.

2.2.4 Cell Surface Biotinylation

Twenty-four hours following transfection with 2-4ug of receptor DNA, balanced with pcDNA3.1, HEK-293T cells were washed in cold PBS+Ca^{2+} and incubated with 5mM Sulfo-NHS-Biotin (Thermo Scientific) or vehicle (PBS+Ca^{2+}) for 1hr on ice. Biotinylation was quenched with 100mM Glycine and cells were harvested in cold PBS. Membranes were ruptured with a rapid freeze-thaw and pelleted at 17,000xg for 15 min at 4°C. The membrane pellet was then re-suspended in 1% Triton X-100 buffer (25mM HEPES, 150mM NaCl, 10mM $MgCl_2$, 1mM EDTA, 1x HALT protease and phosphatase inhibitor (Thermo)) and rotated end-over-end for 45 min to solubilize membrane proteins. The insoluble fraction was then pelleted at 17,000xg for 15min at 4°C and solubilizates were incubated with streptavidin agarose (Thermo) for 30 min to precipitate biotinylated proteins. Agarose was washed with 1% Triton buffer and proteins were eluted in 2x Laemmli buffer. Biotinylated proteins were detected via Western Blot (above).

2.2.5 Co-immunoprecipitation

Cells were transfected with 1-4ug of receptor DNA (balanced with empty vector (EV) pcDNA3.1) and 1ug of G protein, arrestin, or ubiquitin. The following day, cells were harvested and membrane proteins were solubilized in 1% Triton X-100 buffer. Solubilizates were separated (above) and incubated with anti-HA agarose (Sigma), magnetic anti-Flag (Sigma), or protein A/G agarose (Thermo) beads with the indicated primary antibody for 1 hr end-over-end at 4°C. Samples were then washed, eluted, and Western blotted as described above.

2.2.6 Luciferase assays

HEK-293T cells were seeded in clear-bottom white 96-well plates 20-24 hours prior to transfection. Each well was transfected with 10ng of empty vector (EV) or receptor DNA, 50ng NFAT-luciferase (pGL4.30, Promega, Madison, WI), 1ng Renilla luciferase (pRL-SV40, Promega). Dual-Glo luciferase assays (Promega) were performed 48hr post-transfection and plates were read on a BMG Omega plate reader. The ratio of firefly:Renilla was calculated for each well and normalized to the mean of the EV-transfected controls.

2.3 Results

2.3.1 Removal of the NT does not impair receptor expression or signaling

To begin these studies, we first sought to determine whether an extremely truncated form of BAI1/ADGRB1 (B1) would be expressed and trafficked to the cell surface. When over-expressed in HEK-293T cells, B1 does not readily undergo autoproteolysis and most of the receptor is detected around 200 kDa, which corresponds to the full-length protein (Stephenson et al. 2013). The cleavage mimetic form of the receptor (B1ΔNT) is found near 75kDa and has been shown to reside on the cell surface (Stephenson et al. 2013). Thus, we expressed full-length B1 (a.a. 1-1584), and B1ΔNT (927-1584) alongside B1-SL (944-1584) and used a surface biotinylation-streptavidin pull-down approach to determine whether the receptors are abundantly expressed in the cells and if they are adequately trafficked to the cell membrane. We found that all three forms of the receptor are found at relatively abundant levels in both total cell lysates and on the cell surface, indicating that they are suitable for comparison in side-by-side signaling assays (Fig. 7).

Previous work in our laboratory has characterized B1 as a $G\alpha_{12/13}$-coupled receptor that activates the Rho pathway (Stephenson et al. 2013). Serum Response Factor (SRF) and Nuclear Factor of Activated T cells (NFAT) are transcription factors that can be activated downstream of many different upstream signaling events including $G\alpha_{12/13}$ (Nishida et al. 2007). We therefore utilized these outputs as luciferase reporters to further evaluate the signaling activity of B1. Advantages of the gene reporter approach included

increased signal-to-noise ratio, higher capacity to screen multiple conditions, and improved replicability.

Initially, the NFAT assay was meant to test the hypothesis that B1ΔNT might also have some $G\alpha_q$ activity, because NFAT is typically thought of as being downstream of $G\alpha_q$, phoshoplipase C, and Ca^{2+} signaling (Hill, Baker, and Rees 2001). However, what we found with this reporter aligns much closer with our initial hypothesis – only the cleavage mimetic B1ΔNT receptor activates NFAT-luciferase. The full-length receptor has no activity to this pathway.

We sought to test the tethered cryptic agonist hypothesis in ADGRB1 by truncating this stalk region away from the B1ΔNT receptor. We utilized a forward primer which placed a new start methionine before amino acid 944 to engineer a new B1-SL (stalk-less) receptor. Hydropathy plots and reference sequences suggested that the first TM domain of B1 begins around amino acid 946-7, so this B1-SL receptor essentially lacks the entire N terminus.

We hypothesized that full-length B1 would have some activity to SRF but that B1ΔNT would be much stronger in its activation. Surprisingly, we found B1 full-length, ΔNT, and B1-SL all strongly activated SRF, revealing the first major difference between the SRF-luciferase assay and the RhoA pull-down approach (Fig. 8A). Additionally, we found that only the two truncated forms of B1 – ΔNT and B1-SL – significantly activated the NFAT-luciferase reporter (Fig. 8B).

Figure 7. B1-SL is expressed on the cell surface

B1 receptor isoforms were over-expressed in HEK-293T cells. The next day, cells were washed, treated with a membrane impermeable biotinylation reagent, and biotinylated surface proteins were isolated by streptavidin agarose pull-down (bottom). Samples of total cell lysates were also blotted (top). All three forms of B1 – full-length, ΔNT, and SL were abundant in the cell surface fraction.

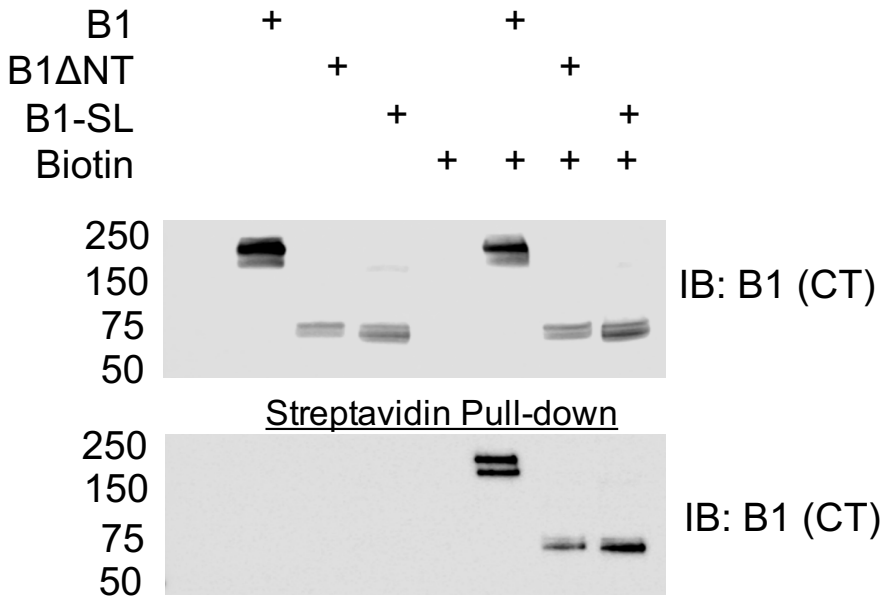
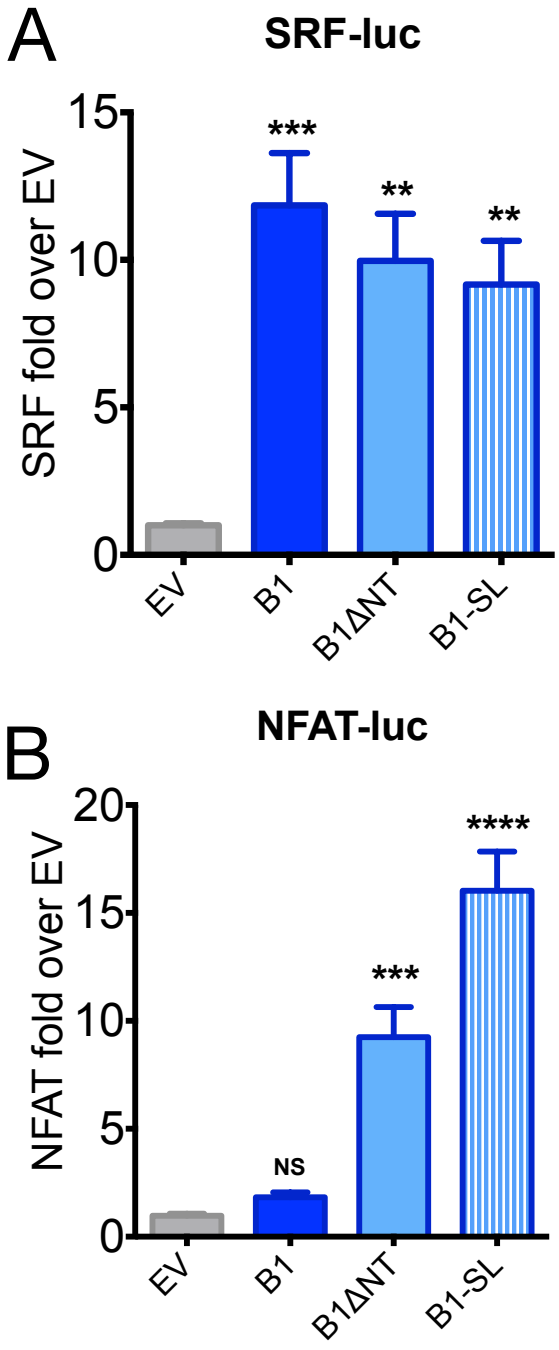


Figure 8. ADGRB1 N terminus is dispensable for signaling activity

B1, B1ΔNT, and B1-SL were expressed in HEK cells along with luciferase reporters (NFAT or SRF and *Renilla* control). All three forms of B1 significantly activated SRF-luciferase (n=5) but only the N-terminally truncated B1ΔNT, and B1-SL activated NFAT-luciferase (n=4). Data are expressed as a ratio of firefly:*Renilla* counts normalized to EV reporter alone controls (One-way ANOVA, **$p<0.01$, ***$p<0.001$ vs EV).

Figure 8. ADGRB1 N terminus is dispensable for signaling



2.3.2 B1ΔNT and B1-SL bind to β-arrestin2 and are ubiquitinated

One potential weakness of gene reporter assays is that there can be many mechanistic steps between G protein activation at the cell membrane and the induction of gene expression in the nucleus. Therefore, we sought to perform assays that were more membrane proximal to alleviate doubts that the signaling we observed was, in fact, the result of constitutively active receptors. One way to assess the activity state of a receptor is to test whether it interacts with β-arrestin. The β-arrestins are regulatory proteins that, as their name suggests, can bind to active receptors and arrest their G protein signaling activity (Luttrell and Lefkowitz 2002). Most GPCRs can only be observed interacting with β-arrestins in the presence of the receptor's agonist and perhaps also with the help of chemical crosslinking agents to stabilize the interaction. Nonetheless, we have previously shown that B1ΔNT (but not full-length B1) strongly interacts with β-arrestin2 (Stephenson et al. 2013). Similarly, we found that in addition to B1ΔNT, B1-SL strongly co-immunoprecipitated with β-arrestin2 when co-expressed in HEK-293T cells (Fig. 9).

An additional proxy measure for receptor activity can be its ubiquitination state. Ubiquitin is a small polypeptide that is added to lysine residues by E3 ubiquitin ligases and highly active receptors are often ubiquitinated to mark them for proteasomal degradation as a mechanism of downregulation (Marchese and Trejo 2013). Stephenson and colleagues had previously reported that similar to the β-arrestin2 results above, B1ΔNT is heavily ubiquitinated but the full-length receptor is not (Stephenson et al. 2013). Likewise, we replicated this result and found that B1-SL is also ubiquitinated in cells (Fig. 10).

Figure 9. ADGRB1ΔNT and B1-SL bind to β-arrestin2

All three isoforms of B1 were expressed with Flag- or HA-tagged β-arrestin2 or with EV DNA as a mock control. Experimental results with alternate forms of B1ΔNT (929-1584) and B1-SL (941-1584) were indistinguishable and were thus pooled. Only B1ΔNT and B1-SL were found to significantly interact with β-arrestin2, suggesting that these are both active forms of the receptor (One-way ANOVA, n=3, *$p<0.05$, **$p<0.01$).

Figure 9. ADGRB1ΔNT and B1-SL bind to β-arrestin2

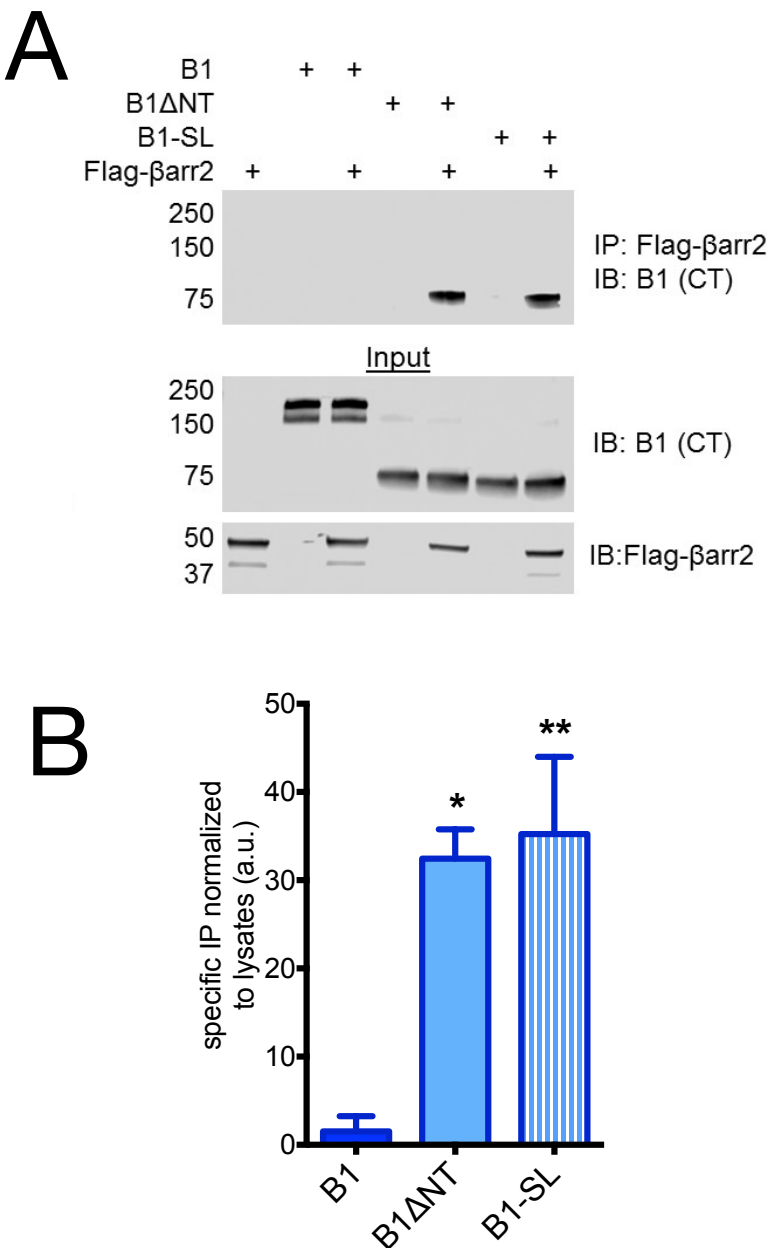
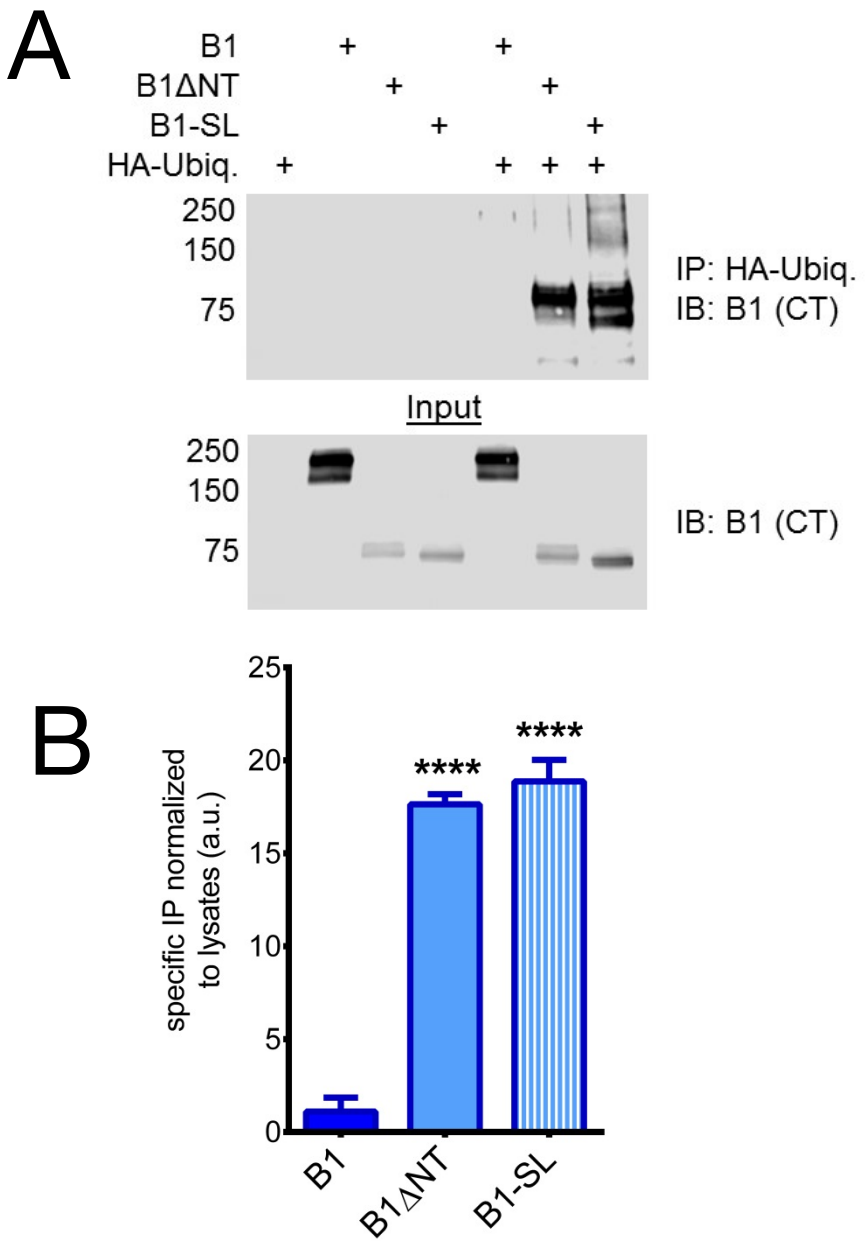


Figure 10. ADGRB1ΔNT and B1-SL are heavily ubiquitinated

The three isoforms of B1 – full-length (B1), B1ΔNT, and B1-SL were transfected into HEK-293T cells with empty vector control DNA or with HA-tagged ubiquitin. Tagged ubiquitin was immunoprecipitated on HA-agarose resin and interacting proteins were eluted and immunoblotted. Despite higher expression in total cell lysates, B1 was scarcely ubiquitinated. By contrast, B1ΔNT and B1-SL were heavily ubiquitinated (A, example blots, B, quantification, $p<0.0001$ for B1ΔNT and B1-SL vs full-length B1, n=3).

Figure 10. ADGRB1ΔNT and B1-SL are heavily ubiquitinated



2.3.3 $G\alpha_{13}$ binds to B1ΔNT and B1-SL and potentiates their signaling activity

Ubiquitination and interaction with β-arrestins are useful proxy measures for receptor activity but do not definitively demonstrate receptor activity. Therefore, we next attempted to directly assess whether B1-SL does indeed adopt an active conformation by attempting to co-immunoprecipitate the receptors with $G\alpha_{13}$. Receptor – G protein interactions can be fleeting and, similar to β-arrestin binding, typically require an abundance of agonist and biochemical tools such as cross-linking agents to be visualized on Western blots. However, since cleavage mimetic aGPCRs do not behave like prototypical GPCRs in β-arrestin binding, we reasoned that we might also be able to observe a relatively stable complex forming with their cognate G proteins. Indeed, using the internal Glu-Glu (EE) tag on $G\alpha_{13}$, we immunoprecipitated the G protein and found B1ΔNT and B1-SL but not full-length B1 in the IP fraction (Fig. 11).

To further demonstrate that B1 is a $G\alpha_{12/13}$-coupled receptor, we co-expressed $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$ with the receptors in NFAT-luciferase assays. Here, we found that both $G\alpha_{12}$ and $G\alpha_{13}$ significantly boosted the signaling activity of B1ΔNT (Fig. 12). Interestingly, $G\alpha_q$ significantly inhibited B1ΔNT NFAT activity, which may indicate that an overabundance of $G\alpha_q$ can act as a dominant negative and inhibit the receptor's activity by obstructing $G\alpha_{12/13}$ from interacting with the receptor.

Figure 11. ADGRB1ΔNT and B1-SL bind to $G\alpha_{13}$

To determine if B1ΔNT and B1-SL represent active forms of the receptor, all three DNA plasmids (B1, B1ΔNT, and B1-SL) were transfected into HEK-293T cells with EE-tagged $G\alpha_{13}$ or with control (EV) DNA. Receptors were co-immunoprecipitated with monoclonal anti-EE antibody and protein A/G agarose beads and immunoblotted. Only B1ΔNT and B1-SL were found in the co-IP fraction suggesting that both of these isoforms are recognized by $G\alpha_{13}$ as active conformations of the receptor (B, quantification of Western blots, B1ΔNT *$p<0.05$ and B1-SL **$p<0.01$ vs full-length B1, n=4).

