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Phosphatidylserine Exposure Modulates BAI1 (ADGRB1) Signaling Activity

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Phosphatidylserine Exposure Modulates Adhesion GPCR BAI1 (ADGRB1) Signaling Activity

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Advisor: Randy A. Hall, Ph.D.

An abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate Division of Biological and Biomedical Science's Neuroscience Program

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By Trisha Lala

The adhesion G protein-coupled receptor family consists of several dozen receptors containing massive N-terminal fragments (NTFs), which confer these receptors with adhesive properties. Recent advances in the field have revealed that some family members can be modulated by small molecules, mechanosensory forces and adhesive ligands, suggesting that these receptors function as massive integrators of multimodal signaling.

The body of work described here provides new insights into the signaling properties of the adhesion GPCR BAI1 (also known as ADGRB1 or "B1"). This receptor has previously been shown to bind phosphatidylserine (PS) via its NTF, but it remains unknown whether this interaction alters the signaling activity of the receptor. B1 in most highly expressed in the brain and can couple to G proteins and other pathways to regulate postsynaptic function and dendritic spine morphology. We investigated G protein-dependent signaling by B1 in the absence and presence of the PS-flippase ATP11A, which can modulate the amount of PS available for B1 to bind in the outer leaflet of the plasma membrane. We mainly used the HEK293T cell system for these studies, as we found that these cells exhibit a quantifiable baseline level of PS exposure that can be modulated by ATP11A overexpression. We observed that ATP11A expression dramatically reduced B1 G protein-dependent signaling for the wild-type receptor but not a truncated mutant lacking the large extracellular NTF, suggesting that the NTF of B1 is required for PS sensing. The flippase activity of ATP11A was found to be essential for regulation of B1 signaling, and co-immunoprecipitation experiments revealed that ATP11A not only modulates B1 signaling but also forms complexes with B1. To characterize regulation of B1 signaling by PS exposure using an independent method, we studied B1 signaling in cells with lower PS externalization due to deletion of the endogenous PS scramblase ANO6 and found that this manipulation also resulted in lowered B1 signaling activity. These findings demonstrate that B1 signaling is modulated by PS exposure and therefore implicate B1 as a PS sensor at synapses and in other cellular contexts.

The studies described here provide a deeper understanding of the adhesion GPCR B1 and also contribute to a deeper understanding of NTF-mediated regulation of signaling by other AGPCRs. Additionally, this work elucidates how changes in PS exposure can be detected in the brain, which has implications for synaptic pruning, synaptic plasticity, and other brain processes known to be regulated by externalization of PS.

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Dedication

The following work is entirely dedicated to Rakhee and Deepak Lala, my unwavering support system without which I could not have received this degree. You have demonstrated to me the value of hard work, persistence and belief in oneself. I hope to continue to learn from you and implement the lessons you have taught me. Mom, thank you for taking on my stress and frustrations as your own and giving me the confidence to work through anything. Dad, thank you for your guidance and for always being willing to talk science with me. Thank you to both my parents for your steadfast support in all aspects of my life. Thank you for inspiring my love of learning & science and encouraging me along my path to discovering my true passions, both professionally and personally. Thank you for believing in me.

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Chapter 1: Introduction

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1.1 CELL-CELL COMMUNICATION

1.1.1 Cell surface receptors

"So we may suppose that in all cells two constituents at least are to be distinguished, a chief substance, which is concerned with the chief function of the cell as contraction and secretion, and receptive substances which are acted upon by chemical bodies and in certain cases by nervous stimuli. The receptive substance affects or is capable of affecting the metabolism of the chief substance (Langley, 1905)." Nobel prize winner Robert J. Lefkowitz cited British pharmacologist, J.N. Langley during his Nobel Lecture in 2012 as he described an early account of the existence of a cellular receptor, which Langley hypothesized must first interact via chemicals and stimuli by binding to them and then acting upon intracellular effectors to alter cellular function. Despite Langley's astute observation, decades of skepticism passed before the pharmacological study of receptors would significantly evolve.

Modern advances have revealed that the ability of a cell to sense its external environment is critical to normal cellular function, including intracellular changes at the proteomic and genetic levels, and for communication with other cells. Proteins, lipids and carbohydrates comprise much of the plasma membrane, where external signals are transduced intracellularly. Receptors can function alone in response to ligand engagement, or they can also form multimers with themselves or form complexes with other proteins to facilitate signal transduction, as is the case in specialized compartments such as the postsynaptic density of neurons. Signals can include the extracellular matrix itself, other cells, adhesive ligands, small molecules, peptides, proteins and even mechanosensory stimuli. These

signals are highly variable, with increasing complexity conferred by the receptors transducing the signal itself.

1.1.2 Synaptic communication

Receptors play especially important roles at junctions between cells, for example, synapses in the nervous system. The discovery of synapses stemmed from scientific advances like higher-powered microscopes and staining techniques developed by Camillo Golgi and others to facilitate the visualization of individual neurons. Further work by Santiago Ramón y Cajal elucidated the ultrastructure of the neuron and revealed that unlike other cells in the body, neurons do not form cellular sheaths but instead are independent entities working in concert, setting into motion what became the discovery of synaptic neurotransmission (Sotelo, 2020). Golgi and Ramón y Cajal shared the 1906 Nobel Prize in Physiology or Medicine for their contributions to the understanding and characterization of neuronal structure.

J.N Langley is considered a father of the chemical receptor theory, building upon his hypotheses described at the beginning of this section. Ultimately, his work revealed that neurons, instead of directly touching other neurons, engage with other cells by releasing neurotransmitters that interact with the recipient cell by binding to receptors. In 1936, Henry Dale and Otto Loewi received the Nobel Prize in Physiology or Medicine for discoveries relating to chemical transmission of nerve impulses and for identifying the first neurotransmitter, acetylcholine.

1.1.3 Overview of GPCRs

What we now consider fact regarding receptor pharmacology is the result of a great effort by Lefkowitz and others beginning in the 1970s, when radioligand binding methods were used to study receptors directly (Williams, Mullikin, & Lefkowitz, 1976). These initial studies were critical to

developing an understanding of receptor-agonist binding states and ultimately led to the development of the ternary complex model in 1980, which explains the agonist-specific binding properties of G protein-coupled receptors (De Lean, Stadel, & Lefkowitz, 1980). Lefkowitz, Kobilka and colleagues then elucidated the first cloned sequence of a ligand-binding G protein-coupled receptor (GPCR), allowing for the establishment of features now considered canonical for this family (Dixon et al., 1986).