Figure 11. ADGRB1ΔNT and B1-SL bind to $G\alpha_{13}$

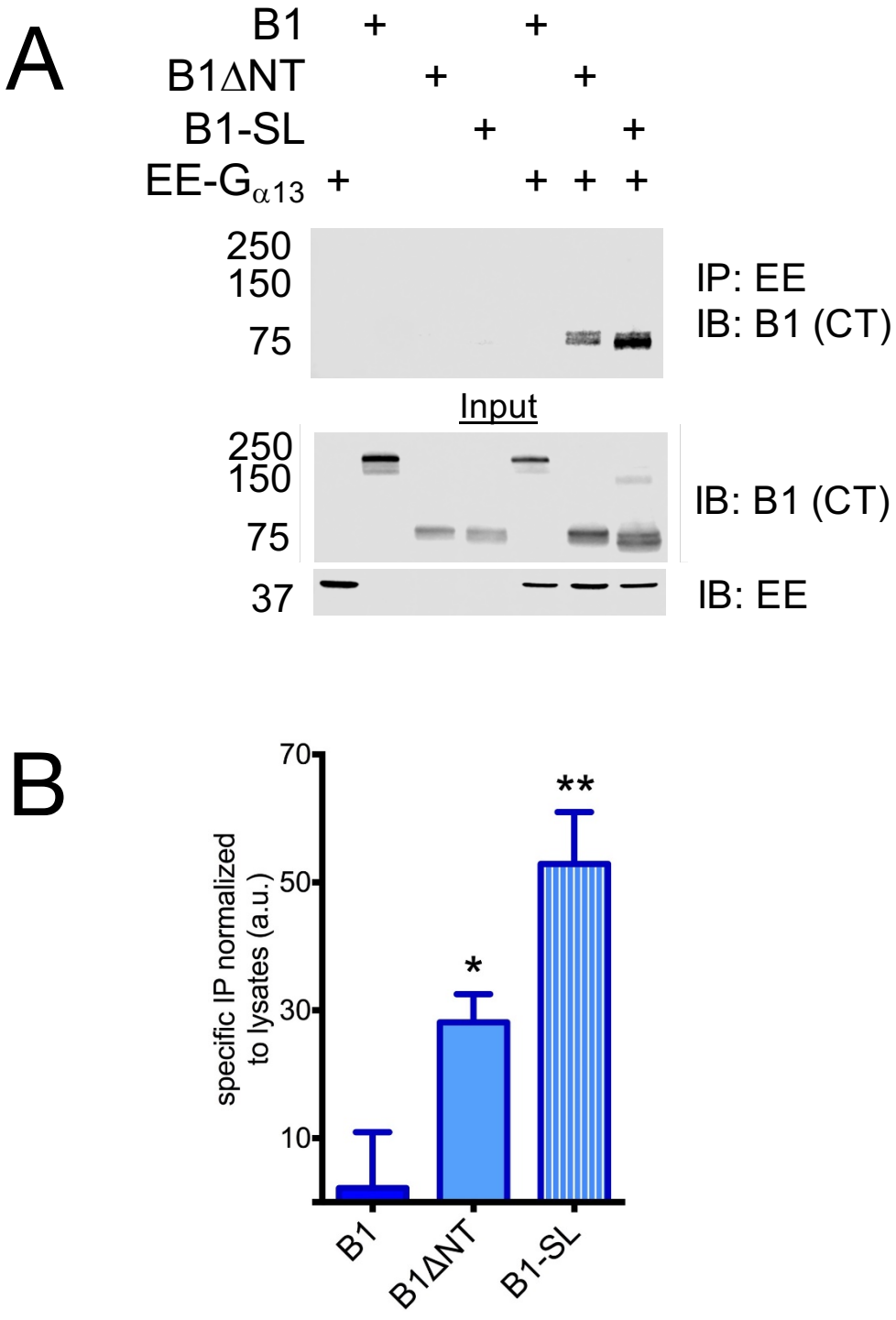


Figure 12. $G\alpha_{12}$ and $G\alpha_{13}$ potentiate signaling activity to NFAT luciferase

$G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$ were co-expressed with all three isoforms of B1 in NFAT luciferase assays. Only $G\alpha_{12}$ and $G\alpha_{13}$ potentiated signaling by B1ΔNT and $G\alpha_q$ significantly inhibited it suggesting that $G\alpha_{12}$ and $G\alpha_{13}$ are the cognate G proteins for B1 (*$p<0.05$, n=5).

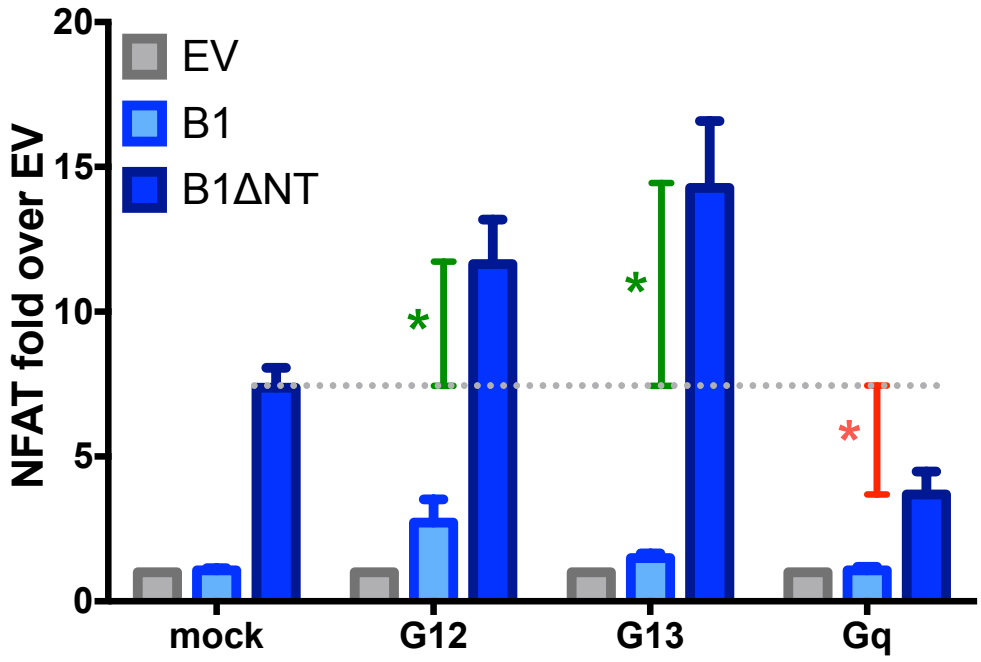
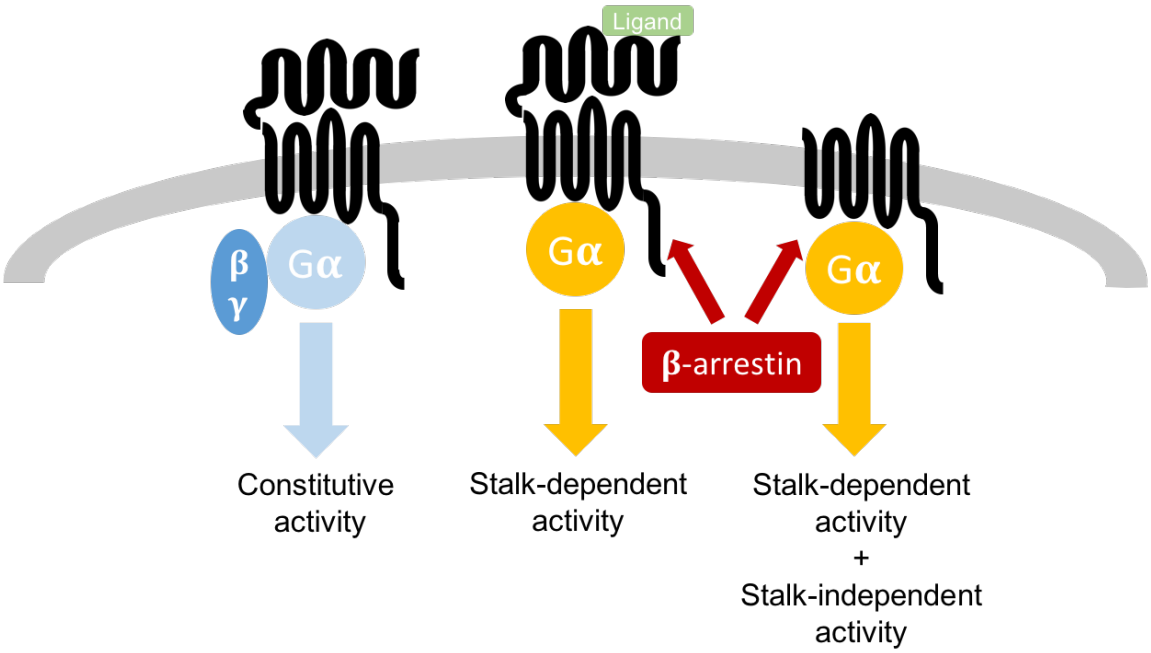


Figure 13. Allosteric antagonist model

From left to right, a depiction of three states of receptor activity. In the unstimulated state, some aGPCRs including ADGRB1 have constitutive activity to certain pathways (SRF in the case of B1). A ligand can bind (center) and stimulate stalk-dependent activity by subtly shifting the conformation of the receptor without relieving the inhibitory constraint of the NTF on the 7TM. If the NTF is completely removed, than the intrinsic, stalk-independent activity of the receptor is expressed along with any stalk-dependent activity.



2.4 Discussion of ADGRB1 activation mechanisms

Substantial recent progress has been made in understanding the mechanisms of aGPCR activation. These mechanisms are important to understand given the association of these receptors with several human diseases and the potential value of these receptors as pharmaceutical targets (Langenhan, Aust, and Hamann 2013). Specifically, it has been found for a number of aGPCRs that truncation of the receptors' N termini up to the point of predicted GAIN domain cleavage leads to increased constitutive activity (Hu et al. 2014, Okajima, Kudo, and Yokota 2010, Paavola et al. 2011, Stephenson et al. 2013, Stoveken et al. 2015). These observations led to the proposal of the disinhibition model of aGPCR activation, which posits that the NTF exerts an inhibitory constraint on signaling by the 7TM region, with this inhibitory constraint being removed following engagement of the NTF with a large extracellular ligand that results in either dissociation of the NTF from the 7TM and/or a conformational change that reduces NTF-mediated inhibition (Paavola and Hall 2012). Subsequently, more mechanistically specific variations of the disinhibition model have been proposed, including the cryptic agonist model (Liebscher et al. 2014, Stoveken et al. 2015), wherein GAIN domain cleavage and NTF dissociation result in the unveiling of a cryptic agonist peptide on the post-cleavage stalk in a manner analogous to protease-activated receptors (Coughlin 2000).

In the studies described in this Chapter, we tested the cryptic agonist model in the aGPCR ADGRB1 by deleting the stalk region and broadly assessing receptor activity using a variety of downstream outputs. Our results provide evidence that the stalk region is not a requisite agonist for this aGPCR, as we observed that deleting the stalk does not affect signaling in any pathway measured. For ADGRB1/BAI1 ("B1"), removal of the

stalk region made no difference whatsoever for any assessment of receptor activity. In contrast, parallel studies on ADGRG1/GPR56 ("G1") resulted in mixed findings, with removal of the stalk region largely abrogating the receptor's ability to stimulate SRF luciferase but having no effect on the other readouts examined (data not shown, see Kishore et al. 2016). Based on these findings, we propose that aGPCRs can mediate both stalk-dependent and stalk-independent signaling, with the relative contribution of the stalk to receptor activity varying substantially between different receptors as well as between different outputs examined.

In addition, these results provide more support for the $G\alpha_{12/13}$ coupling of B1. This coupling was first posited in 2013 based on the ability of the transfectable $G\alpha_{12/13}$ inhibitor RGSp115 to block B1ΔNT signaling to the Rho pathway (Stephenson et al. 2013) and we replicated this result (Kishore et al. 2016). We also showed that both B1ΔNT and B1-SL interact with $G\alpha_{13}$ and that $G\alpha_{12}$ or $G\alpha_{13}$ can potentiate B1ΔNT signaling to NFAT. Interestingly, $G\alpha_q$, a Gα subunit that would be expected to potentiate the NFAT signal disrupted B1ΔNT signaling, which may indicate that an overabundance of this Gα can interfere with endogenous $G\alpha_{12/13}$ coupling to the receptor.

A recent study suggested that the NT stalk of G1 is necessary for signaling activity (Stoveken et al. 2015). This study reported signaling data from SRE luciferase experiments in transfected cells and GTP loading experiments in a reconstitution system. Our data are in agreement with the findings of Stoveken et al., as we found that the activity of G1-SL was sharply reduced compared to G1ΔNT in the SRF luciferase assay, which is very similar to the SRE luciferase assay (Kishore et al. 2016). However, in other assays in which we assessed G1 activity (TGFα shedding, NFAT luciferase, β-arrestin

recruitment and receptor ubiquitination), we found G1-SL to be in an active conformation and capable of mediating receptor signaling to a similar extent as G1ΔNT. These results suggest that the stalk region of G1 is necessary for certain aspects of receptor signaling activity but dispensable for others.

There have been prior indications that the cryptic agonist model may represent an incomplete description of aGPCR activation. For example, studies on the *C. elegans* aGPCR lat-1 demonstrated that mutations blocking cleavage of the receptor's GAIN domain exerted no effect on the *in vivo* function of the receptor (Promel, Frickenhaus, et al. 2012). These *in vivo* data find a parallel in the *in vitro* findings reported here regarding the non-cleaving G1-T383A mutant, which we found to exhibit no change in signaling activity relative to wild-type G1 (Kishore et al. 2016). According to the cryptic agonist model, GAIN-mediated cleavage should be essential for exposure of the agonistic peptide portion of the stalk region. Thus, observations that the activity of at least some aGPCRs is not modulated by GAIN cleavage obviously run counter to this model. Moreover, there is convincing evidence that some aGPCRs do not undergo GAIN-mediated cleavage (Promel, Waller-Evans, et al. 2012), an observation that needs to be taken into account in general models of aGPCR activation.

Another challenge faced by the cryptic agonist model is the uncertainty surrounding how aGPCR NTF regions become dissociated from their cognate 7TM regions. In the cryptic agonist model, it is envisioned that the N-terminal portion of a cleaved GAIN domain can be released from the receptor's stalk region in a regulated manner, thereby exposing the agonistic stalk peptide sequence (Liebscher et al. 2014, Stoveken et al. 2015). However, descriptions of the first crystal structures of GAIN

domains have cast doubt on whether GAIN domains can actually exist as stable folded protein units in the absence of the hydrophobic stalk peptides (Arac et al. 2012). Thus, while it is clear that aGPCR NTF regions can become dissociated from their cognate 7TM regions (Hamann et al. 2015), it is uncertain whether dissociated GAIN domains leave the stalk behind or take the stalk with them. Interestingly, studies on ADGRL1/CIRL/latrophilin-1 provided evidence that the release of this receptor's NTF region is dependent on two proteolytic steps, with GAIN domain cleavage followed by a second cleavage event that cleaves the receptor's stalk region to release the GAIN domain and stalk together (Krasnoperov et al. 2009). According to the cryptic agonist model, the resultant 7TM region of such a twice-cleaved aGPCR would be devoid of signaling activity, as the stalk region containing the agonistic peptide would have been lost with the second cleavage event. However, our studies on stalkless versions of G1 and B1 demonstrate that stalkless receptors can still exert significant downstream signaling, albeit signaling that is altered in some cases (as in the case of G1) relative to receptors that possess an intact stalk (Kishore et al. 2016).

Understanding the natural mechanism(s) of aGPCR activation is a critical step toward facilitating drug development efforts aimed at these receptors. For example, the elucidation of agonistic peptide sequences on the N-terminal stalks of certain aGPCRs (Liebscher et al. 2014, Stoveken et al. 2015) has provided insights that may lead to the development of peptidomimetic small molecules with agonistic activity at these receptors. Similarly, the findings reported here that cryptic agonist sequences on aGPCR stalks do not account for the entirety of aGPCR signaling are important because these observations suggest an additional antagonistic effect of tethered GAIN domains on

aGPCR activity beyond the simple masking of the stalk region. Therefore, we propose an allosteric antagonist model of aGPCR activation, in which the NTF can antagonize receptor activity in two distinct ways: *i)* by masking the stalk region and *ii)* by directly antagonizing the inherent stalk-independent constitutive activity of the 7TM region (Fig. 13). This model is consistent with the data presented here as well as in previous studies (Liebscher et al. 2014, Stoveken et al. 2015) and furthermore is consistent with the possibility that aGPCRs may still signal even if they are not cleaved at the GAIN domain or lose their stalk following GAIN cleavage.

Further insights into the structural determinants of the antagonistic relationship between aGPCR NTF and 7TM regions may help to facilitate discovery of distinct classes of small-molecule aGPCR modulators that either block or potentiate NTF-mediated suppression of aGPCR 7TM signaling. Additionally, a model in which aGPCRs can mediate both stalk-dependent and stalk-independent signaling has clear implications for the future development of biased agonists targeting these receptors (Luttrell, Maudsley, and Bohn 2015). In many cases, it is therapeutically desirable for a molecule to have functional selectivity and target some but not all pathways downstream of a given receptor. Thus, it will be of interest going forward to study the various members of the aGPCR family on a receptor-by-receptor basis in order to understand the structural determinants of receptor coupling to different downstream signaling pathways in order to facilitate the discovery of biased ligands possessing therapeutic potential.

Chapter 3. A disease-associated C-terminal mutation in ADGRB2 potentiates receptor signaling activity

3.1 Introduction

Genomic approaches are increasingly utilized as clinical strategies to detect genetic variants that underlie pathologies. These approaches are valuable for developing targeted therapeutics and have also yielded a wealth of information related to the function of unfamiliar genes and proteins (Hamburg and Collins 2010). To date, very little is known about most of the adhesion family of GPCRs but genetic data has been useful in ascribing functions to several of these receptors. For example, mutations in ADGRG1/GPR56 result in the cortical malformation bilateral frontoparietal polymicrogyria (Piao et al. 2004), mutations in ADGRG6/GPR126 severely disrupt peripheral myelination (Ravenscroft et al. 2015), and mutations to ADGRV1/VLGR1 cause deafness and retinitis pigmentosa (Weston et al. 2004).

As described in the previous chapters, much of the focus of adhesion GPCR research has been concentrated on their strikingly long extracellular amino (N) termini. These regions contain multiple domains – including adhesion folds – and nearly all of the more than 30 human aGPCRs contain a juxtamembrane GPCR Autoproteolysis Inducing (GAIN) domain. This hallmark aGPCR feature has autoproteolytic ability and can sever the receptors into two non-covalently associated protomers – an extracellular N-terminus and a C-terminal portion containing the archetypal seven transmembrane (7-TM) domain (Arac et al. 2012).

ADGRB2 (B2) is one of three ADGRB sub-family receptors, which were previously known as Brain-specific Angiogenesis Inhibitor 1-3 (BAI1-3) (Stephenson, Purcell, and Hall 2014). As was reviewed in Chapter 1, these receptors are expressed in (not limited to) brain tissue and ADGRB1 and ADGRB3 have been shown to have

important roles at synapses (Bolliger, Martinelli, and Sudhof 2011, Duman et al. 2013, Sigoillot et al. 2015). At this point, much less is known about the function of ADGRB2. In fact, mice lacking B2 were found to have no gross deficits but had increased hippocampal neurogenesis and displayed a resilience to learned-helplessness behavior (Okajima, Kudo, and Yokota 2011).

While most aGPCRs remain orphan receptors with no known endogenous ligands, substantial progress has been made in understanding the activation mechanisms and signaling activity of many of these receptors. As discussed in Chapter 2, the extraordinarily long N-termini have an inhibitory effect on the constitutive signaling activity of the 7-TM domain in most aGPCRs studied thus far. For many aGPCRs, including ADGRB1 (Stephenson et al. 2013) and ADGRB2 (Okajima, Kudo, and Yokota 2010), and at least six other aGPCRs from five different sub-families (Kishore and Hall 2016), removal of the NT results in a constitutively active receptor. One of the earliest reports of this phenomenon demonstrated that removing the N-terminus of B2 unveils the constitutive activity of the receptor, as measured by the NFAT luciferase reporter (Okajima, Kudo, and Yokota 2010) but it remains unclear which G protein alpha subunit (Gα) primarily couples to the receptor.

Despite substantial progress in understanding how aGPCRs are activated, at this point, very little is known about how these receptors are regulated. Previous reports from our lab have shown C-terminal interactions with many different proteins containing PDZ domains (Kreienkamp et al. 2000, Tobaben, Sudhof, and Stahl 2000) and have demonstrated that with the constitutively-active forms of ADGRB1 and ADGRG1 co-immunoprecipitate with β-arrestins (Paavola et al. 2011, Stephenson et al. 2013).

However, it remains unclear whether these interactions can fully explain how the receptors are internalized and undergo post-endocytic trafficking.

The present deeper investigation into the signaling activity and regulation of B2 was spurred by the discovery of a *de novo* mutation in a human patient suffering from progressive spastic paraparesis among other symptoms (Arg1465Trp), by the NIH Undiagnosed Diseases Program. We engineered this Arg to Trp substitution into B2 expression constructs and studied the effect of this mutation on receptor signaling, trafficking and protein-protein interactions. Our data reveal that this mutation increases B2 signaling activity and surface expression, and also disrupts interaction with the regulatory protein endophilin A1. Moreover, our results provide evidence that B2 predominantly couples to $G\alpha_z$, with the mutation promoting improved $G\alpha_i$ coupling.

3.2 Experimental procedures

3.2.1 Cell culture

HEK293T/17 cells (ATCC, Manassas, VA) were maintained in a humid, 5% CO_2, 37°C incubator with standard growth medium (DMEM (Gibco), 10% FBS (Atlanta Biologicals), 1% penicillin/streptomycin (Sigma)). Transfections utilized Mirus TransIT-LT1 (Madison, WI).

3.2.2 DNA constructs

Human ADGRB2 wild-type and R1465W plasmids were synthesized in pcDNA3.1 vectors (Genscript, Piscataway, NJ). B2ΔNT and B2ΔNT-RW (912-1584) were sub-cloned into pcDNA3.1+ between 5' KpnI (AGA CCA TCT ACA TTT GCT GTA CTA GCT CAA CCT CCT) and 3' EcoRI (AGA CCA GAA TTC TCA AAC TTC TGT CTG GAA GTC ACC ATC AGG) from each of these templates and sequences were verified (Eurofins Genomics, Louisville, KY). In a separate set of plasmids, the Flag epitope (DYKDDDDK) was added to the C terminus of each receptor by an additional round of PCR in order to differentiate between transfected and endogenous B2.

GFP-EndophilinA1 was a gift from Kozo Kaibuchi (Nagoya University), GST-EndoA1-SH3 was provided by Harvey McMahon (Cambridge University), EE-tagged G protein α subunits were a gift from John Hepler (Emory University) and RGS20 (splice variant 2) were purchased from the cDNA Resource Center (cdna.org), HA-ubiquitin was

a gift from Keqiang Ye (Emory University), and HA-βarrestin2 (Luttrell et al. 1999) was a gift from Robert Lefkowitz (Addgene plasmid # 14692).

3.2.3 Western Blot

Protein samples were reduced in 1x Laemmli buffer, electrophoresed in 4-20% Tris-glycine gels and transferred to nitrocellulose membranes (Bio-rad, Hercules, CA). Non-specific binding was blocked with 5% milk (in 50mM NaCl, 10mM HEPES pH 7.3, 0.1% Tween-20 (Sigma)) and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. The B2 C-terminal antibody was purchased from Mab Technologies (Stone Mountain, GA), mouse monoclonal EE antibody from Abcam (Cambridge, MA), and Flag-HRP from Sigma. Blots were then washed, incubated with HRP-conjugated secondary antibodies (GE Healthcare) and visualized with Thermo Scientific SuperSignal West solutions on a Li-Cor Odyssey Imager.

3.2.4 Cell Surface Biotinylation

Twenty-four hours following transfection with 2ug of receptor DNA, HEK-293T cells were washed in cold PBS+Ca^{2+} and incubated with 5mM Sulfo-NHS-Biotin (Thermo Scientific) or vehicle (PBS+Ca^{2+}) for 1hr on ice. Biotinylation was quenched with 100mM Glycine and cells were harvested in cold PBS. Membranes were ruptured with a rapid freeze-thaw and pelleted at 17,000xg for 15 min at 4°C. The membrane pellet was then re-suspended in 1% Triton X-100 buffer (25mM HEPES, 150mM NaCl, 10mM $MgCl_2$, 1mM EDTA, 1x HALT protease and phosphatase inhibitor (Thermo)) and rotated end-over-end for 45 min to solubilize membrane proteins. The insoluble fraction was then pelleted at 17,000xg for 15min at 4°C and solubilizates were incubated with

streptavidin agarose (Thermo) for 30 min to precipitate biotinylated proteins. Agarose was washed with 1% Triton buffer and proteins were eluted in 2x Laemmli buffer. Biotinylated proteins were detected via Western Blot (above).

3.2.5 Co-immunoprecipitation

Cells were transfected with 2ug of receptor and 1ug of G protein, arrestin, or ubiquitin DNA. The following day, cells were harvested and membrane proteins were solubilized in 1% Triton X-100 buffer. Solubilizates were separated (above) and incubated with anti-HA agarose (Sigma), magnetic anti-Flag (Sigma), or protein A/G agarose (Thermo) beads with the indicated primary antibody for 1 hr end-over-end at 4°C. Samples were then washed, eluted, and Western blotted as described above.

3.2.6 Luciferase assays

HEK-293T cells were seeded in clear-bottom white 96-well plates 20-24 hours prior to transfection. Each well was transfected with 50ng of empty vector (EV) or receptor DNA, 50ng NFAT-luciferase (pGL4.30, Promega, Madison, WI), 1ng Renilla luciferase (pRL-SV40, Promega). Dual-Glo luciferase assays (Promega) were performed 48hr post-transfection and plates were read on a BMG Omega plate reader. The ratio of firefly:Renilla was calculated for each well and normalized to the mean of the EV-transfected controls.

3.3 Results

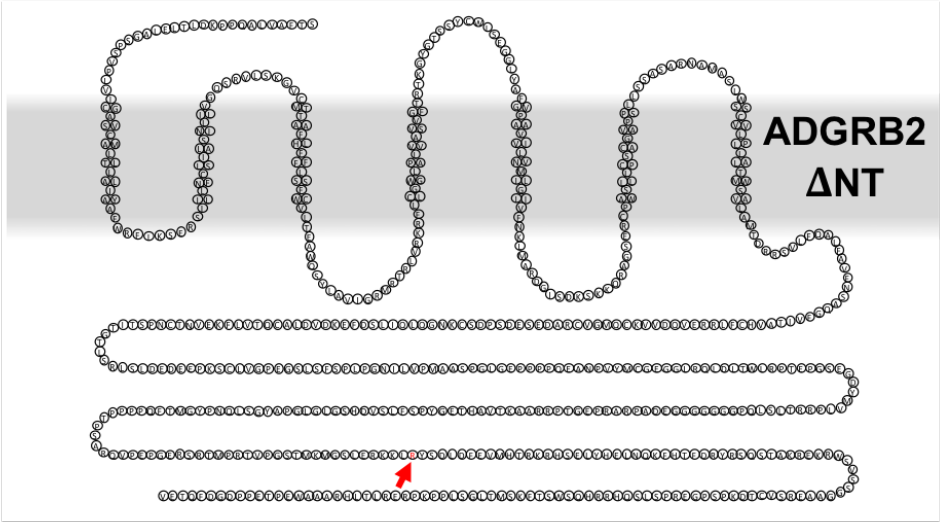
3.3.1 ADGRB2 R1465W

The NIH Undiagnosed Diseases Program (UDP) performed whole exome sequencing on a 46-year-old female who presented with spastic quadriparesis. This patient's symptoms began with visual disturbances at age 16, followed by a progressive spastic paraparesis that necessitated a wheelchair at age 21. Other symptoms included atrophy of the cervical and thoracic spinal cord, with reflexes intact, and an abnormal EMG suggestive of length-dependent motor neuropathy or neuronopathy with signs of chronic denervation. Somatosensory-evoked potentials were also abnormal and pulmonary function was decreased, with significant reductions in maximal inspiratory and expiratory pressures. Whole exome sequencing of the patient and her parents by the NIH-UDP revealed a *de novo* mutation in ADGRB2/BAI2 (B2) in the patient: NM_001703.2(BAI2):c.4393C>T, Arg1465Trp. This R1465W mutation is located in the middle of the B2 C-terminus (Fig. 14A) and has a CADD score of 22.7, indicating a high likelihood that the mutation is deleterious. Thus, given that the whole exome sequencing did not reveal any other mutations that might plausibly account for the observed pathology, we engineered this mutation into human B2 expression plasmids and explored whether the R1465W mutation might alter B2 function.

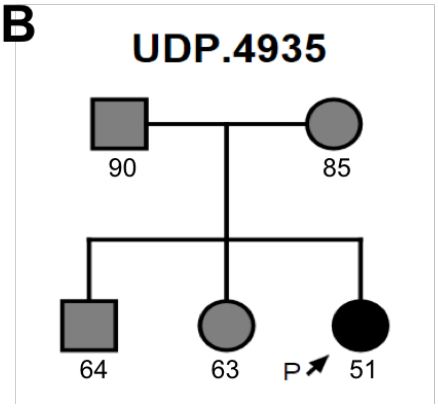
Figure 14. ADGRB2 R1465W

The NIH Undiagnosed Diseases Program discovered a missense mutation in ADGRB2/BAI2 by whole exome sequencing of a patient presenting with an unexplained neuromuscular disorder. The exchange of cytosine for thymine at position 4393 (NM_001703.2) results in a substitution of tryptophan for arginine at amino acid 1465 as indicated by the topological diagram (A, red arrow). B, Family pedigree, patient indicated by black arrow. C, Region surrounding R1465 is highly conserved.

A



B



C

Species	Alignment	Overall Identity
Human	GSLERKKLRYSDLD	
P. troglodytes	GSLERKKLRYSDLD	97%
B. taurus	GSLERKKLRYSDLD	95%
M. musculus	GSLERKKLRYSDLD	93%
G. gallus	GSLERKRLRYSDLD	71%
X. tropicalis	GSLERKRLRYSDLD	60%

3.3.2 ADGRB2 R1465W increases signaling activity and surface expression

To investigate the role of the R1465W mutation on ADGRB2 (B2) signaling activity, we over-expressed full-length and cleavage-mimicking (ΔNT) forms of the wild-type (WT) and mutant receptors and assessed activation of a panel of luciferase reporters. Serum response factor (SRF) and nuclear factor of activated T cells (NFAT) were the most likely outputs based on homology to ADGRB1/BAI1 (Stephenson et al. 2013) and a previous report about B2 (Okajima, Kudo, and Yokota 2010). We found that transfection of full-length B2 into HEK-293T cells did not result in activation of either reporter, either for the WT or mutant forms of the receptor (Fig. 15). However, we found that B2ΔNT robustly activates NFAT-luciferase (One-way ANOVA, $F(4, 15) = 66.61$, Sidak *post-hoc* test vs EV, $p<0.0001$, $n=4$), consistent with previous findings (Okajima, Kudo, and Yokota 2010). Furthermore, we observed that the R1465W mutation significantly potentiates this signaling activity to NFAT luciferase ($p=0.0001$ vs B2ΔNT, $n=4$).

To test potential effects of the B2 R1465W mutation on receptor insertion in the plasma membrane, we assessed cell surface expression of the WT and mutant forms of the receptor using a surface biotinylation approach. These studies revealed that the B2ΔNT R1465W mutation exhibited significantly increased surface expression over WT B2ΔNT (Fig. 16; one sample t test of surface expression normalized to WT B2ΔNT: B2ΔNT-RW=2.06 ±0.15, $p=0.0019$, $n=5$).

Figure 15. ADGRB2 R1465W increases signaling activity

WT and R1465W mutant forms of full-length (B2) and cleavage-mimicking (ΔNT) forms of the receptor were transfected into HEK-293T cells along with luciferase reporter plasmids. Assays were performed 48hrs post-transfection. The full-length WT receptor (B2) had no observable activity in this assay but B2ΔNT strongly activated the NFAT reporter (****p<0.0001 vs EV, n=4). The RW mutation did not increase signaling of the full-length receptor (B2-RW) but significantly augmented signaling by B2ΔNT (### p<0.001 vs WT B2ΔNT, n=4).

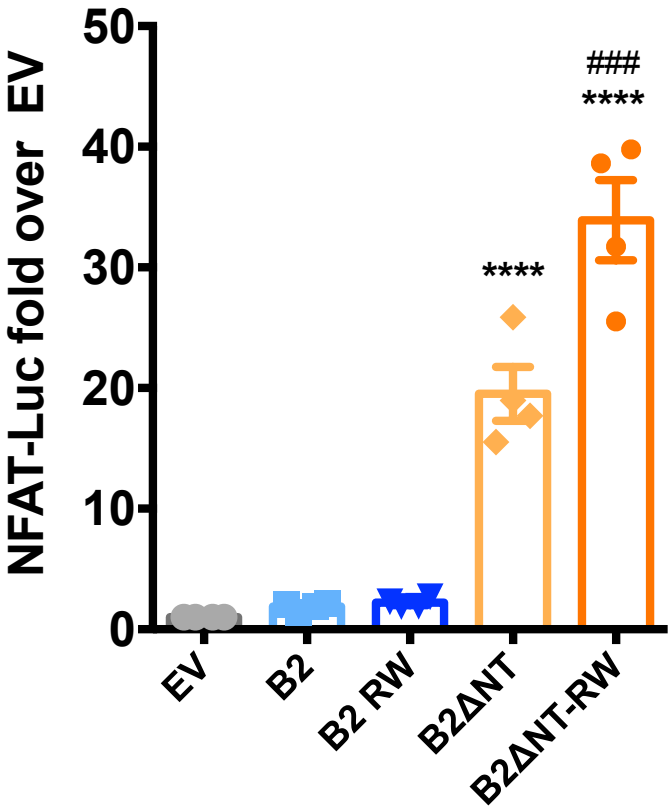
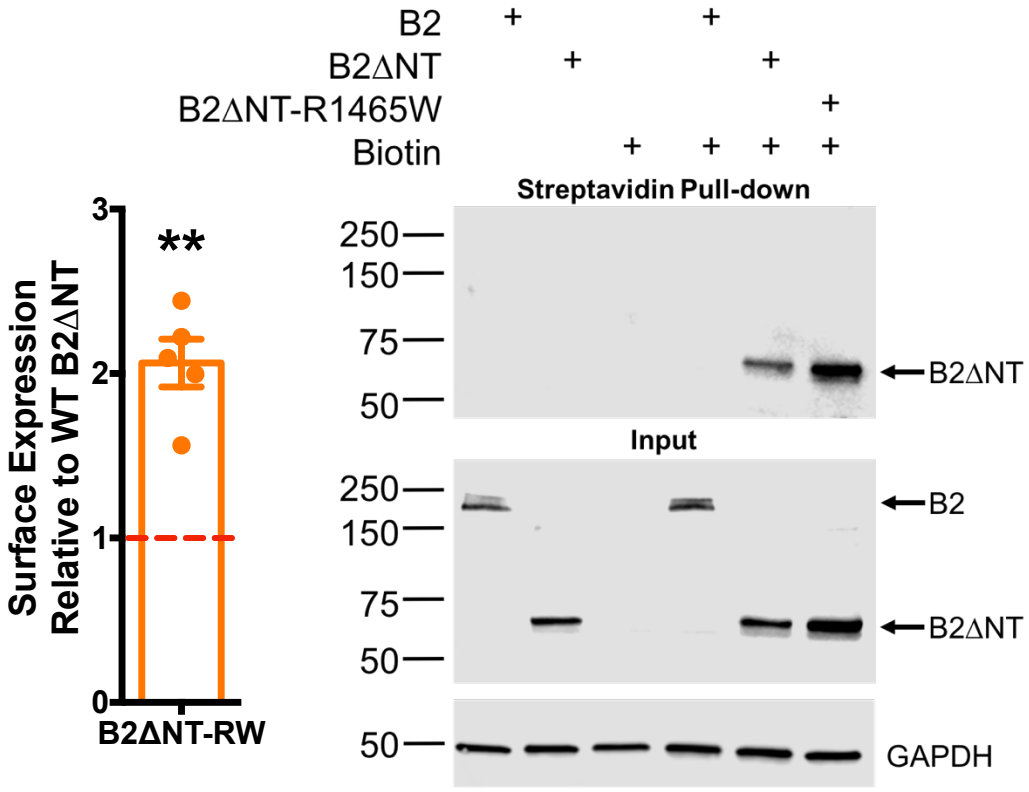


Figure 16. B2 R1465W increases surface expression

Cells were treated with a plasma membrane-impermeable biotinylation reagent to label all free amines on the cell surface and then these biotinylated proteins were purified out of soluble cell lysates with streptavidin beads. The R1465W mutation significantly increased the amount of B2ΔNT found in the surface fraction approximately two-fold (Inset, **p<0.01 normalized to WT B2ΔNT, n=5).



3.3.3 B2ΔNT signals to NFAT luciferase via Gβγ and a calcium channel

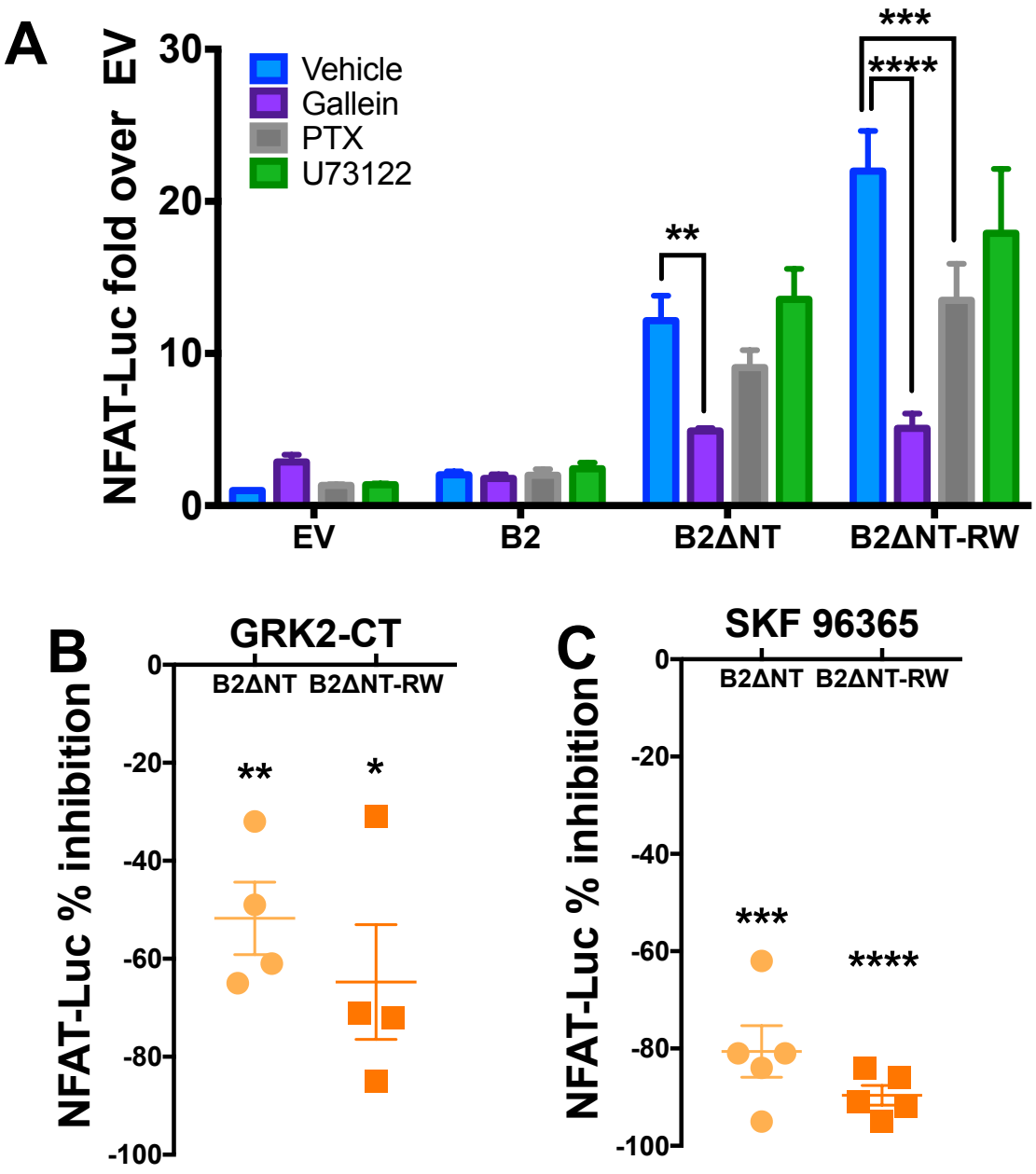
To shed light on the signaling pathway by which B2ΔNT activates NFAT luciferase, a number of different inhibitors were deployed. The B2ΔNT signal to NFAT luciferase was strongly inhibited by the Gβγ subunit inhibitor gallein (Lehmann, Seneviratne, and Smrcka 2008) (Fig. 17; Two-way ANOVA, main effect of inhibitor treatment, $F(3, 56) = 10.06$, Holm-Sidak *post-hoc* test B2ΔNT $p<0.001$ vs vehicle for gallein, B2ΔNT-RW $p<0.0001$ vs vehicle for gallein, n=4). Signaling by neither WT nor mutant B2ΔNT was inhibited by the PLCβ inhibitor U73122, which blocks signaling by $G\alpha_q$-coupled receptors, and only signaling by the mutant receptor B2ΔNT-RW was sensitive to pertussis toxin (PTX) (Fig. 17A; B2ΔNT-RW $p<0.01$ vs vehicle, n=4), which inhibits $G\alpha_{i/o}$-mediated signaling.

To further test the possibility that the B2ΔNT signal to NFAT luciferase is largely mediated by Gβγ, we co-expressed GRK2-CT (βARKct), which can bind to and inhibit the activity of Gβγ subunits (Koch et al. 1993). Indeed, GRK2-CT also inhibited the activity of both B2ΔNT and B2ΔNT-RW (Fig. 17B; one sample t test of percent inhibition: B2ΔNT = -51.75±7.41%, $p=0.0060$ vs. 0.00%; B2ΔNT-RW = -64.75±11.69%, $p=0.0116$ vs. 0.00%, n=4). The activation of NFAT luciferase by both WT and R1465W mutant B2ΔNT was almost completely blocked by the calcium channel inhibitor SKF96365 (Fig. 17C; B2ΔNT $p<0.05$ vs vehicle, B2ΔNT-RW $p<0.001$ vs vehicle). These results indicate that the NFAT reporter activation by B2ΔNT is almost entirely due to Gβγ signaling and the activation of a calcium channel.