We now appreciate that there are a wide variety of receptor classes, and among the largest and most diverse is the GPCR family, with nearly 1,000 receptors encoded in the human genome, a far shift in the understanding of pharmacology since Langley's days (Takeda, Kadowaki, Haga, Takaesu, & Mitaku, 2002). GPCRs are ubiquitously expressed in the body and activated by a wide range of ligands including proteins, lipids, carbohydrates and even light. Canonical ligand activation of various GPCRs triggers activation of the G protein α subunit and the subsequent dissociation of the $\beta\gamma$ subunits of the complex that then initiates signaling via secondary messengers. G protein signaling pathways are turned off by GTPase-activating proteins (GAPs) known as Regulators of G protein signaling (RGS) proteins. These proteins activate the GTPase activity of Gα subunits to turn off G protein signaling activity. RGS proteins play important roles in many aspects of physiology, including synaptic plasticity (Gerber, Squires, & Hepler, 2016). Alternative signaling pathways mediated by GPCRs include pathways involving GPCR kinases (GRKs), β-arrestins, and other intermediates. GPCRs are major drug targets, with roughly one-third of all Food and Drug Administration-approved drugs targeting this receptor class (Eiger, Pham, Gardner, Hicks, & Rajagopal, 2022).

Canonical GPCR signaling is mediated by the subsequent signaling triggered by heterotrimeric G proteins. However, this activity must be tightly regulated to avoid aberrant signaling which could lead to cytotoxicity. As mentioned above, GRKs and β-arrestins regulate GPCR activity and can recognize the receptor depending on its activity state. GRK-mediated phosphorylation of serine and threonine residues intracellularly generates binding sites for β-arrestins (Premont, Inglese, & Lefkowitz, 1995). The binding of β-arrestins sterically occludes heterotrimeric G protein-binding and can halt excessive G protein-mediated signaling (Tian, Kang, & Benovic, 2014). Novel single-molecule fluorescence resonance energy transfer imaging has demonstrated that β-arrestins are strongly autoinhibited in their basal state, and that agonist-promoted receptor activity is needed for concurrent self-disinhibition of β-arrestins (Asher et al., 2022). β-arrestin binding can also facilitate alternate signaling pathways; for example, they act as scaffolds for the MAP kinase, Src and Akt pathways (Violin & Lefkowitz, 2007). GPCR physiology can also be regulated by key protein-protein interactions with a variety of different scaffold and regulatory proteins that can alter receptor function (Ritter & Hall, 2009).

Monumental advances in the GPCR field were made possible with the advent of crystallography and cryo-electron microscopy. The first GPCR for which a crystal structure was obtained was the bovine rhodopsin receptor (Palczewski et al., 2000). The Palczewski group was then able to generate a photoactivated deprotonated intermediate GPCR crystal structure of 4.15 angstroms (Salom et al., 2006). In 2011, the Kobilka group was able to generate a crystal structure of the active state ternary complex of the agonist-bound monomeric β-adrenergic receptor in complex with G proteins, a 14-angstrom structure (Rasmussen et al., 2011). As more advanced techniques are developed, the GPCR field will continue to exponentially grow and generate even more therapeutics to improve patient lives.

1.2 GPCR MULTIMERIZATION

Although initially controversial, it is now well-established that GPCRs can form functionally relevant multimers. Furthermore, multimerization (or oligomerization) of GPCRs plays a role in protein maturation and cell surface trafficking. Multimerization may also facilitate heterotrimeric G protein association and β-arrestin binding (Milligan, 2008). This discovery has great clinical significance as well, as multimerization can inform drug design. The Palczewski group was the first to generate biophysical evidence of GPCR multimerization of the bovine rhodopsin GPCR first using atomic-force microscopy (Fotiadis et al., 2003), then using electron crystallography (Ruprecht, Mielke, Vogel, Villa, & Schertler, 2004) and finally by X-ray crystallography (Lodowski et al., 2007).

The clinical relevance of this phenomenon is underscored by the example of the regulation of the dopamine D1 and D2 receptor heterodimer by clozapine. The existing treatment for schizophrenia, clozapine is known to bind to two sites on the D1 and D2 receptor, with the affinity of the D1 receptor dependent on whether the D2 receptor is also present (Faron-Gorecka, Gorecki, Kusmider, Wasylewski, & Dziedzicka-Wasylewska, 2008). The Dziedzicka-Wasylewska group demonstrated that clozapine may antagonize the effect of dual stimulation of both D1 and D2 receptors and modulate downstream calcium signaling. Such crosstalk between receptor subtypes, mediated by multimerization, will likely be applied to understanding the pharmacology of other clinically-relevant GPCRs in the future.

1.2.1 GPCR Allosteric Modulation

Allosteric modulation of GPCRs expands the available toolbox of drug discovery. Traditional agonist or antagonist drug design results in the persistent activation or blockade of a given receptor, but in many cases persistent activation or complete shutdown of a receptor may be undesirable.

Allosteric modulation, on the other hand, is a method of modulating receptor activity without binding to the orthosteric site but instead to a completely different location on the receptor (Wold, Chen, Cunningham, & Zhou, 2019). This method also allows for higher specificity (potentially based on receptor multimer-state) and subsequently precise pharmacological modulation (Foster & Conn, 2017). This has a particular appeal for central nervous system (CNS)-related disorders, where tight control of specific receptor subpopulations is needed. Allosteric modulators can be synthetic drugs, but endogenous allosteric modulators of GPCRs also exist, including heterotrimeric G proteins themselves and a variety of ions, lipids, amino acids, peptides and accessory proteins (van der Westhuizen, Valant, Sexton, & Christopoulos, 2015). Positive allosteric modulators (PAM) increase receptor responsiveness to the orthosteric agonist, while negative allosteric modulators do the opposite.

1.3 ADHESION GPCR FAMILY

Mammalian GPCRs have typically been classified as either Rhodopsin-like, Secretin-like, Glutamate-like or Frizzled-like (Kolakowski, 1994). In this classification system, adhesion GPCRs (AGPCRs), marked by their large N-termini containing various adhesion domains, are part of the Secretin-like family. Evolutionary studies have revealed that AGPCRs are an ancient family, predicted to have appeared approximately 1275 million years ago, although these prehistoric AGPCRs generally had shorter N-termini (Kovacs & Schoneberg, 2016). Elongation of the N-termini of receptors may have been prompted by a need for increased interactions with the extracellular environment, necessitating receptors with larger extracellular domains (Hamann & Petrenko, 2016). These evolutionary studies on AGPCRs have prompted suggestions that AGPCRs should represent their own family of GPCRs, as in the "GRAFS" classification system, which divides GPCRs into Glutamate,

Rhodopsin, Adhesion, Frizzled and Secretin families (Fredriksson, Lagerstrom, Lundin, & Schioth, 2003).

Among the adhesion GPCRs with resolved structures are ADGRD1 and ADGRF1 (Qu et al., 2022), ADGRG3 (Ping et al., 2021) and ADGRG6 (Leon et al., 2020). As more structures are generated, AGPCR study will accelerate as will the development of therapeutics targeting these receptors.