Figure 17. B2ΔNT signals to NFAT-luciferase via Gβγ and a calcium channel

The NFAT signal from B2ΔNT and B2ΔNT-RW is abolished by Gβγ inhibitor gallein (A, **p<0.01 vs B2ΔNT-vehicle condition, ****p<0.0001 vs B2ΔNT-RW vehicle n=4). The mutant receptor is sensitive to pertussis toxin (PTX, 100ng/ml) but WT is not (***p=0.001 vs B2ΔNT-RW vehicle condition, n=4). Neither receptor is inhibited by 10uM U73122. A transfectable Gβγ inhibitor, GRK2-CT, significantly inhibited the NFAT signal from B2ΔNT and B2ΔNT-RW (B, One-sample t test, B2ΔNT **p<0.01 and B2ΔNT-RW *p<0.05 compared to 0.00% inhibition, n=4). Calcium channel inhibitor SKF96365 (50μM) completely blocks the NFAT signal from both receptors (C, One-sample t test B2ΔNT ***p=0.0001, B2ΔNT-RW ****p<0.0001 compared to 0.00% inhibition, n=5).

Figure 17. B2ΔNT signals to NFAT-luciferase via Gβγ and a calcium channel



3.3.4 B2 is linked to $G\alpha_z$

NFAT-luciferase is a common readout of $G\alpha_q$ activity (Hill, Baker, and Rees 2001) but can also report activity from $G\alpha_{12/13}$ (Nishida et al. 2007). In addition, $G\beta\gamma$ activity is most typically due to $G\alpha_i$ activation (Smrcka 2008). We have previously reported that ADGRB1/BAI1, a $G\alpha_{12/13}$-coupled receptor, activates NFAT and therefore, given a high degree of similarity in the 7TM region, considered $G\alpha q$, $G\alpha_{12/13}$, and $G\alpha_i$ as the most likely candidates to be the cognate G protein for B2 (Kishore et al. 2016). However, we observed no interaction between B2ΔNT and $G\alpha_q$ or $G\alpha_{13}$ in co-immunoprecipitation experiments (Fig. 18A). In contrast, we observed that the $G\alpha_{i/o}$-family member $G\alpha_z$ robustly co-immunoprecipitated with B2ΔNT, with WT and R1465W mutant B2ΔNT immunoprecipitating $G\alpha_z$ to a similar extent (Fig. 18B).

To further test the possibility that $G\alpha_z$ might be the cognate G protein for ADGRB2, we assessed B2ΔNT signaling to NFAT luciferase in the presence of co-transfection with the $G\alpha_z$-specific regulator of G protein signaling RGS20 (RGSZ1) (Glick et al. 1998). As a control, we co-expressed a related RGS protein, RGS2, which acts specifically on $G\alpha_q$ (Heximer et al. 1997). RGS2 had no effect on B2ΔNT or B2ΔNT-RW signaling to NFAT, whereas RGS20 strongly increased the activity of B2ΔNT and B2ΔNT-RW (Fig. 19; Two-way ANOVA $F(3, 36) = 54.56$, $p<0.0001$ n=4, Tukey *post-hoc* test B2ΔNT vs mock $p<0.0001$, B2ΔNT-RW vs mock $p<0.001$).

Based on the findings described above showing that signaling by the R1465W mutant but not WT B2ΔNT was significantly inhibited by the $G\alpha_{i/o}$-inhibitor pertussis toxin (PTX), we hypothesized that R1465W mutant B2ΔNT might possess an enhanced ability to couple to $G\alpha_{i/o}$ in addition to $G\alpha_z$. To test this hypothesis, we performed co-

immunoprecipitation experiments assessing WT B2ΔNT and B2ΔNT-RW interactions with $G\alpha_{i1}$. No interaction was observable between WT B2ΔNT and $G\alpha_{i1}$, but in contrast substantial co-immunoprecipitation of $G\alpha_{i1}$was observed with the B2ΔNT R1465W mutant (Fig. 20).

Figure 18. ADGRB2 interacts with $G\alpha_z$

Among a panel of G protein α subunits, only Gαz co-immunoprecipitated with B2ΔNT (A, representative of 4 independent experiments). Both WT and R1465W forms of B2ΔNT co-immunoprecipitated with Gαz (B) and there was no significant difference in their ability to interact (n=5).

Figure 18. ADGRB2 interacts with $G\alpha_z$

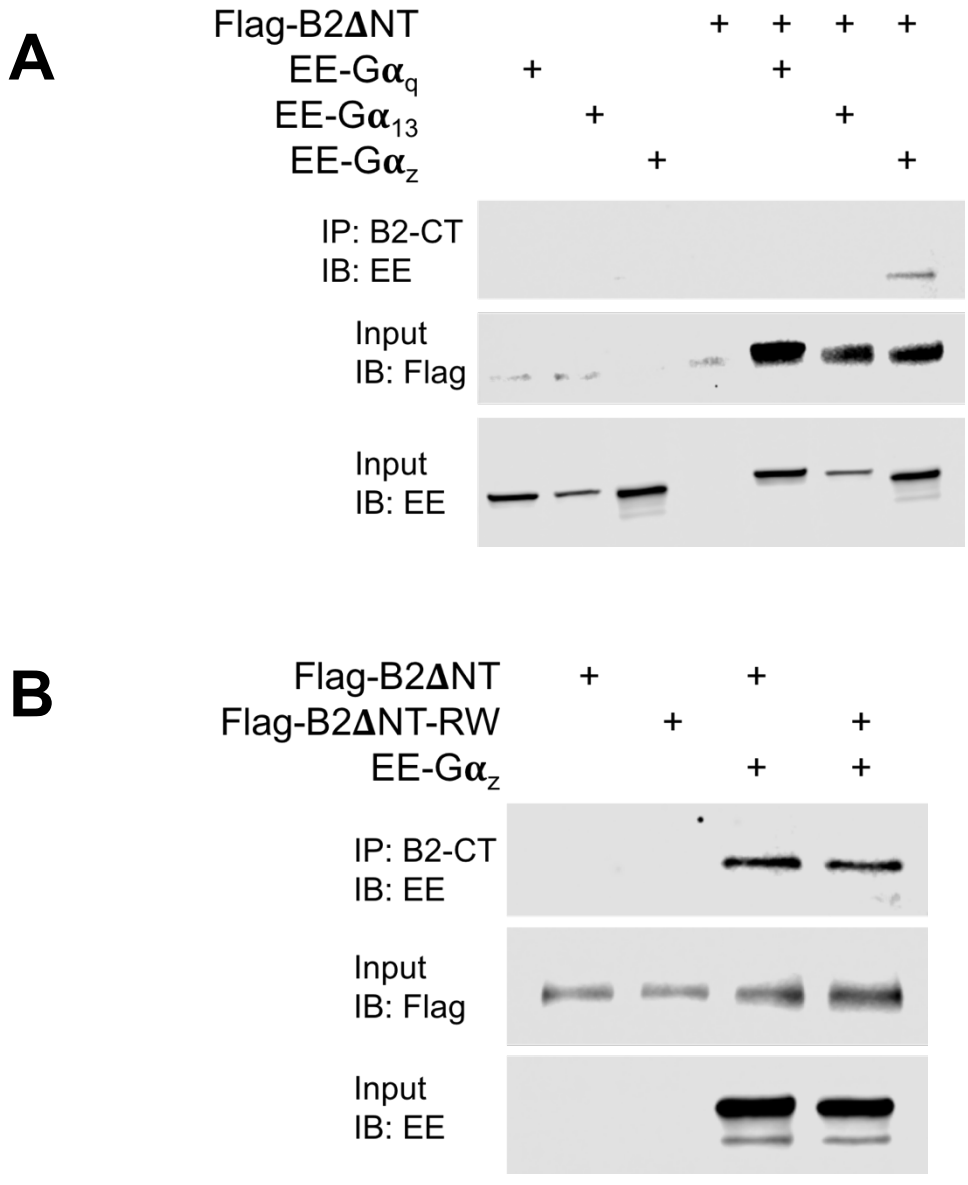


Figure 19. $G\alpha_z$-specific inhibitor RGS20 amplifies B2ΔNT signaling

Co-expression of the Gαq-specific RGS protein RGS2 had no effect on WT or RW signaling but the Gαz-specific RGS20 increased both B2ΔNT and B2ΔNT-RW activity (Two-way ANOVA, Tukey test B2ΔNT-RGS20 ****p<0.0001 vs B2ΔNT-mock, B2ΔNT-RW ***p=0.001 vs B2ΔNT-RW-mock, n=4).

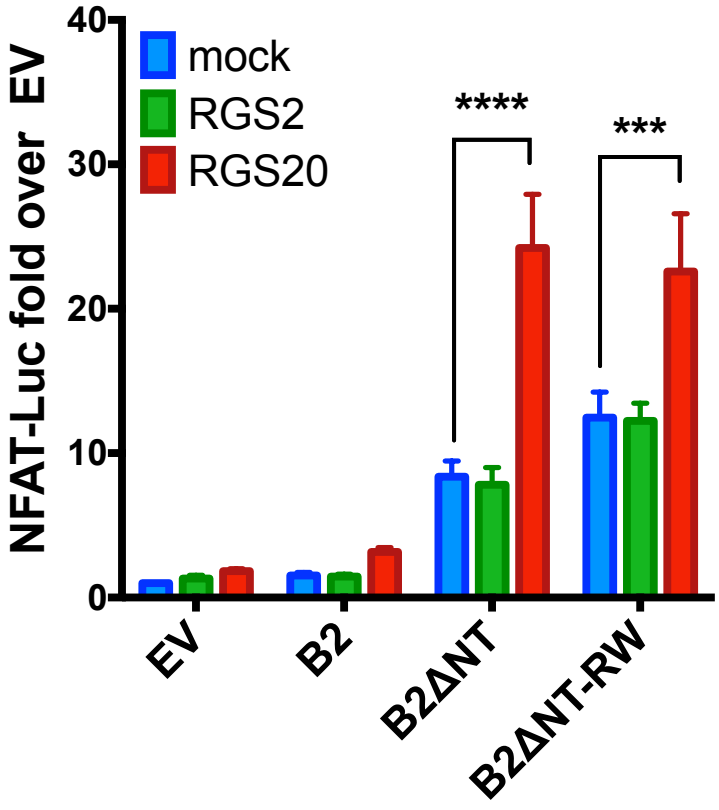
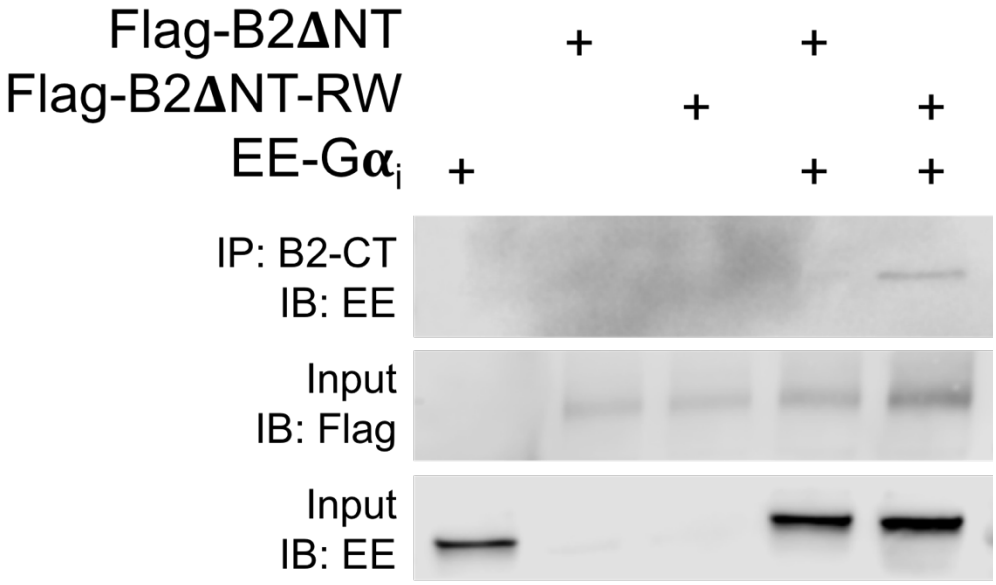


Figure 20. B2ΔNT-R1465W interacts with Gαi

Co-immunoprecipitation experiments revealed that mutant B2ΔNT-RW interacts with Gαi1 but an interaction with the WT receptor was undetectable (n=3).



3.3.5 R1465W mutation disrupts B2 interaction with Endophilin A1

The activity and surface expression of GPCRs can be regulated by β-arrestins, which bind to active receptors and often mediate their internalization and desensitization (Reiter and Lefkowitz 2006). Moreover, we have previously found that cleavage-mimicking forms of ADGRB1 and the unrelated adhesion receptor ADGRG1 strongly interact with β-arrestins (Paavola et al. 2011, Stephenson et al. 2013). Indeed, we found that B2ΔNT binds robustly to β-arrestin2, but the R1465W mutation did not have any effect on this interaction (Fig. 21, n=3). An additional hallmark of constitutively-active aGPCRs is that they are heavily ubiquitinated (Paavola et al. 2011, Stephenson et al. 2013). We hypothesized that the B2-R1465W mutation might impair ubiquitination, which might slow receptor degradation and prolong surface residence. However, no differences in ubiquitination were observed between WT and R1465W forms of B2ΔNT (Fig. 22).

The β-arrestin-mediated pathway is not the sole mediator of GPCR internalization (Ferguson 2001). The membrane-binding BAR- and SH3-domain-containing protein endophilin A1 (SH3-GL2) has been shown to interact with GPCRs (Tang et al. 1999) and can mediate GPCR internalization in a rapid, clathrin-independent manner (Boucrot et al. 2015). Therefore, we tested whether endophilins could interact with B2 by performing pull-down assays using the SH3 domain of endophilin A1 fused to glutathione-S-transferase (GST), or GST alone as a control, to pull down B2ΔNT. We found that WT B2ΔNT robustly interacted with the endophilin A1 SH3 domain, whereas interaction with B2ΔNT-RW was significantly reduced relative to WT (Fig. 23; unpaired T test, WT binding=6.46±0.59 vs RW=0.48±0.22, $p<0.0001$, n=4).

We hypothesized that if endophilin A1 is in fact an important regulator of B2 signaling, then co-transfection should limit the signaling activity of B2ΔNT. Indeed, we found that in 96-well format NFAT luciferase assays, as little as 2ng of endophilin A1 DNA significantly reduced the signaling of B2ΔNT and B2ΔNT-RW (Fig. 24; Two-way ANOVA, main effect of endo1transfection $F(1, 12)=22.45$, $p=0.0005$, Sidak *post-hoc* test B2ΔNT and B2ΔNT-RW not significantly different from EV with endo1 co-transfection, n=3). Interestingly, both B2ΔNT WT and RW receptors were inhibited by endophilin A1 over-expression, suggesting that even low levels of endophilin over-expression are sufficient to overcome the binding deficit of the mutant receptor.

The members of the brain-specific angiogenesis inhibitor (BAI1-3/ADGRB1-3) sub-family of receptors are highly enriched in brain tissue. Among the three endophilin A proteins, endophilin A1 has the most brain-enriched expression (Kjaerulff, Brodin, and Jung 2011). To determine whether endophilin A1 can interact with endogenous ADGRB2 from brain tissue, we incubated GST-endoA1-SH3 domain with mouse brain lysates and probed pull-down fractions for B2. We observed a robust interaction with ADGRB2 (Fig. 25, n=3).

Figure 21. B2ΔNT interacts with β-arrestin2

Based on the increased surface expression of B2ΔNT-RW, we assessed the association of B2ΔNT and B2ΔNT-RW with β-arrestin2 by co-immunoprecipitation but found both receptors to associate equally well (n=3).

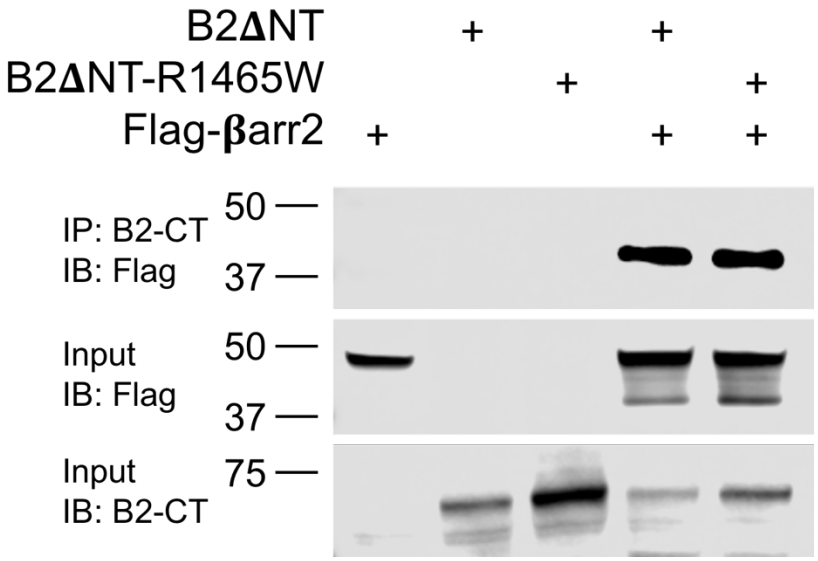


Figure 22. B2ΔNT-WT and B2ΔNT-RW are each heavily ubiquitinated

An additional possible explanation for the increased surface expression of B2ΔNT-RW is that it has a deficit in ubiquitination. Therefore, we tested this hypothesis by co-expressing B2ΔNT and B2ΔNT-RW with HA-ubiquitin and performed a co-immunoprecipitation. However, both forms of the receptor were found to be heavily ubiquitinated (n=3).

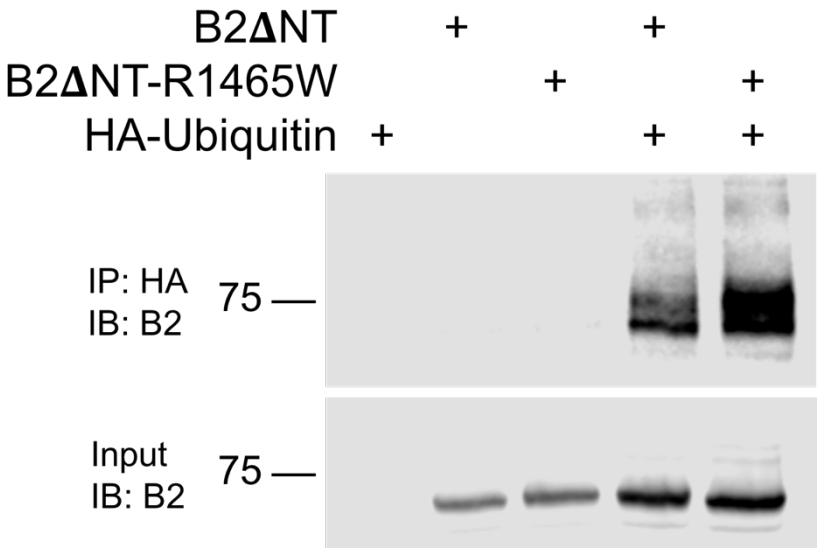


Figure 23. R1465W mutation disrupts interaction with endophilin A1

Endophilin A1 is another protein that can mediate the endocytosis of GPCRs. We produced the SH3 domain of endophilin A1 as a GST-fusion protein and performed a pull-down assay with B2ΔNT and B2ΔNT-RW using GST-agarose as a control condition. We found that B2ΔNT robustly interacted with the endophilin A1 SH3 domain but that B2ΔNT-RW scarcely did (t test, ****p<0.0001 RW vs WT, n=4).

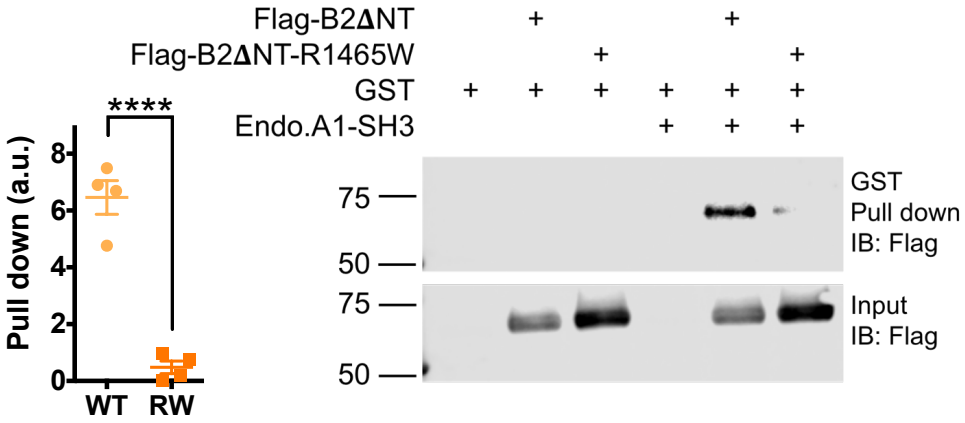


Figure 24. Endophilin A1 inhibits B2ΔNT signaling activity

To test whether the interaction of endophilin A1 with B2 has a functional consequence, we co-expressed endophilin with B2ΔNT and B2ΔNT-RW in NFAT luciferase assays. Surprisingly, we found that even a small amount of endophilin (2ng per well) strongly inhibited both the signal of B2ΔNT and B2ΔNT-RW such that neither receptor was significantly active over baseline (EV, n=3).

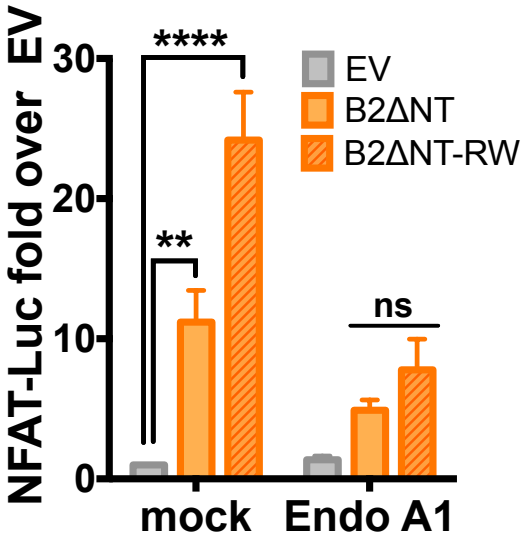


Figure 25. Endophilin A1 interacts with B2 and ADGRB3 in mouse brain

To begin to understand whether the interaction between endophilin A1 and the ADGRB sub-family receptors may be important in the brain, we performed the same endophilin A1-SH3 domain fusion protein pull-down experiment with tissue samples from mouse brain. We found that both B2 and ADGRB3 were pulled-down by the Endo1-SH3 domain suggesting that they may be able to interact in the brain *in vivo* (n=3).