1.3.1 AGPCR Nomenclature

There are 33 AGPCR genes expressed in humans, and the traditional names for many of these receptors are idiosyncratic, relating to details associated with the initial discovery of each receptor. Several years ago, the Human Gene Nomenclature Committee worked with IUPHAR and the Adhesion GPCR Consortium to develop a unified nomenclature for AGPCRs. In this official nomenclature, the name of each family member begins with "ADGR", a unique prefix referring to *ad*hesion *G* protein-coupled *r*eceptor (Table 1.1). ADGR is then followed by letters and numbers relating to the subfamilies of the receptors (Hamann et al., 2015). In situations where the name of a receptor is used repeatedly in written articles or oral presentations, the "ADGR" can be dropped and the last letter and number can be used alone for ease of reference (i.e., ADGRB1 can be referred to simply as "B1"). This nomenclature will be used in this dissertation, along with references to the traditional names of the receptors at the first mention of each receptor in each section.

TABLE 1.1: Annotated names and chromosomal locations of AGPCRs

ADG Nomenclature name	Alternative Name(s)	Human Gene ID	Location (Chromosome)	Exon Count
ADGRA1	GPR123	84435	10q26.3	9
ADGRA2	GPR124	25960	8p11.23	19
ADGRA3	GPR125	166647	4p15.2	21
ADGRB1	BAI1	575	8q24.3	35
ADGRB2	BAI2	576	1p35.2	32
ADGRB3	BAI3	577	6q12-q13	32
ADGRC1	CELSR1	9620	22q13.31	38
ADGRC2	CELSR2	1952	1p13.3	34
ADGRC3	CELSR3	1951	3p21.31	35
ADGRD1	GPR133/PGR25	283383	12q24.33	30
ADGRD2	GPR144/PGR24	347088	9q33.3	21
ADGRE1	EMR1/TM7LN3	2015	19p13.3-p13.2	23
ADGRE2	CD97/VBU/EMR2/CD312	30817	19p13.12	24
ADGRE3	EMR3	84658	19p13.12	17
ADGRE5	CD97/TM7LN1	976	19p13.12	20
ADGRF1	PGR19; GPR110; KPG_012; hGPCR36	266977	6p12.3; 6	16
ADGRF2	GPR111, PGR20, hGPCR35	222611	6p12.3	12
ADGRF3	GPR113, PGR23	165082	2p23.3	19

ADGRF4	GPR115, PGR18	221393	6p12.3	10
ADGRF5	GPR116, KPG_001	221395	6p12.3	25
ADGRG1	BFPP, BPPR, GPR56, TM7LN4, TM7XN1	9289	16q21	23
ADGRG2	CBAVDX, EDDM6, GPR64, HE6, TM7LN2	10149	Xp22.13	32
ADGRG3	GPR97, PB99, PGR26	222487	16q21	13
ADGRG4	GPR112, PGR17, RP1-299I16	139378	Xq26.3	28
ADGRG5	GPR114, PGR27	221188	16q21	13
ADGRG6	APG1, DREG, GPR126, LCCS9, PR126, PS1TP2, VIGR	57211	6q24.2	28
ADGRG7	GPR128	84873	3q12.2	16
ADGRL1	CIRL1, CL1, LEC2, LPHN1	22859	19p13.12	27
ADGRL2	CIRL2, CL2, LEC1, LPHH1, LPHN2	23266	1p31.1	39

ADGRL3	CIRL3, CL3, LEC3, LPHN3	23284	4q13.1	32
ADGRL4	ELTD1, ETL, KPG_003	64123	1p31.1	15
ADGRV1	FEB4; GPR98; MASS1; USH2B; USH2C; VLGR1; VLGR1b	84059	5q14.3	91

1.3.2 AGPCR Structure

The general structural features of most AGPCRs include an extracellular N-terminal domain, a GPCR autoproteolysis-inducing (GAIN) domain, the seven-transmembrane (7TM) domain common to all GPCRs, and a cytoplasmic C-terminus (Figure 1.1). The domain architectures of the large N-termini of AGPCRs have led to the categorization of the 33 family members into 9 subfamilies based on the conserved domains: ADGRL (group 1, latrophilins; LPHLs), ADGRE (group 2, EMRs), ADGRA (group 3), ADGRC (group 4, CELSRs), ADGRD (group 5), ADGRF (group 6), ADGRB (group 7, BAIs), ADGRG (group 8), and ADGRV (group 9, GPR98). While this classification system is based on sequence homology and domain conservation, recent analyses have questioned whether this system might need to be reevaluated (Scholz, Langenhan, & Schoneberg, 2019).

Figure 1.1 AGPCRs exhibit great structural diversity.

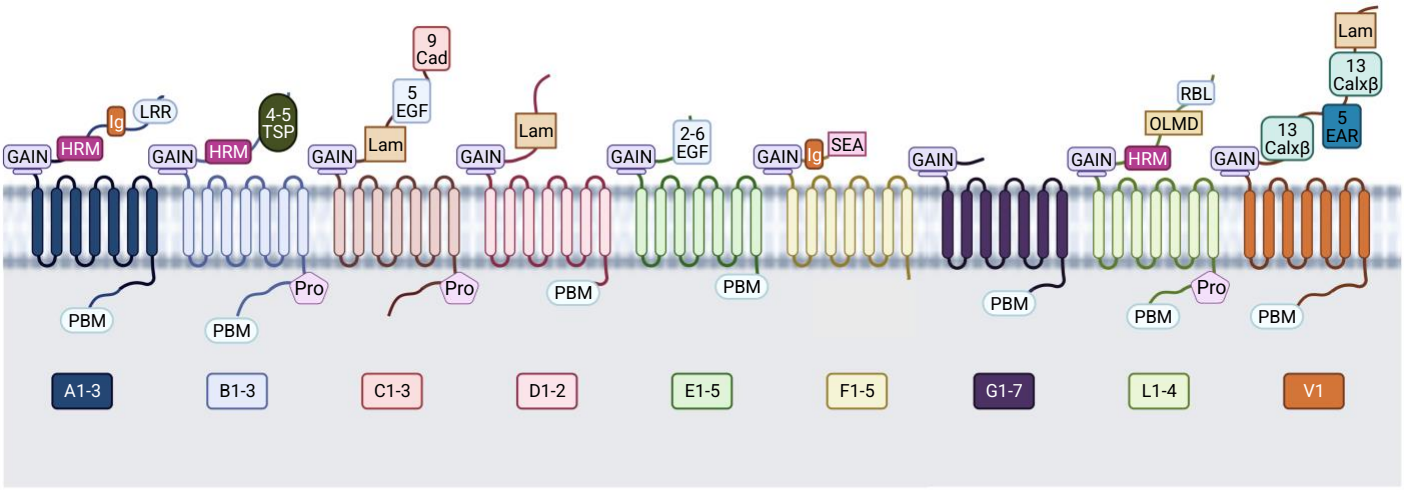

Figure 1.1 AGPCRs exhibit great structural diversity.