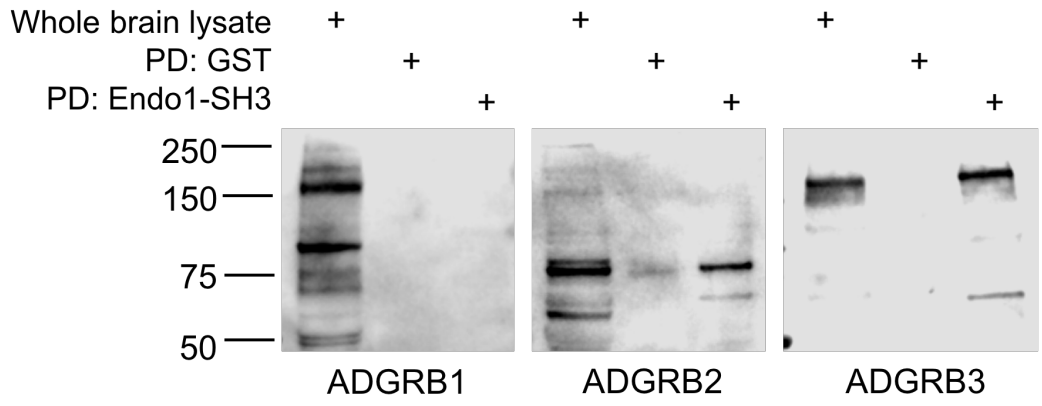
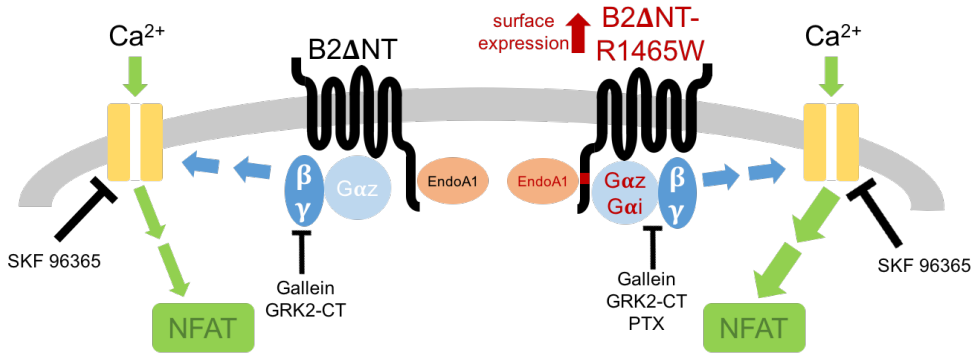


Figure 26. ADGRB2 signaling pathway model

Shedding of the N terminus results in a constitutively active form of ADGRB2 (B2ΔNT). B2ΔNT couples to Gαz, which liberates Gβγ subunits leading to calcium influx and activation of the NFAT-luciferase reporter. However, the R1465W mutation in B2ΔNT confers an additional ability to couple to Gαi. The mutation also disrupts interaction with endophilin A1 (EndoA1). These differences may explain why B2ΔNT-RW is found at higher levels on the cell surface and is significantly more active than the WT receptor.



3.4 Discussion of ADGRB2 R1465W

The aim of the present study was to investigate the signaling activity and regulation of the aGPCR ADGRB2 (B2) and assess the potential functional effects of a *de novo* disease-associated B2 mutation (R1465W) found in a human patient. We determined that this mutation in the C-terminal region of B2 potentiates the receptor's signaling activity and enhances receptor surface expression. In agreement with a previous study, we found that a truncated form of B2, corresponding to the predicted B2 polypeptide after GAIN domain autoproteolysis and NT shedding, robustly activates the NFAT luciferase reporter (Okajima, Kudo, and Yokota 2010). Most GPCRs that activate NFAT luciferase do so via coupling $G\alpha_q$ (Hill, Baker, and Rees 2001), but the signaling to NFAT by B2 was found in the present study to be almost entirely dependent on $G\beta\gamma$-mediated activity. In terms of the $G\alpha$ subunit involved, WT B2 exhibited a preferential coupling to the $G\alpha_{i/o}$-family member $G\alpha_z$, whereas the R1465W mutant exhibited significant coupling to both $G\alpha_z$ and $G\alpha_i$. Thus, the data presented here suggest two mechanisms by which the R1465W mutation increases signaling activity: enhancement of receptor surface expression and increased flexibility of G protein coupling to encompass other $G\alpha_{i/o}$ family proteins.

Gain-of-function mutations in other GPCRs have been informative in understanding critical residues for receptor activation. For example, any substitution at position 293 in the α_{1b}-adrenergic receptor results in constitutive activity (Kjelsberg et al. 1992). There are clinical consequences as well; missense mutations in the retinal-binding lysine-296 of rhodopsin can result in a constitutively active receptor and lead to the deterioration of rod cells in retinitis pigmentosa (Robinson et al. 1992) and the

substitution of aspartate for glycine at residue 578 in the luteinizing hormone receptor imparts constitutive activity, which can induce precocious puberty (Shenker et al. 1993). Thus, the investigation of these mutations has been informative for clinicians as well as for receptor biologists.

In our model (Fig. 26), removal or rearrangement of the 911-amino-acid N-terminus of B2 relieves an inhibitory constraint on the 7TM region, which allows the receptor to adopt its active conformation and associate with heterotrimeric G proteins. Our data indicates that the B2ΔNT signal to the NFAT luciferase reporter is almost entirely mediated by Gβγ subunits because co-expressing the GRK2-CT or treating cells with gallein abolishes the signal. We also found that B2 can be co-immunoprecipitated with $G\alpha_z$, and furthermore observed that the $G\alpha_z$-specific RGS protein, RGS20, significantly increased B2ΔNT signaling. Thus, these data implicate $G\alpha_z$ as a mediator of B2 signaling, although the findings are somewhat paradoxical in that a $G\alpha_z$-specific RGS protein should inhibit $G\alpha_z$-mediated signaling rather than potentiate it. However, it is important to point out that B2ΔNT is a highly constitutively-active receptor and thus is presumably highly desensitized. It may be the case that toning down the $G\alpha_z$-mediated signal downstream of B2 results in less desensitization and therefore more sustained signaling, resulting in a paradoxical increase in the 48-hour luciferase reporter assay. Without an identified ligand or any other tool to activate B2 in a temporally-controllable manner, overexpression of the cleavage-mimetic ΔNT form of B2 is the most effective way we have at present to study its signaling activity, so this represents a limitation of the present study.

There are several other potential explanations of the paradoxical effect of RGS20 on B2ΔNT signaling. One possibility is that activated $G\alpha_z$ may have unknown functions that limit signaling to NFAT, such that RGS20 relieves this inhibition and thereby potentiates the $G\beta\gamma$-mediated NFAT activation. We demonstrated that nearly all of the activity that we observed to the NFAT reporter was dependent on calcium influx. A previous study found that $G\alpha_z$ can modulate ion channel function including that of N-type calcium channels in a pertussis toxin-insensitive manner (Jeong and Ikeda 1998). Therefore, inhibiting $G\alpha_z$ with RGS20 may relieve inhibition on calcium channels and thereby increase activity to NFAT.

Alternatively, it appears that RGS20 was found to increase the signaling activity of the WT receptor somewhat more than for the B2ΔNT R1465W mutant such that the baseline difference in their signaling activity evaporates in the presence of RGS20. It is possible that inhibiting $G\alpha_z$ activity via RGS20 promotes somewhat more $G\alpha_{i/o}$ coupling to WT B2, which normally does not couple strongly to $G\alpha_i$. It is conceivable that $G\alpha_{i/o}$ activation results in liberation of pools of $G\beta\gamma$ subunits that are more effective at activating the pathway to NFAT luciferase than $G\beta\gamma$ released from $G\alpha_z$. Since the B2ΔNT R1465W mutant already couples efficiently to $G\alpha_i$ (as indicated by co-immunoprecipitation and sensitivity to pertussis toxin), signaling by the mutant receptor would not be expected to change as much as WT if the balance was shifted from $G\alpha_z$ to $G\alpha_i$. Further studies will be required to differentiate between these possibilities and to determine what role(s) $G\alpha_z$ might have once it is activated downstream of B2 in native cell types *in vivo*.

In the studies reported here, B2 activation was found to provoke a Gβγ-dependent influx of Ca^{2+} through an as-yet-unidentified calcium channel, leading to the activation of the NFAT luciferase reporter. SKF 96365 is most commonly used as an antagonist of transient receptor potential canonical type (TRPC) channels, but at the concentration we utilized (50μM) it can block several other types of calcium channels as well (Singh et al. 2010). Interestingly, the *Drosophila* homolog of the adhesion GPCR ADGRL1/latrophilin (*dCIRL*) was recently found to modulate the action of a TRP channel to influence mechanosensation (Scholz et al. 2015). Further studies will be required to determine how exactly B2 activation impacts calcium channel function and whether this regulation depends on direct channel association with Gβγ subunits.

We observed that expression of B2ΔNT-R1465W is significantly higher than WT B2ΔNT on the cell surface, with the magnitude of this effect being comparable to the extent by which the mutation increases receptor signaling activity. Additionally, we found that association with β-arrestins and the levels of receptor ubiquitination were unchanged by the R1465W mutation, which suggested other mechanisms may play a role in dictating B2 surface expression. Endophilin A1 was recently found to bind to a number of GPCRs and mediate their internalization via a pathway independent of β-arrestins, ubiquitination or clathrin coat proteins (Boucrot et al. 2015). We found that the SH3 domain of endophilin A1 avidly interacts with WT B2ΔNT, but interacts less robustly with the B2-R1465W mutant. However, the mutation does not completely abrogate binding, and overexpression of endophilin A1 in heterologous cells can overcome the binding deficit and inhibit receptor signaling activity similar to its effect on the WT receptor.

Src homology 3 (SH3) domains (like that in endophilin A1) typically interact with proline-rich sequences and the classic P-X-X-P motif (where X can be any amino acid) (Li 2005). However, while there are six distinct P-X-X-P motifs in the long C terminus of B2, there are none in the vicinity of R1465. If the endophilin A1 SH3 domain binds directly to the B2 CT, then there are two apparent explanations for why the R1465W mutation disrupts the interaction: 1. The tertiary structure of B2 positions R1465 in close proximity to a classic P-X-X-P site but the substitution of a W disrupts this structure, or 2. The endophilin A1 SH3 domain actually interacts with a string of positive charges. Arginine 1465 is found in a highly conserved region of positive charges in B2 and K-K-L-**R** (where R=1465) could in fact be an atypical SH3 binding sequence (Li 2005). In B1 and ADGRB3, the matching sequence is R-K-S-R (B1: 1473-6, B3: 1415-8), which could also mediate SH3 domain binding. We found that both B2 and B3 can be precipitated out of mouse brain lysates with the same endophilin A1 SH3 domain fusion protein, indicating that endophilin A1 may in fact associate with B2 and B3 in the brain. However, the apparent lack of binding to ADGRB1 does not necessarily mean that endophilin does not interact with this receptor (further data on this topic can be found in Chapter 4). It is plausible that in mouse brain tissue, the C-termini of most B1 receptors are bound to other proteins (possibly including endophilins) which occlude the site where the endophilin A1-SH3 fusion protein would interact. Thus, the present studies identify endophilin A1 as a novel binding partner of B2, even though it is uncertain whether the reduced binding of endophilin A1 by the B2 R1465W contributes to the altered trafficking and activity of this receptor. A complicating factor in these studies is that there are three closely-related endophilins (A1-3) that are all widely expressed, so further

studies will be needed to dissect the potential regulation of B2 by the various members of the endophilin family.

Mutations to GPCRs that increase constitutive activity are frequently toxic (Parnot et al. 2002). Our data indicate that the B2 R1465W mutation, which was discovered in a patient with a progressive neuromuscular disorder, significantly increases B2 signaling activity. Given the preferential expression of B2 in the nervous system, it is conceivable that heightened or prolonged activity from B2 could lead to neuromuscular disease. Deletion of B2 in mice has been reported to have no obvious negative consequences, with B2 null animals actually displaying increased hippocampal neurogenesis and exhibiting resistance to learned helplessness behavior (Okajima, Kudo, and Yokota 2011). Together, these data suggest that B2 may be an attractive target for the development of antagonists, and a recent report regarding the development of a small molecule antagonist for another adhesion receptor (ADGRG1/GPR56) demonstrates the feasibility of this approach (Stoveken et al. 2016). Future studies will also be needed to shed further light on the function(s) of B2 *in vivo* and the mechanisms by which B2 signaling is regulated.

Chapter 4. C-terminal determinants of ADGRB1 activity

4.1 Introduction

Adhesion GPCRs are exceptionally large receptors, and the massive ectodomains have received far more attention than their intracellular counterparts due to their adhesion and GPCR-activating functions. The post-7TM cytoplasmic domains of aGPCRs range from 13 (ADGRF2/GPR111) to more than 500 (ADGRC3/CELSR3) amino acids with 9 aGPCRs, including ADGRB1-3, having C termini longer than 300 amino acids. These cytoplasmic C-termini are much larger than the C-termini of most other GPCRs. ADGRB1/BAI1 (B1) has a nearly 400 amino acid intracellular C terminus (CT) with several interesting sites of potential protein-protein interaction including an extended polyproline region and an extreme C-terminal PDZ (PSD95/Discs-large, ZO-1) domain. These features are not unique among the ADGRB receptors, as 13 aGPCRs end in class I PDZ-binding motifs and three other receptors have poly-proline sequences (Table 3).

The first report on the role of the B1 CT in the regulation of signaling pathways was related to its effects on small GTPases rather than heterotrimeric G protein signaling (Park et al. 2007). B1 was found to recruit the RacGEF complex ELMO1/DOCK180 dependent on a cytoplasmic RKR motif (a.a. 1489-91). More recently, B1 was reported to interact with another RacGEF, Tiam1, via its C-terminal PDZ binding motif. In addition to activation of the Rac pathway, it was demonstrated that B1 recruits the neuronal polarity protein par3, which is critical for normal dendritic spine development (Duman et al. 2013). Concurrent with these data, another report showed that the B1 CT interacted with a number of PDZ-domain containing proteins, many of which are enriched in the post-synaptic density (Stephenson et al. 2013).

One of the first proteins that was described to interact with B1, IRSp53, is also known to be an important factor at the post-synaptic density (PSD). However, since its initial discovery, there have been no descriptions of its functional relationship to B1. The human form of IRSp53 or BAI-associated protein 2 (BAIAP2) was discovered in 1999 due to its SH3 domain interaction with the C-terminal tail of BAI1/ADGRB1 (Oda et al. 1999). At the time, very little was known about either of these proteins, but IRSp53 was subsequently shown to be enriched in the PSD (Abbott, Wells, and Fallon 1999). Since then, the BAI-associated protein IRSp53 has been found to be a key regulator of actin dynamics and a critical PSD protein in complex with PSD-95 and Shank scaffold proteins (Soltau et al. 2004). It was presumed that IRSp53 interacts with the extensive C-terminal proline-rich region of B1 due to the propensity for SH3 domains to bind to proline-rich sequences (Li 2005), though this was never formally tested.

Importantly, IRSp53 has been associated with several neuropsychiatric conditions. One study found that the *BAIAP2* gene (which codes for IRSp53) is asymmetrically expressed in the cortex of individuals with ADHD (Ribases et al. 2009) and another reported several single nucleotide polymorphisms in this gene that are also significantly associated with ADHD (Liu et al. 2013). Others have found that asymmetric expression (Toma et al. 2011) and copy-number variation (Levy et al. 2011) in *BAIAP2* are linked to autism spectrum disorders. Lastly, IRSp53 protein levels were found to be significantly reduced in post-synaptic density fractions from post-mortem Alzheimer's disease brain tissue (Zhou et al. 2013). Together, these data strongly suggest that IRSp53 is a key synaptic protein that may be a common link underlying a number of neuropsychiatric disorders.

Interestingly, mice lacking IRSp53 strongly resemble *Adgrb1*$^{-/-}$ mice in several key areas. First, both IRSp53 and B1 null animals were found to have smaller PSDs than WT animals and to display paradoxical increases in hippocampal LTP (Sawallisch et al. 2009, Zhu et al. 2015, Kim et al. 2009). Behavioral studies found that both IRSp53 and B1 knockout mice have deficits in spatial learning as assessed by the Morris water maze (Zhu et al. 2015, Kim et al. 2009), and additionally IRSp53 KO mice exhibit impaired fear conditioning (Sawallisch et al. 2009) and social learning (Chung et al. 2015). Based on these data, we hypothesized that B1 and IRSp53 are functionally connected in post-synaptic dendrites and that some of the behavioral and physiological deficits in IRSp53 KO animals could be due to a loss of regulation or modulation of B1.

While the respective literatures on B1 and IRSp53 have grown substantially since these two proteins were originally identified as binding partners, there have been no studies whatsoever of the functional consequences of this interaction since its discovery aside from a report that protein levels of IRSp53 are unchanged in *adgrb1* null mice (Zhu et al. 2015). Therefore, we hypothesized that IRSp53 is a key regulator of B1 signaling and sought to test that hypothesis in the present study. Furthermore, we investigated the role of the proline-rich region in the cytoplasmic domain of B1 based on the hypothesis that this region mediates the interaction with IRSp53 as well as other SH3 domain containing proteins.

Finally, we report initial work on three putative mutations that were identified in *ADGRB1* with a potential link to schizophrenia. Alterations in gene dosage (such as copy number variants – CNVs) at the synapse have emerged as an important and consistent risk factor for neuropsychiatric conditions such as autism and schizophrenia (Kirov

2015). The 3q29 microdeletion is rare but is one of the strongest known risk factors for schizophrenia (SZ) and is also associated with autism spectrum disorders (ASD) and intellectual disability (Mulle 2015, Mulle et al. 2010). Thus far, the biological mechanisms underlying the increased risk for SZ and ASD associated with this microdeletion have not been elucidated. However, of the 20 annotated genes in this chromosomal region, two stand out as having great potential for involvement in these mechanisms: *DLG1* and *PAK2*. DLG1 (aka SAP97) is a synaptic PDZ-domain containing protein that our laboratory has previously identified as a B1 interacting protein (Stephenson et al. 2013). PAK2 (p21-activated kinase 2) is ubiquitously expressed and known to be activated by Rac1 downstream of GPCRs (Knaus et al. 1995). As described above, B1 can activate Rac1 in at least two separate ways – via an RKR motif midway into its CT (Park et al. 2007) and also via recruitment of the RacGEF Tiam1 at its C-terminal PDZ-binding motif (Duman et al. 2013). Thus, as a common link between these proteins, we hypothesized that B1 might be an upstream mediator of the neuropathology.

ADGRB1 was sequenced in multiplex SZ families and three mutations with high CADD scores were identified: Pro947Leu, Arg1473Trp, and Ser1566Leu. The location of each of these mutations was immediately interesting. First, Proline 947 is located right at the junction of the extracellular stalk region and first transmembrane domain. We hypothesized that this mutation could alter stalk-dependent signaling by changing the orientation of the stalk peptide with respect to the rest of the 7TM protomer. Arginine 1473 is found a homologous region to ADGRB2 R1465 (but is not exactly equivalent to this arginine in B2) and therefore could drastically alter the signaling and regulation of B1. Lastly, Serine 1566 is near the extreme CT and we postulated that this residue could

be an important phosphorylation site, which would be disrupted by the substitution of a leucine residue. In the final section of this chapter, we report initial results from the study of the effect of these three mutations on B1ΔNT.

Table 3. Adhesion GPCR cytoplasmic domain features

Receptor	PDZ-binding Motif	Poly-proline Region (a.a. number)	A.A. length of cytoplasmic domain
ADGRA1	- TTV		255
ADGRA2	-TTV		270
ADGRA3	-TTV		276
ADGRB1	-TEV	1411-1422, 1425-30	397
ADGRB2	-TEV	1425-30	411
ADGRB3	-TEV		376
ADGRC2	---	2878-84	311
ADGRD1	-SAV		69
ADGRE5	-SGI		46
ADGRG1	-SRI		36
ADGRL1	-TSL	1303-14, 1410-20	378
ADGRL2	-TSL		376
ADGRL3	-TSL		343
ADGRV1	-THL		152

4.2 Experimental procedures

4.2.1 Cell culture

HEK293T/17 cells (ATCC, Manassas, VA) were maintained in a humid, 5% CO_2, 37°C incubator with standard growth medium (DMEM (Gibco), 10% FBS (Atlanta Biologicals), 1% penicillin/streptomycin (Sigma)). Transfections utilized Mirus TransIT-LT1 (Madison, WI).

4.2.2 DNA constructs

Human ADGRB1 provided by Dr. Erwin Van Meir was used as a template to delete the C-terminal proline-rich region (del. a.a. 1393-1437) (Genscript, Piscataway, NJ). ADGRB1ΔNTΔPRR (927-1393,1437-1584) was subsequently sub-cloned from this template into pcDNA3.1 between EcoRI and XbaI as described above.

B1 schizophrenia-linked (SZ) mutations were introduced into the B1ΔNT plasmid by site-directed mutagenesis using the Quikchange XL kit (Agilent) and primers P947L (5'-AAG GCG ACT CTG CTG TCG GTG ACG CTC-3', 5'-GAG CGT CAC CGA CAG CAG AGT CGC CTT-3'), R1473W (5'-CCC TGG AGC GGT GGA AGT CGC GG-3', 5'-CCG CGA CTT CCA CCG CTC CAG GG-3'), S1566L (5'-GTG GCG CCC AAC CTC TCC CAC TCC AC-3', 5'-GTG GAG TGG GAG AGG TTG GGC GCC AC-3') and verified by Sanger sequencing (Eurofins Genomics).

GFP-EndophilinA1 was a gift from Kozo Kaibuchi (Nagoya University), GST-EndoA1-SH3 was provided by Harvey McMahon (Cambridge University), EE-Gα_z and

HA-RGS20 were purchased from the cDNA Resource Center (cdna.org), HA-ubiquitin was a gift from Keqiang Ye (Emory University), and HA-βarrestin2 (Luttrell et al. 1999) was a gift from Robert Lefkowitz (Addgene plasmid # 14692).

4.2.3 Western Blot

Protein samples were reduced in 1x Laemmli buffer, electrophoresed in 4-20% Tris-glycine gels and transferred to nitrocellulose membranes (Bio-rad, Hercules, CA). Non-specific binding was blocked with 5% milk (in 50mM NaCl, 10mM HEPES pH 7.3, 0.1% Tween-20 (Sigma)) and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. Two primary C-terminal rabbit polyclonal antibodies to B1 were utilized with minor differences. The first antibody was provided by Dr. Erwin Van Meir (Emory University) and has been described previously (Stephenson et al. 2013). The second was found to be slightly better at detecting mouse ADGRB1, and had less nonspecific background in GST pull-down experiments (Thermo Scientific). The mouse monoclonal EE antibody from Abcam (Cambridge, MA), and Flag-HRP from Sigma. Blots were then washed, incubated with HRP-conjugated secondary antibodies (GE Healthcare) and visualized with Thermo Scientific SuperSignal West solutions on a Li-Cor Odyssey Imager.

4.2.4 Cell Surface Biotinylation

Twenty-four hours following transfection with 2ug of receptor DNA, HEK-293T cells were washed in cold PBS+Ca^{2+} and incubated with 5mM Sulfo-NHS-Biotin (Thermo Scientific) or vehicle (PBS+Ca^{2+}) for 1hr on ice. Biotinylation was quenched with 100mM Glycine and cells were harvested in cold PBS. Membranes were ruptured

with a rapid freeze-thaw and pelleted at 17,000xg for 15 min at 4°C. The membrane pellet was then re-suspended in 1% Triton X-100 buffer (25mM HEPES, 150mM NaCl, 10mM $MgCl_2$, 1mM EDTA, 1x HALT protease and phosphatase inhibitor (Thermo)) and rotated end-over-end for 45 min to solubilize membrane proteins. The insoluble fraction was then pelleted at 17,000xg for 15min at 4°C and solubilizates were incubated with streptavidin agarose (Thermo) for 30 min to precipitate biotinylated proteins. Agarose was washed with 1% Triton buffer and proteins were eluted in 2x Laemmli buffer. Biotinylated proteins were detected via Western Blot (above).

4.2.5 Co-immunoprecipitation

Cells were transfected with 2ug of receptor and 1ug of IRSp53 or GEF-endophilin A1 DNA. The following day, cells were harvested and membrane proteins were solubilized in 1% Triton X-100 buffer. Solubilizates were separated (above) and incubated with anti-HA agarose (Sigma), magnetic anti-Flag (Sigma), or protein A/G agarose (Thermo) beads with the indicated primary antibody for 1 hr end-over-end at 4°C. Samples were then washed, eluted, and Western blotted as described above.

4.2.6 Luciferase assays

HEK-293T cells were seeded in clear-bottom white 96-well plates 20-24 hours prior to transfection. Each well was transfected with 10ng of empty vector (EV) or receptor DNA, 50ng NFAT-luciferase (pGL4.30, Promega, Madison, WI), 1ng Renilla luciferase (pRL-SV40, Promega). Dual-Glo luciferase assays (Promega) were performed 48hr post-transfection and plates were read on a BMG Omega plate reader. The ratio of

firefly:Renilla was calculated for each well and normalized to the mean of the EV-transfected controls.

4.2.7 Mutation discovery

Multiplex SZ families were ascertained as previously described (Blouin et al. 1998). In each of 245 multiplex families, the two most distantly-related affected members of the pedigree were selected for whole-exome sequencing at the Broad Institute. Target sequences were generated as described (Fromer et al. 2014) using either Agilent hybrid capture or Nimblegen array-based capture, and libraries were then subjected to paired-end sequencing. Sequence variants were annotated using the SeqAnt annotater developed by and housed at Emory University (Shetty et al. 2010). Variants were prioritized by Combined Annotation-Dependent Depletion (CADD) scores (Kircher et al. 2014), which takes multiple sources of sequence annotation into account to measure the likely deleteriousness of a given genetic variant.

4.3 Results

4.3.1 IRSp53 increases surface expression and activity of ADGRB1

To initially assess a functional connection between IRSp53 and B1, we expressed the two proteins in HEK-293T cells. Interestingly, co-expression of IRSp53 significantly increased the amount of B1ΔNT found on the cell surface but had no significant effect on B1 (Fig. 27, Two-way ANOVA $F(1, 8) = 6.925$, $p<0.05$, Sidak *post-hoc* test surface B1ΔNT *$p<0.05$, n=3).

Next, we tested the hypothesis that the addition of IRSp53 would increase the signaling activity of B1ΔNT. Indeed, we found that co-expressing IRSp53 significantly augmented the activity of B1ΔNT to the NFAT reporter but had no effect on full-length B1 (Fig. 28, Two-way ANOVA $F(1, 24) = 6.692$, $p=0.0162$, Sidak *post-hoc* test mock vs IRSp53 B1ΔNT ***$p<0.001$, n=5). Interestingly, despite the increased surface expression, we found no evidence for an increase in activity of either receptor isoform to SRF luciferase (n=3) indicating that the effect of IRSp53 on B1 signaling is at least somewhat pathway specific.

Figure 27. IRSp53 improves the surface expression of B1ΔNT

A cell-surface biotinylation approach revealed that the addition of IRSp53 significantly increases the amount of B1ΔNT found on the cell surface (*p<0.05 vs mock, n=3).

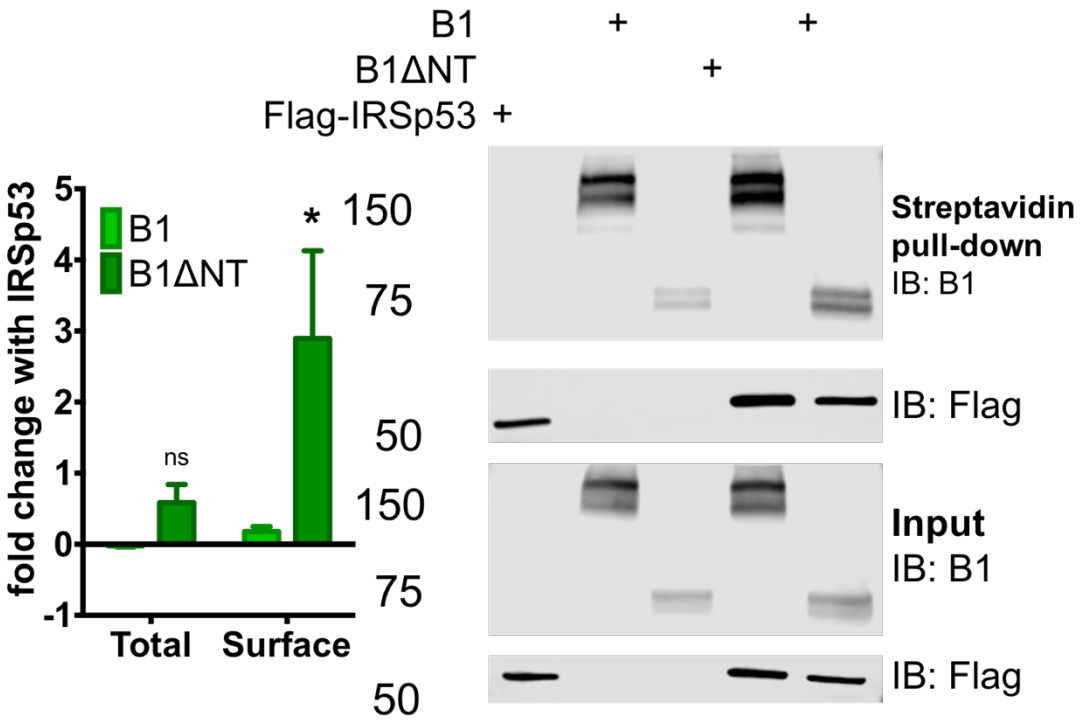
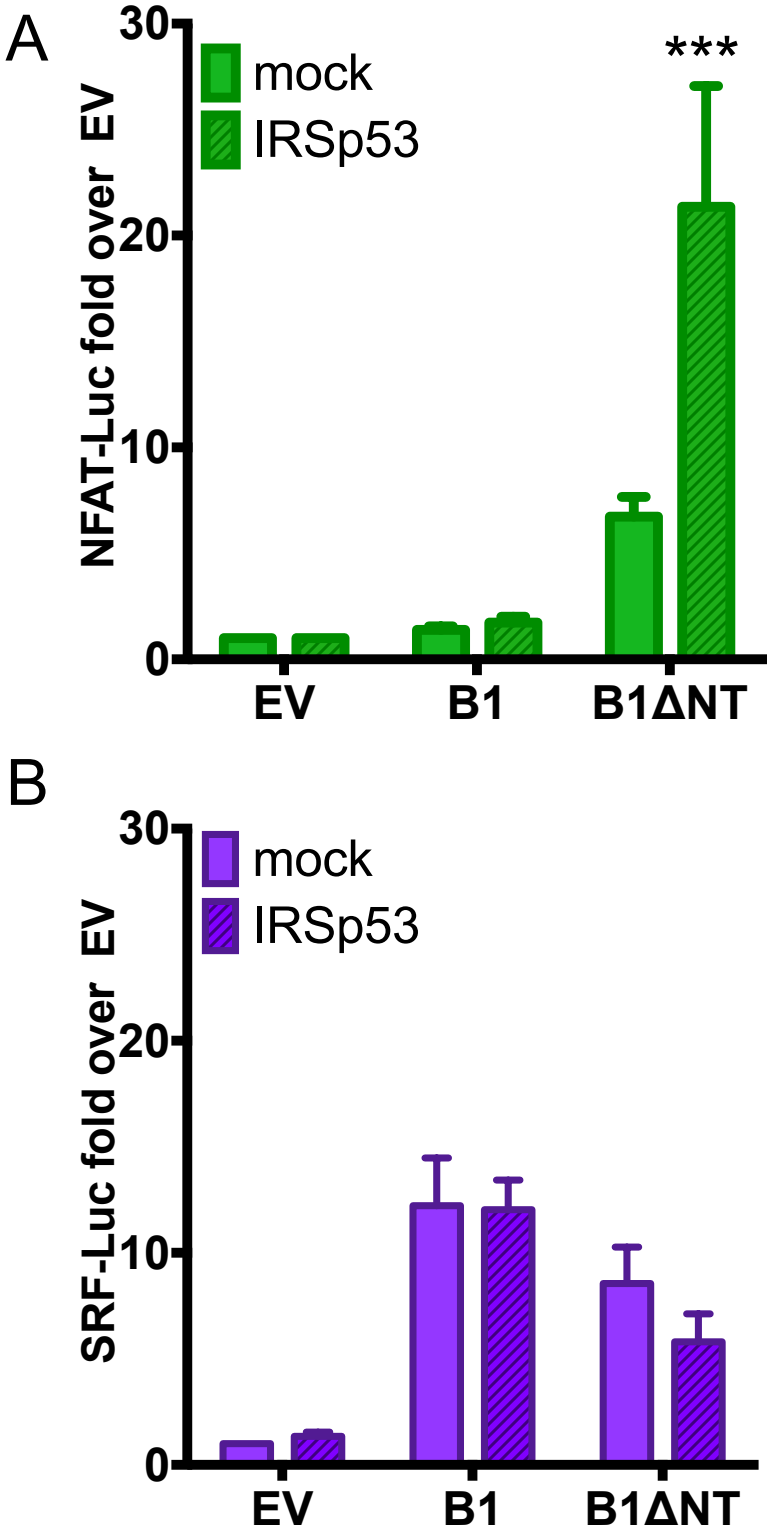


Figure 28. IRSp53 selectively increases the activity of B1ΔNT to NFAT-luciferase

B1 and B1ΔNT were expressed alone or in combination with IRSp53 in HEK cells along with NFAT- or SRF-luciferase and *Renilla* reporters. The addition of IRSp53 significantly increased the signaling activity of B1ΔNT to NFAT luciferase (A) but had no effect of full-length B1 (B1ΔNT-IRSp53 ***$p<0.001$ vs mock-B1ΔNT, n=5). Additionally, IRSp53 had no effect on either isoform of the receptor's activity to SRF-luciferase (B, n=3).

Figure 28. IRSp53 selectively increases the activity of B1ΔNT to NFAT-luciferase



4.3.2 Proline-rich region deletion does not interrupt IRSp53 binding but drastically biases B1 signaling activity

The original report of the identification of IRSp53 as a B1-binding partner indicated that its interaction with the cytoplasmic domain of B1 was dependent on the Src homology 3 (SH3) domain of IRSp53 (Oda et al. 1999). SH3 domain interactions often depend on polyproline sequences (Li 2005), and thus we hypothesized that the interaction between B1 and IRSp53 could be disrupted by deleting the extensive proline-rich region found on the C-terminus of B1.

We engineered B1 expression plasmids lacking amino acid 1393-1437, which removes 28 proline residues. Surprisingly, though, we found that B1ΔNTΔPRR retains its ability to interact with IRSp53 in a co-immunoprecipitation experiment (Fig. 29). Next, we tested whether this mutant for the B1 lacking the main proline-rich region could interact with endophilin A1 in a co-immunoprecipitation experiment. In the case of WT B1 and B2, a co-immunoprecipitation experiment with endophilin A1 is not technically feasible because co-expression of endophilin dramatically reduces receptor protein levels such that the interaction is not observable. Interestingly, the same phenomenon was observed in this experiment (Fig. 30) where WT B1ΔNT is essentially absent from whole cell lysate input samples (as is GFP-endophilin A1). However, B1ΔNTΔPRR is not noticeably reduced and strongly co-immunoprecipitates with GFP-endophilin A1. This result suggests that endophilin is not sufficient for the down-regulation and degradation of the receptor and that perhaps loss of the PRR abrogates binding to an additional, as yet unidentified protein, that mediates this process.

Next, we tested what effect the loss of the PRR has on receptor signaling in NFAT and SRF luciferase assays. Surprisingly, deletion of this polyproline region eliminated signaling activity to SRF luciferase (Fig. 31, One-way ANOVA $F(4, 5) = 19.86$, $p=0.0029$, Sidak *post-hoc* test B1 vs B1ΔPRR *$p<0.05$, B1ΔNT vs B1ΔNTΔPRR **$p<0.01$, n=5) and strongly amplified the NFAT reporter pathway in the active form of the receptor B1ΔNTΔPRR (One-way ANOVA $F(4, 27) = 33.09$, $p<0.0001$, Sidak *post-hoc* test B1ΔNT vs B1ΔNTΔPRR ****$p<0.0001$, n=4-7). These results suggest that this key cytoplasmic protein-protein interaction region strongly influences receptor signaling activity.

Figure 29. Loss of proline-rich region does not abrogate IRSp53 binding

B1ΔNT and B1ΔNTΔPRR were expressed alone or with Flag-IRSp53 in HEK cells. IRSp53 was immunoprecipitated with Flag resin and blotted with B1-CT antibody. Both B1ΔNT and B1ΔNTΔPRR immunoprecipitated specifically and to a similar extent with IRSp53.

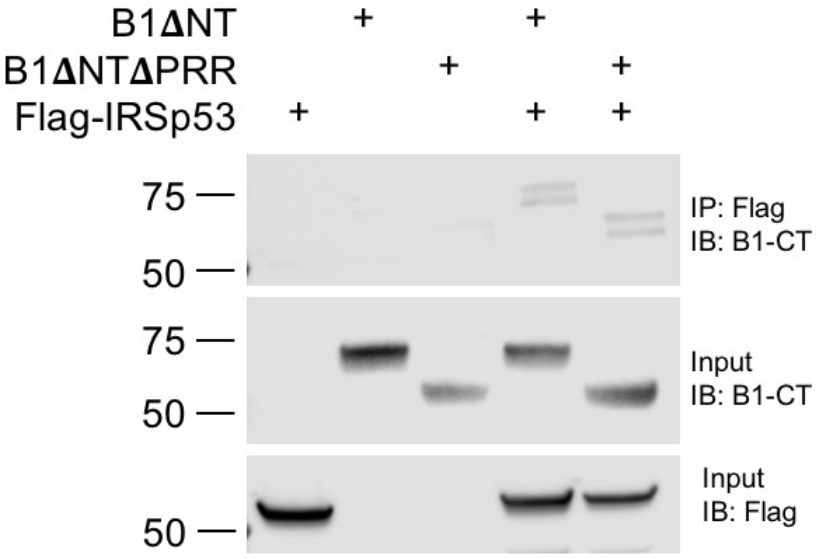


Figure 30. B1ΔNTΔPRR co-immunoprecipitates with endophilin A1

B1ΔNT and B1ΔNTΔPRR were co-expressed with GFP-endophilin A1 in HEK cells. A monoclonal anti-GFP antibody and protein A/G beads were utilized to immunoprecipitate endophilin A1 and this fraction was probed with a B1-CT antibody. Co-expression of Endo1 dramatically reduced protein levels of B1ΔNT but not B1ΔNTΔPRR. B1ΔNTΔPRR robustly co-immunoprecipitated with endophilin A1.

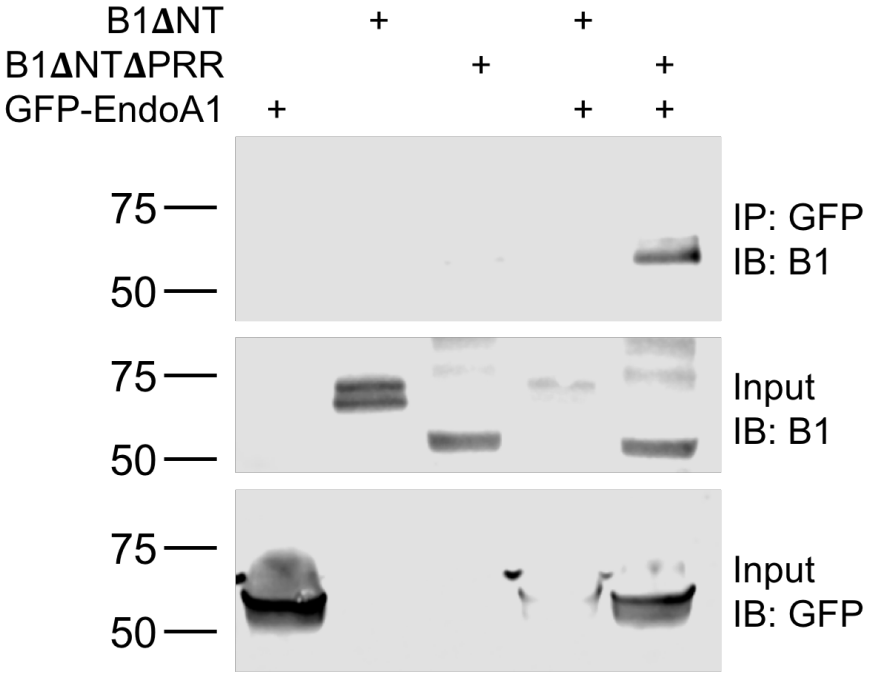
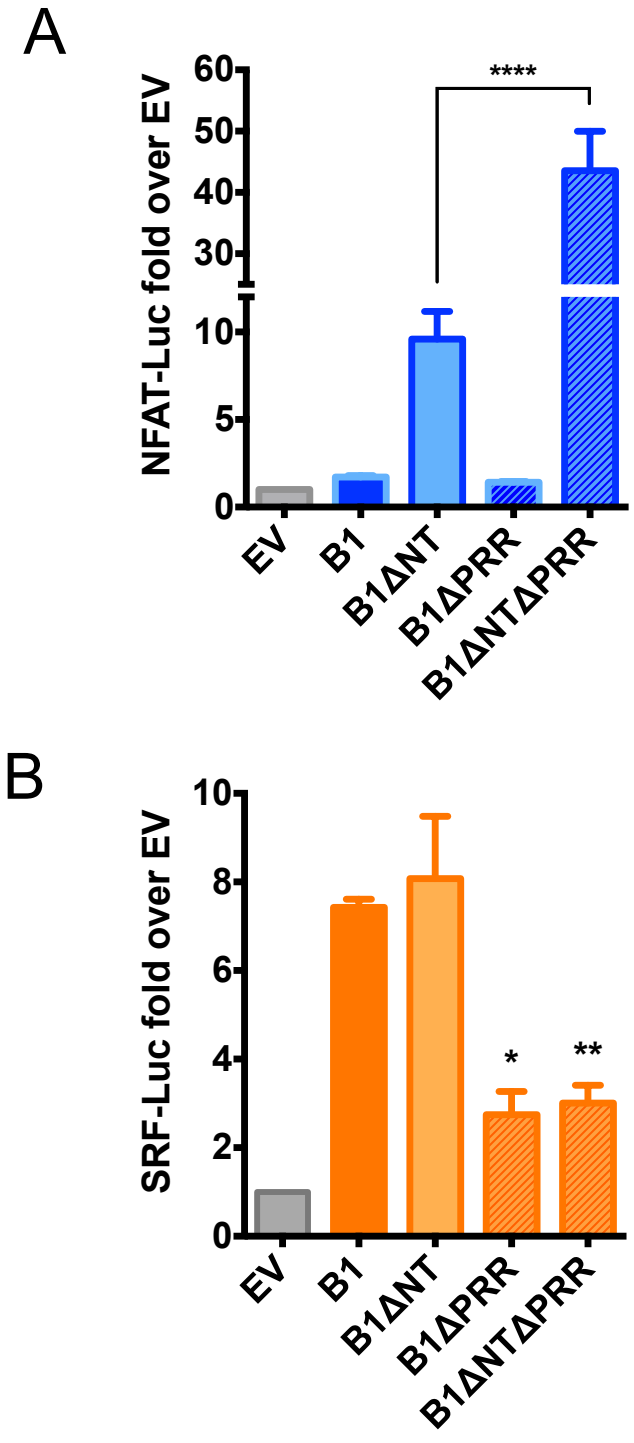


Figure 31. PRR loss silences SRF but potentiates NFAT activity

Full-length (B1) and truncated forms (ΔNT) of WT and ΔPRR mutant receptors were expressed in HEK cells and assayed for NFAT and SRF reporter activity. Deletion of the proline-rich region significantly increased the activity of B1ΔNT (A, B1ΔNT vs B1ΔNTΔPRR ****$p<0.0001$, n=7) but did not change the activity of B1 (n=4). This deletion reduced the activity of full-length B1 and B1ΔNT to SRF luciferase (B, B1 vs B1ΔPRR *$p<0.05$, B1ΔNT vs B1ΔNTΔPRR **$p<0.01$, n=5).