The various adhesion GPCR sub-families are depicted with key motifs labeled. Abbreviations: GAIN: GPCR autoproteolysis-inducing domain, LRR: Leucine-rich repeat, Ig: Immunoglobulin-like, HRM: Hormone receptor motif, PBM: PDZ binding motif, TSP: Type-1 thrombospondin repeats, Cad: Cadherin repeat, EGF: Epidermal Growth Factor-like, Lam: Laminin, Pro: polyproline sequence, SEA: Sperm protein/Enterokinase/Agrin doman, RBL: rhammose-binding lectin, OLMD: olfactomedin-like, EAR: Epilepsy-associated repeat.

Most AGPCRs possess large extracellular N-terminal domains of hundreds to thousands of residues in length, in addition to membrane-spanning seven-transmembrane (7TM) domains and intracellular C-terminal domains (de Graaf, Nijmeijer, Wolf, & Ernst, 2016). Almost all AGPCRs contain GPCR autoproteolysis-inducing (GAIN) domains in the juxtamembrane region of their N-termini, and these domains possess intrinsic autoproteolytic activity (Promel, Langenhan, & Arac, 2013). Following self-cleavage of the GAIN domain, adhesion GPCRs exist as two fragments that remain non-covalently associated for at least some period: an N-terminal fragment (NTF), which consists of the N-terminus up to the site of GAIN domain cleavage, and a C-terminal fragment (CTF), which is comprised of the 7TM region plus the intracellular domains and the small extracellular N-terminal stalk that remains following cleavage of the GAIN domain.

Over the past decade, there has been a major push to understand the structures of AGPCRs in greater detail. In 2012, X-ray crystal structures of the GAIN domains from several adhesion GPCRs provided the first high-resolution look at the structures of these domains (Arac, Boucard, et al., 2012). Subsequent X-ray crystallography studies provided novel insights into the structures of the extracellular regions (GAIN domains plus other NTF domains) from ADGRG1 (GPR56) (Salzman et al., 2016) and ADGRG6 (GPR126) (Leon et al., 2020). X-ray crystallography experiments have also visualized the associations of portions of the ADGRL1-3 (latrophilin-1-3) NTFs with their binding partners FLRT2 (Jackson et al., 2016) and teneurin-2 (Del Toro et al., 2020), and, independently, cryo-electron microscopy (cryo-EM) studies have provided a look at the interaction of ADGRL3 (latrophilin-2) with teneurin-2 (J. Li et al., 2020).

Most recently, cryo-EM studies have yielded the first-ever view of an AGPCR-G protein complex, with receptor coupled to Gαo (Ping et al., 2021). One interesting aspect of this structure was that all

three intracellular loops of G3 were found to have extensive interactions with the G protein heterotrimer (Ping et al., 2021), which is unusual relative to other GPCR/G protein structures that have been solved to date (Hilger, Masureel, & Kobilka, 2018). Interestingly, it is known from previous biochemical studies that adhesion GPCRs form surprisingly stable complexes with their cognate G proteins, such that AGPCR/G protein complexes often can be easily immunoprecipitated together without the need for chemical cross-linking (Kishore, Purcell, Nassiri-Toosi, & Hall, 2016; Purcell, Toro, Gahl, & Hall, 2017). The recent ADGRG3/Gαo structure provides insight into the remarkably robust associations of active AGPCRs with the G proteins to which they couple.

Many important questions remain to be answered in future AGPCR structural studies. For example, the ADGRG3/Gαo cryo-EM experiments were performed using a version of G3 with a mutation in the GAIN domain to prevent autoproteolysis (Ping et al., 2021). Furthermore, the conditions of these experiments did not allow for high-resolution visualization of the ADGRG3 NTF. Thus, no insights can be obtained from these studies about the relationship between the NTF of the receptor and CTF fragments following GAIN domain cleavage. Additionally, the palmitoylation on the C-terminus of Gαo was found in these studies to be inserted directly into the ADGRG3 7TM core, a feature of the ADGRG3/ Gαo complex that has not been observed for other GPCR/G protein interactions (Ping et al., 2021). Most Gα subunits have lipid modifications, but these lipid groups do not typically make direct contacts with receptors. Future work will be necessary to determine whether this unusual mode of receptor/G protein association is common to other AGPCRs or unique to the ADGRG3/Gαo complex.

1.3.2.1 Autoproteolysis of AGPCRs

Adhesion GPCRs can autoproteolytically cleave themselves at the GPCR proteolysis site (GPS), which is part of the GAIN domain. The GAIN domain is conserved in all AGPCRs except for ADGRA1 (GPR123), which possesses a short N-terminus devoid of any modular domains (Folts, Giera, Li, & Piao, 2019). The GPS is conserved in all other AGPCRs except for ADGRF2 (GPR111) and ADGRF4 (GPR115), which lack the consensus GPS motif and do not appear to undergo autoproteolysis (Promel, Waller-Evans, et al., 2012). Other than ADGRA1, ADGRF2 and ADGRF4, however, the other 30 members of the human AGPCR family appear to possess intact GAIN domains and GPS motifs, and there is good evidence for most of these receptors that they undergo autoproteolysis as a part of their normal processing (Promel et al., 2013). This autoproteolysis occurs spontaneously, often during receptor trafficking to the plasma membrane, and there is little evidence that it can be modulated by ligand binding (Promel et al., 2013). However, the binding of ligands to AGPCRs can exert conformational forces that may lead to dissociation of the non-covalently-associated NTF and CTF regions that have already been cleaved by autoproteolysis (Promel et al., 2013).

The GAIN domain is both necessary and sufficient for the autoproteolytic process in AGPCRs (Arac, Boucard, et al., 2012). The N-terminal portion of the GAIN domain consists of six α-helixes, while the C-terminal region closer to the transmembrane portion of the receptor consists of a twisted β-sandwich, including 13 β-strands and 2 small α-helices (Arac, Aust, et al., 2012). The GPS motif, which consists of the last five β -strands of the portion of the N-terminus proximal to the transmembrane domain of the receptor, is an integral part of this domain but is not functional by itself (Arac, Aust, et al., 2012). Interestingly, the GAIN domain is also known to be the site of multiple human disease mutations (Tesmer, 2012). For example, mutations of the GAIN domain of

ADGRG1 cause bilateral frontoparietal polymicrogyria (Piao et al., 2005). Additionally, mutations in the GAIN domains of ADGRL1 and ADGRB1 genes are hot spots for human cancers (Arac, Aust, et al., 2012).