Figure 31. PRR loss silences SRF but potentiates NFAT activity



4.3.3 ADGRB1 SZ-linked mutations

Sequencing *ADGRB1* in multiplex SZ families resulted in the initial identification of three *ADGRB1* variants with relatively high combined annotation-dependent depletion (CADD) scores (Fig. 32). We engineered these substitutions into B1 expression plasmids and assessed their ability to express on the plasma membrane using cell surface biotinylation (Fig. 33). All three mutant receptors were found to be robustly expressed on the cell surface.

Next, we sought to test the hypothesis that these mutations may affect receptor signaling activity (Fig. 34). We expressed all three mutants alongside WT B1ΔNT and found that the activity of B1ΔNT-R1473W was dramatically increased over WT to the NFAT reporter (A, One-way ANOVA, $F(5, 18) = 19.24$ $p<0.0001$, *post-hoc* Holm-Sidak multiple comparison B1ΔNT-R1473W vs B1ΔNT ****$p<0.0001$, n=4) but P947L and S1566L did not have a significant effect. Interestingly, none of the mutations significantly altered activity to the SRF pathway (n=6).

The B1-R1473W signaling activity finding described above was similar to the result reported in Chapter 3 describing the effect of an R→W substitution at site 1465 of ADGRB2. These two arginine residues are not exactly homologous, but are located in the same stretch of positive residues. Based on the finding the Chapter 3 that B2ΔNT-R1465W has a reduced ability to interact with endophilin A1, we tested whether this effect also applied to B1ΔNT-R1473W (Fig. 35). Interestingly, we found that all three forms of B1 – WT full-length and B1ΔNT, as well as B1ΔNT-R1473W strongly interacted with the SH3 domain of endophilin A1 in the GST pull-down assay.

Figure 32. B1 schizophrenia (SZ)-associated mutations

Three missense mutations were initially identified in a cohort of individuals with SZ: P947L at the junction of the juxtamembrane stalk and the first transmembrane domain, R1473W in a very similar location in the C terminus as ADGRB2 R1465W, and S1566L in the distal C terminus (Abbreviations: TSP – thrombospondin repeat; GAIN – GPCR autoproteolysis inducing; PRR – proline-rich region; PBM – PDZ-binding motif).

Figure 32. B1 SZ mutations

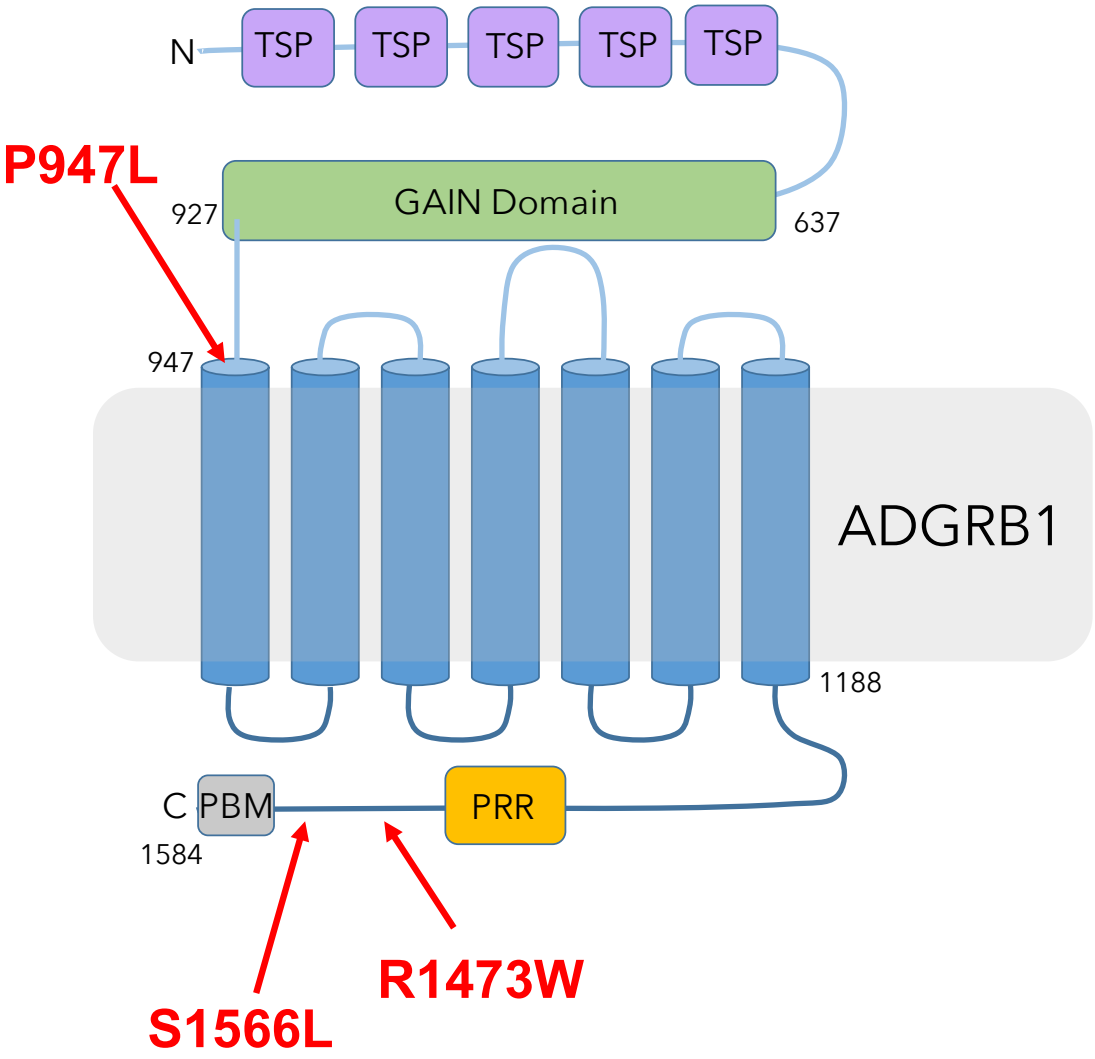


Figure 33. Surface expression of B1 schizophrenia (SZ)-associated mutants

WT and SZ mutant forms of B1ΔNT were expressed in HEK cells. Receptors that were expressed on the cell surface were biotinylated with a cell membrane-impermeable biotinylation reagent and isolated on streptavidin resin. Whole cell lysates and surface pull-down fractions were probed with a B1 CT antibody. All three mutant forms of the receptor were found to be expressed at least as well as WT on the cell surface.

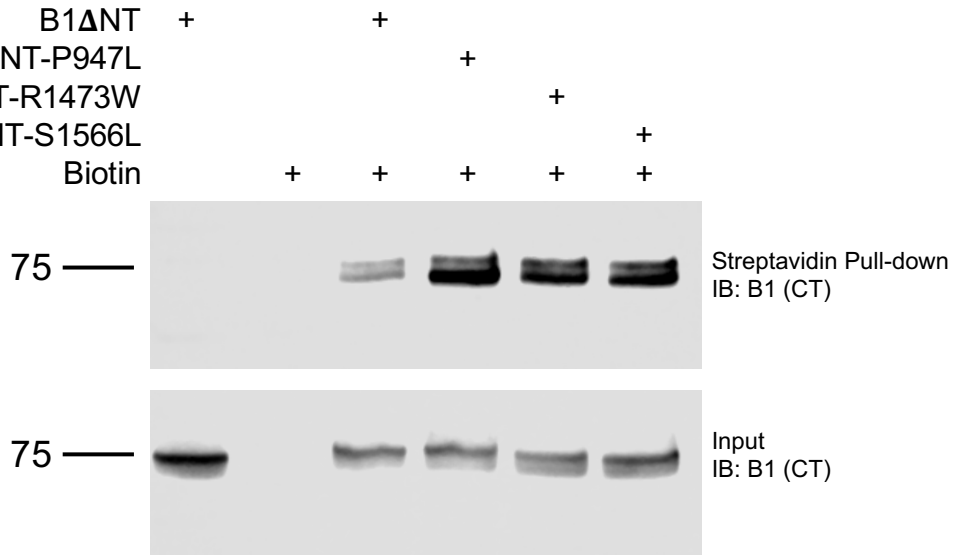


Figure 34. R1473W increases B1ΔNT signaling activity

Analyses of all three B1 schizophrenia-linked mutants in terms of signaling activity in NFAT (A) and SRF (B) luciferase assays revealed a selective effect of the R1473W mutation on signaling to NFAT luciferase (A, ****$p<0.0001$, n=4). None of the mutations significantly altered signaling to SRF luciferase (B, n=6).

Figure 34. R1473W increases B1ΔNT signaling activity

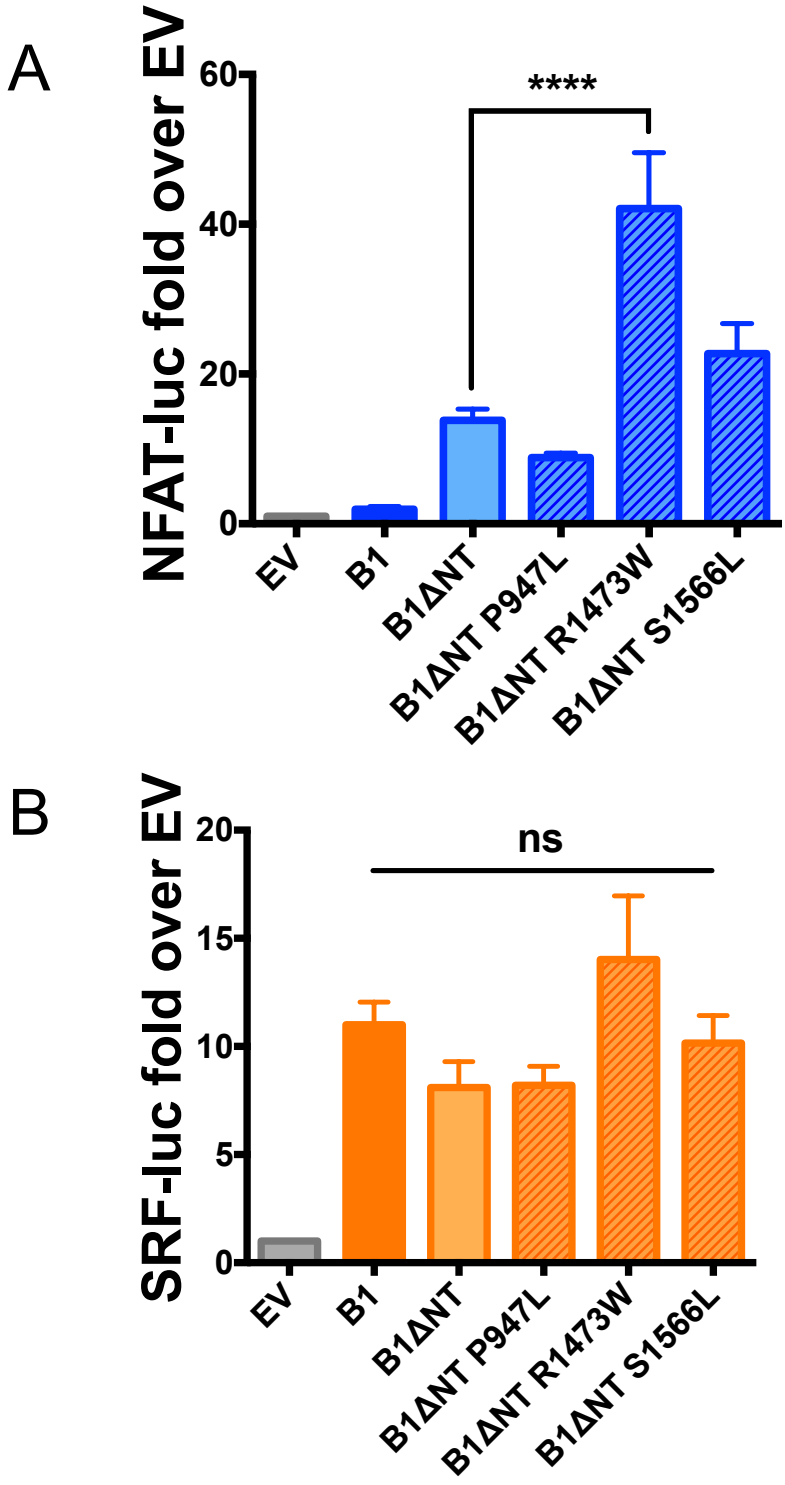


Figure 35. B1 R1473W retains interactions with endophilin A1

Lysates from cells expressing EV, B1, B1ΔNT, or B1ΔNT-R1473W were split and incubated with either control GST or GST-Endophilin A1-SH3 domain fusion protein beads. Input lysates and pull-down fractions were probed with a B1 CT antibody. The R1473W mutation did not impair the ability of B1ΔNT to interact with the SH3 domain of endophilin A1.

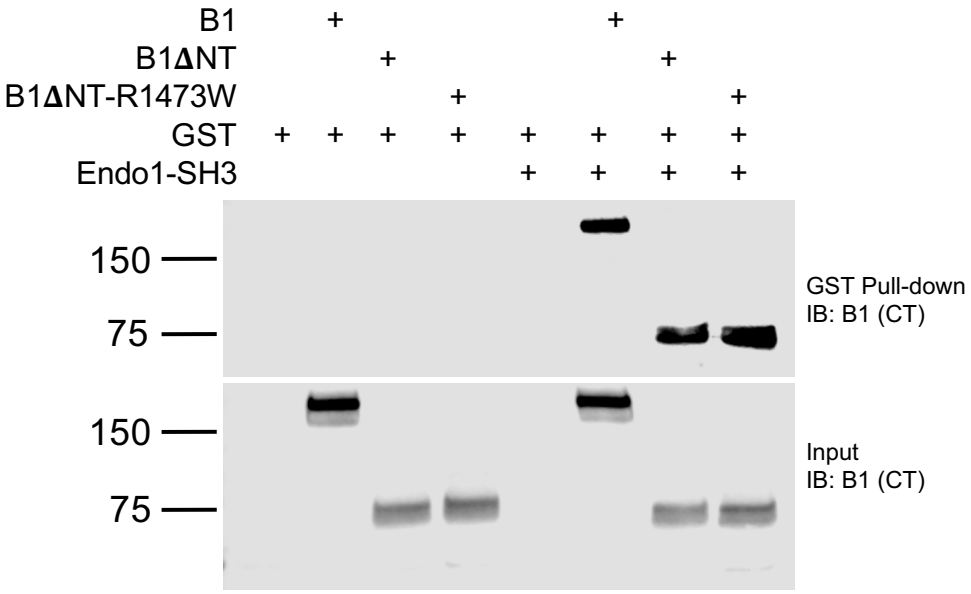
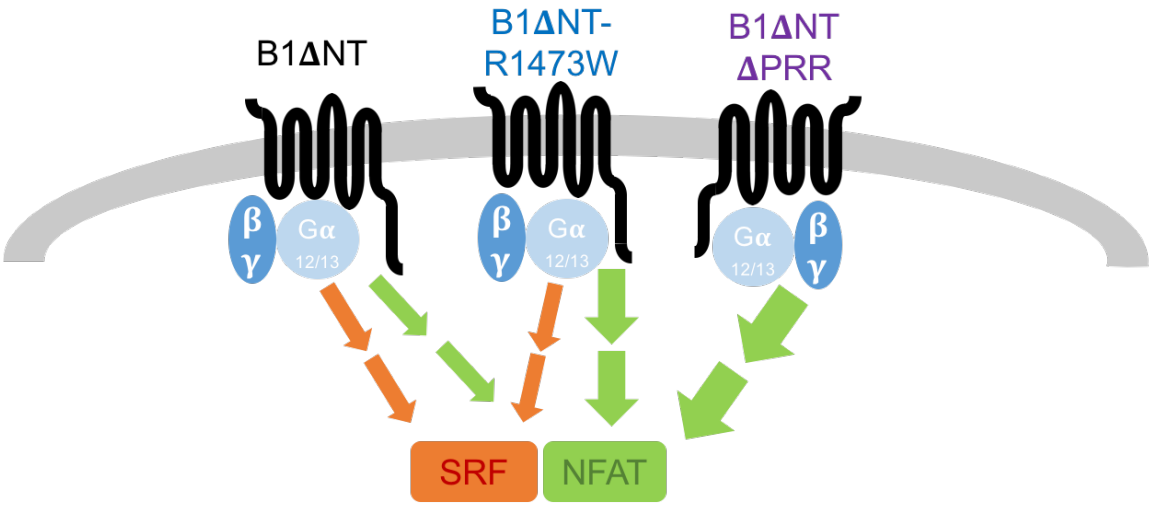


Figure 36. Effects of C-terminal mutations on B1 signaling

B1ΔNT activated both SRF and NFAT-luciferase reporters. The schizophrenia-linked R1473W mutation strongly increased signaling to NFAT but had no significant effect on signaling to the SRF pathway. Deletion of the proline-rich region (PRR) biased the receptor's activity such that SRF tone was completely eliminated but activity to NFAT-luciferase was strongly potentiated.



4.4 Discussion of ADGRB1 C terminal determinants of signaling activity

These studies began with an investigation into the functional relationship between IRSp53 and ADGRB1. IRSp53 was originally discovered as a protein that interacts with the cytoplasmic C terminus of B1, but since then there has been no examination of the functional implications of this interaction. We found that IRSp53 increases the surface expression of the active form of B1 (B1ΔNT) and also strongly increases the receptor's signaling activity to the NFAT but not SRF reporter. Interestingly, co-expression of IRSp53 had no effect on the full-length receptor either in cell surface trafficking or signaling activity.

One notable feature of the C terminus of B1 that is absent in ADGRB2 and ADGRB3 is the polyproline region. Poly-proline sequences are often key sites of protein-protein interaction, specifically with WW and SH3 domains (Li 2005). Moreover, the interaction between B1 and IRSp53 was shown to occur via the SH3 domain of IRSp53, but the region of interaction on the receptor has not been characterized (Oda et al. 1999). We hypothesized that deleting the proline-rich region (PRR) of B1 would abolish this interaction, but surprisingly found that IRSp53 retained the ability to interact with B1ΔNTΔPRR. Perhaps even more surprising was the effect on the interaction between B1 and endophilin A1. As shown in Figure 26, B1ΔNTΔPRR co-immunoprecipitates with full-length endophilin A1. We had been unable to observe this interaction with B1ΔNT in cells because when endophilin is co-expressed with B1, the receptor is rapidly degraded. This result suggests that *i)* the interaction between B1 and the SH3 domain of endophilin A1 does not require the polyproline region of B1 and *ii)* endophilin alone might not be sufficient to degrade the receptor. It could be that an additional protein

(perhaps a protein that binds the B1 PRR) is involved. Results from Chapter 3 indicate that endophilin binding might not depend on polyproline sequences because endophilin binds robustly to ADGRB2 despite many fewer prolines than B1, and moreover the mutation of a positively charged residue in B2 (R1465) disrupts the receptor's interaction with endophilins.

While deleting the PRR did not result in disruption of B1 interaction with IRSp53, the effects of this manipulation on receptor signaling were nonetheless striking. Removal of the PRR in full-length B1 eliminated its signaling ability. Full-length B1 ordinarily exhibits no activity to the NFAT reporter but strongly activates SRF luciferase. Our results indicate that this signaling activity to SRF is in some way dependent on the PRR, which suggests that a RhoGEF might be recruited to this region of the receptor to activate signaling to the Rho pathway. A previous study found that the SRF activity of B1ΔNT was only inhibited ~50% by RGSp115, a $G\alpha_{12/13}$ inhibitor, but the ability of this inhibitor to block signaling by the full-length receptor was not tested (Kishore et al. 2016). This leaves open the possibility that a significant portion of signaling in this pathway is not mediated solely by heterotrimeric G proteins and could also be driven by small GTPases, particularly from the full-length receptor.

The effect of PRR removal on the NFAT pathway was even more profound. This manipulation had no effect on full-length B1 but dramatically increased the activity of B1ΔNT. This finding could be related to the discussion of endophilin binding above. While we did not observe a striking increase in the levels of B1ΔNTΔPRR relative to B1ΔNT, it is possible that removing the PRR disrupts regulatory mechanisms and allows for each receptor to signal longer or more efficiently. The mechanisms underlying this

strong bias in receptor signaling induced by removal of the PRR will require further investigation to thoroughly understand.