Beyond being found in AGPCRs, GAIN domains are also found in polycystin-1 (PKD1) and the PKD1-like family of related transmembrane proteins (Maser & Calvet, 2020; Promel et al., 2013). Mutations in PKD1 are responsible for most cases of autosomal dominant polycystic kidney disease, a leading cause of end-stage renal disease, and several of the disease-causing mutations are located in the PKD1 GAIN domain (Maser & Calvet, 2020). Interestingly, although PKD1 is not a GPCR, it has been shown to regulate G protein signaling in a manner that is influenced by cleavage of the GAIN domain (Maser & Calvet, 2020). Along these same lines, GAIN domain cleavage also plays a key role in regulating signaling by AGPCRs, as described in the next section.

1.3.3 Signaling of AGPCRs

Early work on AGPCRs, and early reviews of the field, focused on the ability of these receptors to mediate adhesive interactions (Bjarnadottir, Fredriksson, & Schioth, 2007; Yona, Lin, Siu, Gordon, & Stacey, 2008). Given that AGPCRs possess 7TM domains, which were known from work on other GPCR families to allow coupling to G proteins, there was speculation that AGPCRs may translate extracellular adhesive interactions into intracellular signaling cascades, but such signaling mechanisms were mostly hypothetical in the early years of the field (Bjarnadottir et al., 2007; Yona et al., 2008). However, the past decade has seen numerous advances in understanding the activation of AGPCR signaling, not only by adhesive interactions but also by mechanosensory forces and secreted small molecule ligands.

1.3.3.1 Canonical G protein-dependent signaling

GPCRs function via their 7TM regions as guanine exchange factors (GEFs) for heterotrimeric G proteins, promoting the exchange of GDP for GTP on the Gα subunit. The first evidence for G protein activation by an AGPCR came from work on ADGRL1, which was shown to bind to alpha-latrotoxin (derived from black widow spiders) and stimulate increases in cyclic AMP and IP3 levels in ADGRL1-transfected COS-7 cells treated with alpha-latrotoxin (Lelianova et al., 1997; Sugita, Ichtchenko, Khvotchev, & Sudhof, 1998). Further work determined that ADGRL1 couples to Gαo to regulate cAMP and IP3 levels (Lelianova et al., 1997) and can also activate phospholipase C by coupling to Gαq (Rahman et al., 1999). Subsequently, many AGPCRs have been shown to stimulate G protein-dependent pathways (Purcell & Hall, 2018), and certain AGPCRs have even been shown to stimulate purified G proteins *in vitro* (Mathiasen et al., 2020; Stoveken et al., 2016; Stoveken, Hajduczok, Xu, & Tall, 2015; Stoveken, Larsen, Smrcka, & Tall, 2018) and co-immunoprecipitate with their cognate G proteins from cells (Kishore et al., 2016; Purcell et al., 2017), thereby providing strong evidence for G protein coupling.

The various members of the AGPCR family all preferentially couple to distinct subsets of G proteins (Figure 1.2A). This fact was vividly illustrated in screening assays performed in 2012 in which the G protein-coupling preferences of a large number of AGPCRs were assessed by measuring second messengers such as cyclic AMP and inositol phosphate, which are traditionally downstream of G protein activation (Gupte et al., 2012). These studies provided insights into the G protein-coupling preferences of several AGPCRs, including ADGRG3, which exhibited a preference for coupling to Gαo (Gupte et al., 2012). Almost a decade later, the aforementioned cryo-EM studies provided a high-resolution view of Gαo in association with the intracellular loops of ADGRG3 (Ping et al., 2021).

Figure 1.2. AGPCRs engage in diverse signaling mechanisms.

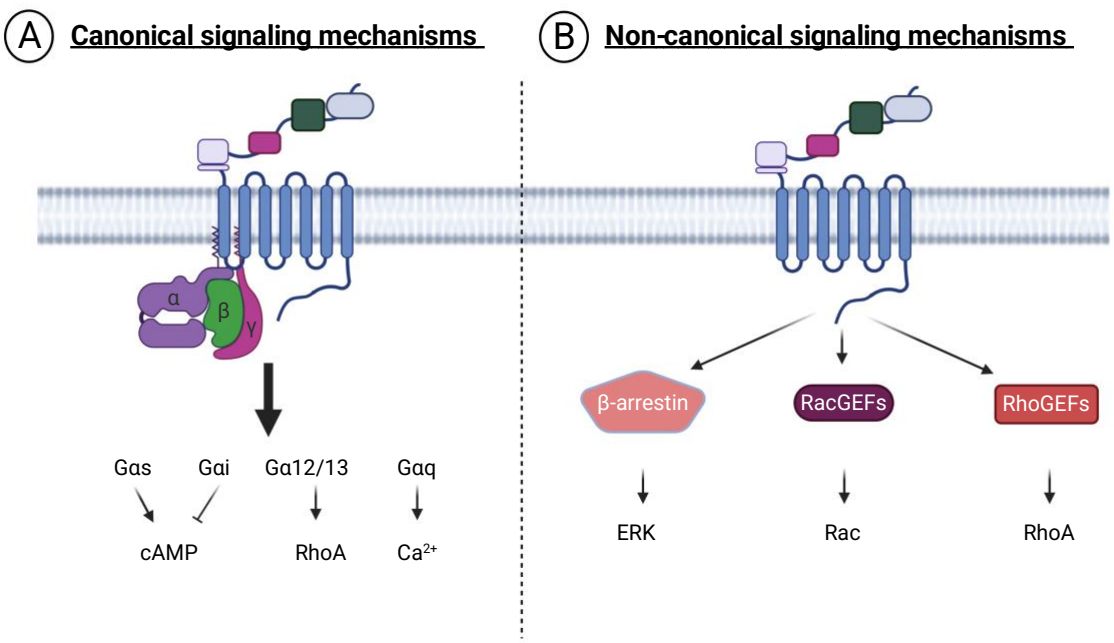

Figure 1.2. AGPCRs engage in diverse signaling mechanisms.

(A) Adhesion GPCRs can engage in canonical G protein-mediated signaling pathways, wherein a receptor engages with heterotrimeric G proteins to trigger G protein-dependent signaling cascades. Shown here are Gαs, Gαi, Gα12/13, Gαq, along with some of their downstream second messengers. (B) AGPCRs can also engage in non-canonical signaling pathways that are independent of heterotrimeric G proteins. For example, AGPCRs can engage with β-arrestins, RacGEFs or RhoGEFs, amongst other signaling proteins, to activate various downstream pathways in a G protein-independent manner.