In the final portion of this chapter, initial findings are shown from the study of three recently identified variants of B1, which may be linked to schizophrenia (SZ). Each of these substitutions are in receptor locations that may be functionally important – P947L is at the interface of the extracellular stalk and first transmembrane domain, R1473W mirrors the positioning of the ADGRB2 R1465W mutation (Chapter 3), and S1566L is a potential phosphorylation site on the distal C terminus of the receptor. Our studies on these mutations indicate that all three mutants are efficiently expressed on the cell surface and exert no significant effects on SRF signaling. However, reminiscent of B2-R1465W, B1-R1473W strongly increased activity to NFAT-luciferase in the cleaved, constitutively-active form of the receptor (B1ΔNT). We hypothesized that this could be due to a loss of binding to endophilin A1, as we observed with B2, but that was not the case. It is perhaps more likely that this mutation increases the flexibility of G protein coupling to B1, similar to the case for B2, and perhaps allows B1 to couple to $G\alpha_i$ or $G\alpha_q$ in addition to coupling to $G\alpha_{12/13}$. Further studies will be needed to assess these possibilities.

In summary, the studies described in this chapter have highlighted how specific portions of the long cytoplasmic C terminus of B1 can powerfully modulate the receptor's signaling activity. It is striking how three separate manipulations – co-expression of IRSp53, deletion of the PRR, and a SZ-linked mutation – all significantly increase the receptor's signaling to the NFAT pathway. The present characterization of these mutant receptors provides the basis for further work in native cells and *in vivo* with

these tools to better dissect the effects of the mutations on the signaling pathways downstream of B1. Additionally, future studies should be aimed at functional analyses of the B1-IRSp53 interaction in a more native context. Further work will be required to fully understand the functional consequences of this interaction between two key synaptic proteins.

Chapter 5. Summary Discussion and Future Directions

5.1 Summary of advances

Rapid progress has been made in the adhesion GPCR field over the past decade in terms of understanding the physiological functions and pharmacology of many receptors across the family. This progress has been catalyzed by three recent breakthroughs: (1) reports in 2010-2013 that multiple receptors from distantly-related subfamilies become constitutively active when the NT is removed (Okajima, Kudo, and Yokota 2010, Paavola and Hall 2012, Paavola et al. 2011, Stephenson et al. 2013, Ward et al. 2011); (2) the crystal structure of the GAIN domain (Arac et al. 2012); and (3) the discovery that several receptors have cryptic tethered agonists (Liebscher et al. 2014, Stoveken et al. 2015). The work presented here has focused on the pharmacology of two closely-related receptors that have surprisingly different downstream signaling activities.

Initial reports of the *stachel* cryptic tethered agonist suggested that this may be a general phenomenon that applies to all receptors in the family. However, there was already evidence that several receptors have GAIN domain cleavage deficiencies (Promel, Waller-Evans, et al. 2012), suggesting that a tethered agonist might not be necessary for the activation of some aGPCRs. The first aim of this project was to determine whether ADGRB1 has a tethered agonist (Chapter 2). We deleted the putative tethered agonist stalk region in B1 and found that this sequence is not required for activation of ADGRB1. The stalk-less receptor was at least as active as B1ΔNT in each signaling assay that we performed and we found that β-arrestin2 and $G\alpha_{13}$ each co-immunoprecipitated with B1-SL, as they do with the constitutively active form of the receptor, B1ΔNT. Parallel studies on ADGRG1 found that this receptor's tethered agonist was required for some but not all pathways downstream of the receptor, and that G

protein alpha subunits and β-arrestins recognized the stalk-less G1 as an active conformation of the receptor (Kishore et al. 2016).

These data have led us to propose a more inclusive model of aGPCR activation whereby the intrinsic activity of aGPCRs is inhibited by the N-terminal fragment (NTF), which holds the receptor in an inactive conformation. A ligand could cause a subtle re-arrangement of the orientation of the tethered agonist such that it could activate the stalk-dependent portion of receptor activity or a larger ligand could cause a more drastic shift in the conformation of the NTF, which could relieve its inhibition and activate the receptor without exposing a tethered agonist. Alternatively, complete removal of the NTF could produce full activity of the receptor both by relieving an inhibitory constraint on the 7-TM and by allowing the tethered agonist to physically push the receptor into an active conformation (Fig. 5, p. 29). This receptor would then likely be internalized and degraded because it is unlikely that the NTF could re-form the original heterodimer conformation.

The second aim of this project was to investigate the functional effects of a disease-associated mutation in ADGRB2 (Chapter 3). We created expression plasmids of wild type and mutant B2 and our data provide a basis for understanding the potential implications of aGPCR signaling gone awry. The NIH Undiagnosed Diseases Program found this *de novo* missense mutation in *ADGRB2* in a patient who had been suffering from an unexplained, degenerative neuromuscular condition for several decades. The mutation was predicted to be highly deleterious based on the residue's extreme conservation and the drastic substitution of a bulky, hydrophobic tryptophan residue in place of a basic arginine. We found that this substitution of a single amino acid out of

nearly 400 in the C terminus of the receptor doubled the surface expression and signaling activity of B2ΔNT. In addition, we were able to dissect the signaling pathway and found that the majority of the signaling we observed occurred via Gβγ liberation. Moreover, contrary to previous assumptions, these studies revealed that B2 predominantly couples to the G protein alpha subunit $G\alpha_z$. This work also identified endophilin A1 as a regulator of B2 and revealed that this interaction is compromised by the R1465W mutation. B2 is the first aGPCR that has been shown to interact with either $G\alpha_z$ or endophilin.

Both of these novel interactions will require further investigation. One aspect of this work that was surprising was that the $G\alpha_z$-inhibitor RGS20 substantially increased the activity of B2ΔNT. We proposed that this could be because active $G\alpha_z$ is engaged in activity that limits the signal to NFAT-luciferase and thus inhibiting $G\alpha_z$ relieves this inhibition. $G\alpha_z$ has been shown to inhibit N-type calcium channels in neurons (Jeong and Ikeda 1998) and therefore inhibiting $G\alpha_z$ could promote calcium influx and more NFAT activity, as we observed. Understanding the functions of $G\alpha_z$ will be critical to fully understanding the physiological role of ADGRB2. Additionally, while the R1465W mutation appeared to drastically disrupt the B2ΔNT interaction with endophilin A1 in a pull-down assay, both WT and mutant receptors appeared to be equally inhibited by co-expression of endophilin. It could be that even slight over-expression of endophilin is able to overcome this binding deficit, or there may be additional proteins involved in a cellular context. For example, data from Chapter 4, demonstrating that deletion of the proline-rich region of ADGRB1 preserves the ability for the receptor to bind to endophilin in cells but prevents its degradation, suggest that an additional polyproline-

binding protein may be involved in the functional interaction of endophilin and these receptors.

The third aim of this project was to test the effects of deleting the cytoplasmic proline-rich region in ADGRB1 (Chapter 4). We hypothesized that, due to the well-known affinity of proteins with WW and SH3 domains to polyproline sequences, that this region is a key site of protein-protein interactions. One of the most notable proteins known to interact with ADGRB1 is IRSp53, but the function of this interaction has not been investigated. Therefore, we explored the functional connection between IRSp53 and ADGRB1 and tested its dependence on the proline-rich region. In addition, we investigated the functional consequences of three mutations in B1 that were discovered in individuals with schizophrenia. This work is still fairly preliminary in nature but suggests exciting future avenues of research. IRSp53 and B1 do indeed appear to have a functional connection, but their physical association does not occur via the conspicuous polyproline sequence on the B1 C-terminus and further work will be required to identify the structural determinants of this interaction. Intriguingly, we found that removing the PRR of B1 dramatically biased the active form of the receptor from the baseline situation where the receptor displays relatively equal activity to both SRF and NFAT pathways to exclusively and robustly activating NFAT luciferase. Finally, we found that one of the B1 SZ-linked mutations that is adjacent to the residue mutated in B2-R1465W also strongly potentiates the receptor's signaling to the NFAT pathway but does not disrupt binding to endophilin A1. The apparent functional importance of this highly positively-charged region of the CT of both B1 and B2 is striking and worthy of further investigation.

5.2 Limitations

These studies have several inherent limitations. First, the vast majority of these experiments were conducted in heterologous cells using protein over-expression to produce effects. Given the dearth of tools to study these proteins, including a lack of pharmacological agents to alter receptor activity in a temporally-controlled manner, this approach has been necessary. Nonetheless, HEK cells have the advantage of expressing a number of neural proteins and among highly-transfectable heterologous cell lines provide a useful vehicle for studying neural receptors (Shaw et al. 2002). In addition, HEK cells provide an excellent model system for interrogating different isoforms of B1 because the receptor is not found to be expressed endogenously in this line and remains uncleaved when over-expressed via transfection. By contrast, endogenous expression of B2 in HEK cells made it somewhat challenging to differentiate between WT B2ΔNT and B2ΔNT-R1465W, so for many experiments we used C-terminally tagged versions of these proteins. However, this approach has its own limitations in that adding an epitope tag to the C terminus compromises the PDZ-binding motif and may substantially alter the receptor interactome. Therefore, future studies should certainly move into primary tissues in order to better evaluate the function of these receptors in a more native environment and in specific cellular compartments such as the synapse.

Similarly, the lack of temporal control over signaling activity in an over-expression gene reporter assay is an extremely limiting factor. It is possible that some results such as the paradoxical increase in signaling that we observed with RGS20 co-expression (Fig. 19, p. 97) could be an artifact of a 48-hour overexpression assay rather than a true biological phenomenon. Over-expression of constitutively-active forms of the

receptors was the only option available to study their downstream signaling but, in some cases, the results of this approach may not accurately reflect the way these receptors signal in response to endogenous ligands.

Finally, our studies fall short of demonstrating causality between any of the mutations under investigation and the diseases that they were initially linked to. We cannot be certain that B2 R1465W causes the neuromuscular symptoms experienced by the patient and do not fully understand how the effects of this mutation on the receptor's expression and activity may underlie these clinical signs. Moreover, the B1 SZ mutations were discovered in a preliminary sequencing effort and control populations have not been assessed for the presence of these mutations. However, even if these mutations are not causally linked to these diseases, they have catalyzed our efforts to understand key regions of these receptors that affect signaling activity.

5.3 Future Directions

The study of the ADGRB sub-family of aGPCRs will likely continue to flourish based on emerging evidence of the important functions of these receptors at synapses and their links to human disease. It will remain important to dissect the signaling pathways downstream of adhesion receptors and determine the relative importance of a tethered agonist for each pathway.

One of the conclusions from this study (Chapter 3) is that an increase in B2 signaling may, over time, lead to human disease – in this case a devastating degenerative neuromuscular condition. However, a previous study suggests that eliminating ADGRB2 signaling may not have any deleterious effects and may, in fact, be beneficial (Okajima,

Kudo, and Yokota 2011). These researchers reported that *adgrb2* null mice exhibited no gross physical or cognitive deficits but displayed an antidepressant-like phenotype based on a resilience to the induction of learned-helplessness behavior. Moreover, post-mortem analysis revealed that the mice lacking ADGRB2 had increased neurogenesis in the hippocampus. Therefore, an exciting possibility for a future aim based on this work would be the development of an antagonist or inverse agonist for ADGRB2ΔNT. Our NFAT luciferase assay would be an efficient platform to screen for an antagonist with its high signal-to-noise ratio and medium to high-throughput capacity. It is unclear how many patients would benefit from a compound that would normalize or reduce ADGRB2 signaling activity, but at the very least such a compound could be a very useful experimental tool. As mentioned above, a similar study recently found an antagonist for ADGRG1, which indicates that this approach is feasible (Stoveken et al. 2016).

If overexpression of ADGRB2 is at the root of the R1465W patient's symptoms, then another approach would be to find a way to reduce receptor expression. We recently found that *ADGRB2* contains an estrogen receptor response element in its promoter and it was reported to be a female biased gene after puberty in a genome-wide study (Shi, Zhang, and Su 2016). Interestingly, *ADGRB2* is also one of the most down-regulated genes when tadpoles are exposed to an estrogen-disrupting chemical (Crump, Lean, and Trudeau 2002). Finally, in a pilot experiment, we found significantly higher levels of ADGRB2 in brain lysates from female compared to male mice (data not shown). Taken together, these data suggest that reducing estrogen levels could be a way to reduce ADGRB2 expression and potentially slow the progression of the disease in the patient. Further *in vitro* work would be required to definitively establish that *ADGRB2* responds

to estrogen levels and a mouse model could be used to test the efficacy of currently-approved estrogen receptor antagonists such as tamoxifen.

Perhaps the most surprising result from our studies has been the finding that deleting the proline-rich region of the intracellular domain of B1 dramatically biases the receptor to the NFAT pathway. However, we do not fully understand the functional implications of the differences between B1 signaling to the SRF and NFAT pathways *in vitro*, and thus do not know what effect this deletion would have in neurons *in vivo*. The finding that one of the SZ-linked mutations in B1 (R1473W) solely increases activity to the NFAT pathway suggests that signaling bias in this direction may have important physiological consequences, but further studies will be required to assess this possibility. This hypothesis could be initially tested by reintroducing B1ΔPRR or B1ΔNTΔPRR into cultured neurons from *adgrb1*$^{-/-}$ mice (Zhu et al. 2015) and assessing effects on dendritic spine number and spine morphology. A similar approach could also be used to assess the functional relationship between IRSp53 and B1.

A notable omission from the studies described here is ADGRB3. Recent work has identified a potential endogenous ligand for B3 (Bolliger, Martinelli, and Sudhof 2011), which has a functional role at synapses (Martinelli et al. 2016, Sigoillot et al. 2015) but the signaling properties and pharmacology of this receptor remain enigmatic. In fact, we do not yet know whether the post-cleavage stalk or *stachel* contributes to the signaling activity of either ADGRB2 or B3. Interestingly, the positively-charged motif that is mutated in B1-R1473W and B2-R1465W is conserved in B3 but at this point it is unknown whether mutations to this motif in B3 exist or affect receptor signaling activity.

Thus, investigating the activation mechanisms, G protein coupling, and regulatory pathways linked to B3 should be a priority of future experiments.

The adhesion GPCR field is moving forward rapidly and additional breakthroughs will likely be reported in the coming years. In particular, further crystallization studies and other structural biology approaches will be required to better understand the conformational dynamics that are involved in receptor activation and what role the *stachel* tethered cryptic agonist has in this process. Antibodies and small molecule antagonists could prove to be useful in stabilizing the large extracellular receptor domains to aid in crystallization.

In addition, the development of small molecule agonists, antagonists, and inverse agonists will be necessary to more fully understand how these receptors signal in response to ligands. Are aGPCRs recycled back to the cell surface following internalization for a second round of signaling like some class A receptors, or are they more like the protease-activated receptors that are simply degraded following internalization? The development of a toolbox of small molecule ligands for the various aGPCRs would help to address many such questions that depend on being able to activate the receptors in a temporally-controlled manner.

5.3.1 Drug targeting opportunities in adhesion GPCRs

Given their extraordinary size and complexity, aGPCRs present a variety of opportunities for therapeutic targeting (Fig. 37). First, like canonical rhodopsin family GPCRs, the 7-TM domain could be modulated by small molecules. Two examples thus far demonstrate the feasibility of this approach; beclomethasone diproprionate was

identified as a small molecule agonist of ADGRG3/GPR97 (Gupte et al. 2012) and dihydromunduletone was found to be an antagonist of G1 (Stoveken et al. 2016). As more of the pathways downstream of aGPCRs are elucidated more aGPCR family members will likely be amenable to large-scale drug screening.

The long, extracellular ectodomains of aGPCRs provide several opportunities for interventions that could alter receptor activity. First, aGPCR ectodomains provide sizeable surfaces for antibody interaction. Indeed, a polyclonal antibody directed at the ectodomain of G1 was found to activate heterotrimeric G protein signaling from the receptor (Iguchi et al. 2008). Antibody-drug conjugates have been successful in specifically delivering cytotoxic compounds to target cells in cancer (Zolot, Basu, and Million 2013) but, in this case, the antibody alone could be sufficient as a therapeutic agent. This action could occur by inducing a rearrangement in the NTF to relieve its inhibition on the 7-TM domain, by promoting ectodomain shedding, or by pushing the tethered agonist into its binding pocket.

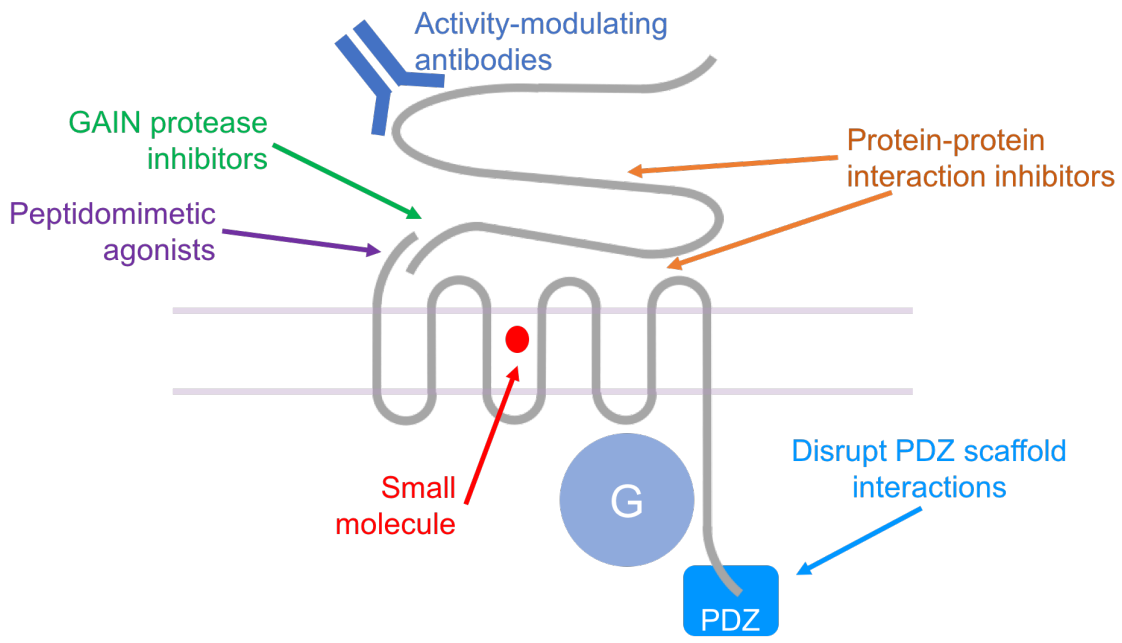
Many of the ligands identified for aGPCRs thus far are large transmembrane or extracellular matrix proteins: neurexins (Boucard, Ko, and Sudhof 2012) and teneurins (Silva et al. 2011) for ADGRL1, collagens and laminins for ADGRG1 (Luo et al. 2011) and ADGRG6 (Petersen et al. 2015, Paavola et al. 2014). Therefore, molecules that disrupt these protein-protein interactions could regulate receptor activity. In addition, the aforementioned disinhibition model of aGPCR activity posits that the NTF exerts an inhibitory constraint on the 7-TM region, possibly through NTF interactions with the extracellular loops. If this interaction could be disrupted, it could also lead to receptor activation. While these targets can be challenging, molecules that target protein-protein

interaction interfaces have entered clinical trials and this appears to be an growth area in drug discovery (Arkin, Tang, and Wells 2014). Advances in crystallizing the ectodomains of aGPCRs will likely aid in these efforts (Arac et al. 2012, Salzman et al. 2016). However, intracellular protein-protein interactions could also be targeted by molecules that can cross the plasma membrane. These molecules could be key mediators of receptor function by disrupting PDZ (PSD-95, Dlg, ZO-1) scaffold protein interactions at the C terminus for example. Thirteen of the 33 human aGPCRs have a C-terminal PDZ binding motif and these have been shown to mediate scaffold interactions as well as recruit signaling proteins like the RacGEF Tiam1 (Duman et al. 2013). Among protein-protein interfaces to target with small molecules, PDZ interactions are actually quite feasible because only the final few amino acids of the receptor C terminus are involved and act almost like a ligand in the binding pocket of the PDZ protein (Blazer and Neubig 2009, Jelen et al. 2003).

The GAIN domain also provides multiple avenues for therapeutic targeting. First, as a protease, it should be possible to inhibit (or encourage) ectodomain proteolysis. Protease inhibitors have been in the clinic since the early 1980s and have been utilized to treat hypertension, cancer, and HIV infection (Drag and Salvesen 2010). Second, a number of aGPCRs can be activated by peptides derived from the post-cleavage receptor stalk or *stachel* sequence (Table 1, p. 31) and peptidomimetic drugs could presumably agonize these receptors as well. Further design of these molecules could also provide greater receptor specificity than is attained with peptides which, due to high conservation of this region throughout the family, can have crossover activity at multiple receptors (Demberg et al. 2017).

Figure 37. Adhesion GPCR targeting strategies

Adhesion GPCRs may offer more opportunities for targeted drug development than classic rhodopsin-like receptors. First, two aGPCRs have been shown to be modulated by a small molecule (red). Each aGPCR has a 7-TM domain and should have binding pockets in this domain for small molecules. Second, the long N termini of aGPCRs offer multiple opportunities including for peptides or peptidomimetic agonists (purple) to act at the tethered agonist interaction site, modulators of GAIN proteolysis (green), antigen surfaces for custom antibodies (dark blue), and regulators of protein-protein interactions (orange). These interactions could also be modulated intracellularly with molecules that could disrupt binding to PDZ scaffold proteins (light blue).



5.4 Conclusion

Many of the diseases with which ADGRB1 and ADGRB2 have been associated – autism, schizophrenia, ADHD and age-related neurodegeneration – either lack pharmacological interventions at present or are treated by therapies that are far from optimal. The development of novel therapeutic approaches for these disorders depends on the identification and characterization of novel drug targets that are physiologically relevant to the pathology. GPCRs are outstanding drug targets with at least 1/3 of approved drugs targeting GPCRs but to date there are no approved drugs targeting adhesion GPCRs (Roth and Kroeze 2015). ADGRB1 and ADGRB2 would seem to be prime targets for novel therapeutics aimed at treating autism, schizophrenia, ADHD and age-related neurodegeneration. The studies presented here provide a fundamental characterization of the signaling activities and regulation of ADGRB1 and ADGRB2, with the goal of setting the stage for the eventual pharmacological targeting of these receptors to treat human disease.

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