Most G protein-coupled receptors can couple to multiple G protein subtypes to activate a diverse array of signaling pathways (Masuho et al., 2015), and AGPCRs are no exception. For example, the promiscuity of ADGRG2 (GPR64, "G2") has been well-documented, with the receptor coupling to Gαs (Azimzadeh, Talamantez-Lyburn, Chang, Inoue, & Balenga, 2019; Balenga et al., 2017; Demberg, Rothemund, Schoneberg, & Liebscher, 2015; Demberg et al., 2017; Y. Sun et al., 2021), Gαq (Azimzadeh et al., 2019; Demberg et al., 2015; Peeters et al., 2015; Y. Sun et al., 2021; Zhang et al., 2018) and Gα12/13 (Azimzadeh et al., 2019; Peeters et al., 2015). In the case of non-adhesion GPCRs that promiscuously couple to multiple G protein pathways, the strength of coupling often varies dramatically depending on cellular context due to the presence of cell-specific scaffold proteins that enhance certain pathways but not others (Ritter & Hall, 2009). Many AGPCRs are known to bind to cytoplasmic scaffold proteins (Duman et al., 2013; Hilbig et al., 2018; Knapp et al., 2019; Kreienkamp, Zitzer, Gundelfinger, Richter, & Bockers, 2000; Reiners et al., 2005; Stephenson et al., 2013; Tobaben, Sudhof, & Stahl, 2000; Zencir et al., 2011) and future studies in this area will undoubtedly shed light on the extent to which these receptor/scaffold interactions confer cell-specificity to the G protein coupling preferences of the receptors.

1.3.3.2 Non-canonical G protein-independent signaling of AGPCRs

Many GPCRs can directly interact with signaling proteins other than heterotrimeric G proteins to mediate G protein-independent signaling (Ritter & Hall, 2009). Several AGPCRs can mediate non-canonical signaling along these lines (Figure 2B). For example, ADGRB1 (BAI1) and ADGRB3 (BAI3) can regulate Rac signaling via interactions with two distinct Rac-GEFs: DOCK180, which associates with these receptors in complex with ELMO1 (Hamoud, Tran, Croteau, Kania, & Cote, 2014; Hochreiter-Hufford et al., 2013), and Tiam1, which associates with ADGRB1 via binding to the distal C-terminus of the receptor (Duman et al., 2013). ADGRB1 has also been shown to

associate with the RhoA-GEF Bcr to activate RhoA activity in hippocampal neurons (Duman et al., 2019). More generally, several AGPCRs, including ADGRG1, ADGRG2, ADGRB1 and ADGRB2 (BAI2) have been shown to robustly couple to β-arrestins (Azimzadeh et al., 2019; Kishore & Hall, 2016; Paavola, Stephenson, Ritter, Alter, & Hall, 2011; Purcell et al., 2017; Stephenson et al., 2013; Y. Sun et al., 2021; Zhang et al., 2018). Activity-dependent GPCR interactions with β-arrestins are a common mode by which GPCRs can mediate G protein-independent signaling (Reiter, Ahn, Shukla, & Lefkowitz, 2012). For example, ADGRG2 signaling through β-arrestin-1 is essential for G2 regulation of fluid reabsorption in the testis (Zhang et al., 2018). The capacity of AGPCRs to signal through G proteins, β-arrestins and other signaling intermediates provides an opportunity for the development of "biased" ligands that preferentially activate one downstream signaling pathway but not others. Such biased ligands can serve as important research tools and also in some cases make for useful therapeutics (Reiter et al., 2012). A summary of all known signaling pathways activated downstream of AGPCRs (including G protein-mediated and non-canonical signaling pathways) is shown in Table 1.2 (note empty areas of the table reflect presently unknown G protein pathways activated by a receptor).

TABLE 1.2: AGPCR G protein-dependent and alternate signaling pathways

CLASS	RECEPTOR	ALT NAME	G PROTEIN PATHWAYS ACTIVATED	OTHER SIGNALING PATHWAYS ACTIVATED
A	**ADGRA1**	**GPR123**		
A	**ADGRA2**	**GPR124**		Wnt7/β-Catenin (Cho, Smallwood, & Nathans, 2017; Eubelen et al., 2018; Posokhova et al., 2015; Vanhollebeke et al., 2015; Y. Zhou & Nathans, 2014); cdc42 (Hernandez-Vasquez et al., 2017; Kuhnert et al., 2010)
A	**ADGRA3**	**GPR125**		Wnt/PCP/β-Catenin (X. Li et al., 2013)
B	**ADGRB1**	**BAI1**	Gα12/13/RhoA (Kishore et al., 2016; Stephenson et al., 2013)	ELMO/Dock180/Rac (D. Park et al., 2007); Tiam1/Rac (Duman et al., 2013); Bcr/RhoA (Duman et al., 2019); mdm2 (D. Zhu et al., 2015; D. Zhu et al., 2018)
B	**ADGRB2**	**BAI2**	Gαz (Purcell et al., 2017); Gα16 (Okajima, Kudo, & Yokota, 2010)	GABPγ (Jeong et al., 2006)
B	**ADGRB3**	**BAI3**		ELMO/Rac1 (Lanoue et al., 2013)
C	**ADGRC1**	**CELSR1**		Wnt/PKC (L. H. Wang et al., 2020); Rho (Nishimura, Honda, & Takeichi, 2012; Yates et al., 2010)
C	**ADGRC2**	**CELSR2**	Gαq/Ca2+ (Shima et al., 2007)	
C	**ADGRC3**	**CELSR3**	Gαq/Ca2+ (Shima et al., 2007)	
D	**ADGRD1**	**GPR133**	Gαs/cAMP (Bohnekamp & Schoneberg, 2011; Frenster et al., 2021; Gupte	

			et al., 2012; Liebscher et al., 2014)	
D	**ADGRD2**	**GPR144**		
E	**ADGRE1**	**EMR1**		
E	**ADGRE2**	**EMR2**	Gα12/Gα13/Gα14/Gαz/ Gαs/Gαi/Gαq (Bhudia et al., 2020); Gα16/PLC (Bhudia et al., 2020; I et al., 2017); Gα15 (Bhudia et al., 2020; Gupte et al., 2012)	
E	**ADGRE3**	**EMR3**		
E	**ADGRE5**	**CD97**	Gαz/Gα14 (Bhudia et al., 2020); Gα12/ Gα13/RhoA (Bhudia et al., 2020; Ward et al., 2011)	
F	**ADGRF1**	**GPR110**	Gαq/IP1 (Demberg et al., 2017; Stoveken et al., 2015); Gαs/cAMP (Demberg et al., 2017; J. W. Lee et al., 2016)	NF-ϰB (T. Park, Chen, & Kim, 2019)
F	**ADGRF2**	**GPR111**		
F	**ADGRF3**	**GPR113**		
F	**ADGRF4**	**GPR115**	Gα15 (Gupte et al., 2012)	
F	**ADGRF5**	**GPR116**	Gαq/RhoA/Rac1 (Tang et al., 2013); Gαq/Gα11/IP1 (K. Brown et al., 2017); Gαs/cAMP (Georgiadi et al., 2021)	ERK1/2 (Georgiadi et al., 2021)
G	**ADGRG1**	**GPR56**	Gα12/Gα13/RhoA (Ackerman, Garcia, Piao, Gutmann, & Monk, 2015; Iguchi et al., 2008; Kishore et al., 2016; Luo et al., 2011; Paavola et al., 2011; Yeung et al., 2020); Gαq/Gα11 (Little, Hemler, & Stipp, 2004); Gαi (Yeung et al., 2020)	
G	**ADGRG2**	**GPR64**	Gα12/Gα13/RhoA (Azimzadeh et al., 2019; Peeters et al., 2015); Gαs/cAMP (Azimzadeh et al., 2019; Balenga et al., 2017; Demberg et al., 2015;	β-arrestin (Y. Sun et al., 2021)

			Demberg et al., 2017; Y. Sun et al., 2021); Gαq (Azimzadeh et al., 2019; Demberg et al., 2015; Peeters et al., 2015; Y. Sun et al., 2021; Zhang et al., 2018)	
G	**ADGRG3**	**GPR97**	Gαo (Gupte et al., 2012; Ping et al., 2021)	RhoA/cdc42 (Valtcheva, Primorac, Jurisic, Hollmen, & Detmar, 2013)
G	**ADGRG4**	**GPR112**	Gα12/Gα14 (Peeters et al., 2016)	
G	**ADGRG5**	**GPR114**	Gαs (Wilde et al., 2016)	
G	**ADGRG6**	**GPR126**	Gαs/cAMP (Demberg et al., 2017; Liebscher et al., 2014; Lizano, Hayes, & Willard, 2021; Mogha et al., 2013; Monk et al., 2009); Gαq/Gα12/α13 (Lizano et al., 2021)	
G	**ADGRG7**	**GPR128**		ELMO (Weng et al., 2019)
L	**ADGRL1**	**Lphn1/CIRL1**	Gαq/Ca2+ (Rahman et al., 1999; Silva et al., 2011); Gαo (Lelianova et al., 1997; Rahman et al., 1999)	
L	**ADGRL2**	**Lphn2/CIRL2**	Gαs/cAMP (Sando & Sudhof, 2021)	
L	**ADGRL3**	**Lphn3/CIRL3**	Gαq/Gα12/α13 (Mathiasen et al., 2020); Gαs/cAMP (Sando & Sudhof, 2021)	
L	**ADGRL4**	**ETL**		
V	**ADGRV1**	**VLRG1**	Gαq/PKC (D. Shin, Lin, Fu, & Ptacek, 2013); Gαs/cAMP/PKA (D. Shin et al., 2013); Gαi (Hu et al., 2014)	

1.3.4 Current hypotheses behind AGPCR G protein-dependent activation

The earliest insights on the activation mechanisms of adhesion GPCRs came from the aforementioned studies on ADGRL1 demonstrating that engagement of the N-terminus of the receptor by alpha-latrotoxin could promote receptor signaling (Boucard, Ko, & Sudhof, 2012; Lelianova et al., 1997; Rahman et al., 1999). Subsequent studies on several different AGPCRs, including ADGRG1 (Paavola et al., 2011), ADGRG4 (GPR112) (Peeters et al., 2016), ADGRB1 (Stephenson et al., 2013), ADGRB2 (Okajima et al., 2010) and ADGRE5 (CD97) (Ward et al., 2011), resulted in the surprising observation that truncation of the N-termini of the receptors, up the point of predicted GAIN domain cleavage, resulted in strong activation of receptor signaling (Bhudia et al., 2020). Taken together, these findings provided the underpinnings for the hypothesis (Paavola & Hall, 2012) that the large N-terminal regions of AGPCRs inhibit signaling by the 7TM regions of the receptors, with NTF engagement resulting in either NTF removal or conformational rearrangement to remove inhibitory constraints and thereby activate receptor signaling.

1.3.4.1 Tethered agonism

Other GPCRs that are known to become activated following removal of N-terminal regions include the members of the protease-activated receptor (PAR) sub-family. For example, PAR1 can be cleaved by the secreted protease thrombin to unveil a cryptic agonist on the N-terminus of the receptor, resulting in receptor activation (Soh, Dores, Chen, & Trejo, 2010). Early work on ADGRL1 signaling led to suggestions that AGPCR signaling might have analogies to PAR signaling (V. Krasnoperov et al., 2002). This hypothesis was explicitly tested in studies on NTF-lacking versions of ADGRG6 (GPR126) and ADGRD1 (GPR133) (Liebscher et al., 2014). Similar to NTF-lacking versions of other AGPCRs, as described above, truncated versions of ADGRG6 and ADGRD1 exhibited high constitutive activation of Gαs to raise cyclic AMP levels, and interestingly

removal of a portion of the post-cleavage stalk (or *stachel*) greatly reduced the signaling activity of these truncated receptors (Liebscher et al., 2014). Moreover, exogenous administration of the *stachel* peptide rescued the activity of these mutant receptors (Liebscher et al., 2014). Similarly, independent studies demonstrated that the removal of portions of the post-cleavage stalk of ADGRG1 and ADGRF1 abolished the activity of these receptors, with this activity being restored following treatment with peptides corresponding to the post-cleavage stalk (Stoveken et al., 2015). Subsequently, similar findings were made in work on ADGRG2 (GPR64) (Demberg et al., 2015; Y. Sun et al., 2021) and ADGRG5 (GPR114) (Wilde et al., 2016).

The studies on the tethered agonist regions of AGPCRs led to questions about how this sequence might get exposed to lead to receptor activation. Do the NTF and CTF regions of a cleaved AGPCR heterodimer need to dissociate to expose the tethered agonist? Studies on mutant versions of AGPCRs that lack intrinsic GAIN domain protease activity (and therefore do not undergo proteolysis) provided evidence against this idea, as such non-cleavable receptors have been shown in many cases to exhibit levels of constitutive signaling activity comparable to wild-type (self-cleaving) receptors (Bohnekamp & Schoneberg, 2011; Kishore et al., 2016; Promel, Frickenhaus, et al., 2012; Scholz et al., 2017). Recent studies employing bioorthogonal labels have revealed that the tethered agonist region can become exposed within the context of an NTF/CTF AGPCR heterodimer through intra-GAIN domain movements (Beliu et al., 2021). These findings suggest a model in which the tethered agonist can become exposed through GAIN domain conformational changes, rather than strictly requiring NTF/CTF dissociation.

1.3.4.2 Beyond tethered agonism

In addition to masking cryptic tethered agonist sequences, AGPCR NTF regions can influence receptor signaling activity in other ways (Figure 1.3). In certain AGPCRs, for example, removal of the tethered agonist/stachel sequence does not appear to impair receptor signaling activity (Kishore et al., 2016). Similarly, mutation of the tethered agonist/stachel sequence in ADGRG1 does not disrupt activation of the receptor by antibodies that bind to the NTF (Salzman et al., 2017). These studies suggest that the NTF controls AGPCR signaling activity in at least two distinct ways: i) modulation of the accessibility of the tethered agonist/stachel region, and ii) interaction with other AGPCR regions (such as perhaps the extracellular loops) to mediate conformational changes that determine receptor signaling activity.

The ability to temporally control AGPCR signaling is crucial for probing the effects of AGPCRs on physiology. In theory, stachel peptides can be useful reagents for temporal control of AGPCR signaling, similar to how SFLLRN and related peptides from the PAR-1 N-terminus have been used for years as ligands to exert temporal control over the activity of PAR-1 (Soh et al., 2010). However, the stachel peptides are fairly well-conserved between different AGPCRs and therefore tend to exhibit a lot of cross-reactivity between receptors, especially at the high concentrations at which these peptides must be used (Demberg et al., 2017). Moreover, stachel peptides often do not activate full-length AGPCRs, instead activating only highly truncated versions of AGPCRs that have had their stalk regions removed or mutated (Stoveken et al., 2015; Vizurraga, Adhikari, Yeung, Yu, & Tall, 2020). A different approach to temporal control of AGPCR signaling has been the development of mutant versions of AGPCRs with the PAR-1 N-terminus fused to the GPS cleavage site to allow for thrombin-dependent exposure of the AGPCR tethered agonist, leading to receptor

activation (Mathiasen et al., 2020). Such PAR/ AGPCR chimeras can be useful tools in allowing for temporally-controlled activation of AGPCR signaling pathways.

Figure 1.3. There are multiple mechanisms by which AGPCRs can be activated.

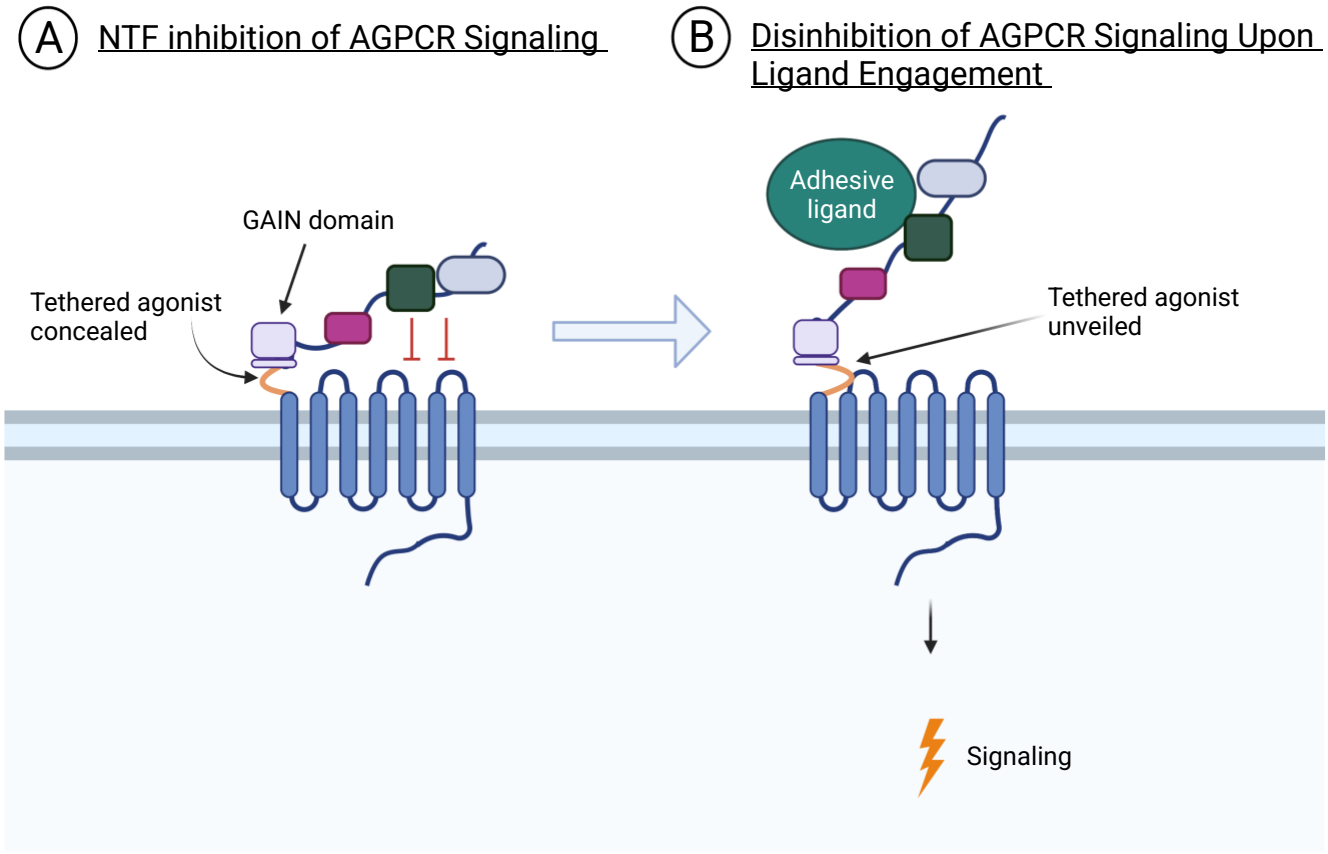

Figure 1.3. There are multiple mechanisms by which AGPCRs can be activated.

(A) For many (if not all) AGPCRs, the NTF inhibits receptor signaling via concealment of the tethered agonist sequence and other actions that suppress receptor activity. (B) Following engagement with an adhesive ligand, the NTF can change conformation such that multiple modes of inhibition are released. For example, the activated receptor may no longer experience allosteric inhibition by the NTF and additionally has an unveiled tethered agonist sequence that can fully activate receptor signaling.

1.3.5 AGPCRs as multimodal signal-transducing platforms

Beyond the use of stachel peptides and PAR/AGPCR chimeras, another way that temporal control over AGPCR signaling can be exerted is via the use of ligands. Most AGPCRs have massive N-termini with multiple conserved domains, suggesting that each receptor possesses the capacity to bind to numerous extracellular partners. Indeed, various binding partners, mostly large adhesion proteins and/or components of the extracellular matrix, have been identified for many AGPCRs (Figure 1.4). Some of these binding partners modulate receptor signaling activity, whereas others seem to solely mediate adhesive interactions. In any case, the elucidation of the interacting partners of the receptor can shed crucial light on the physiological effects of that receptor. For this reason, the various AGPCR ligands/binding partners are summarized in Table 1.3.

Figure 1.4. AGPCRs can engage with a diverse array of ligands to exert their physiological actions.

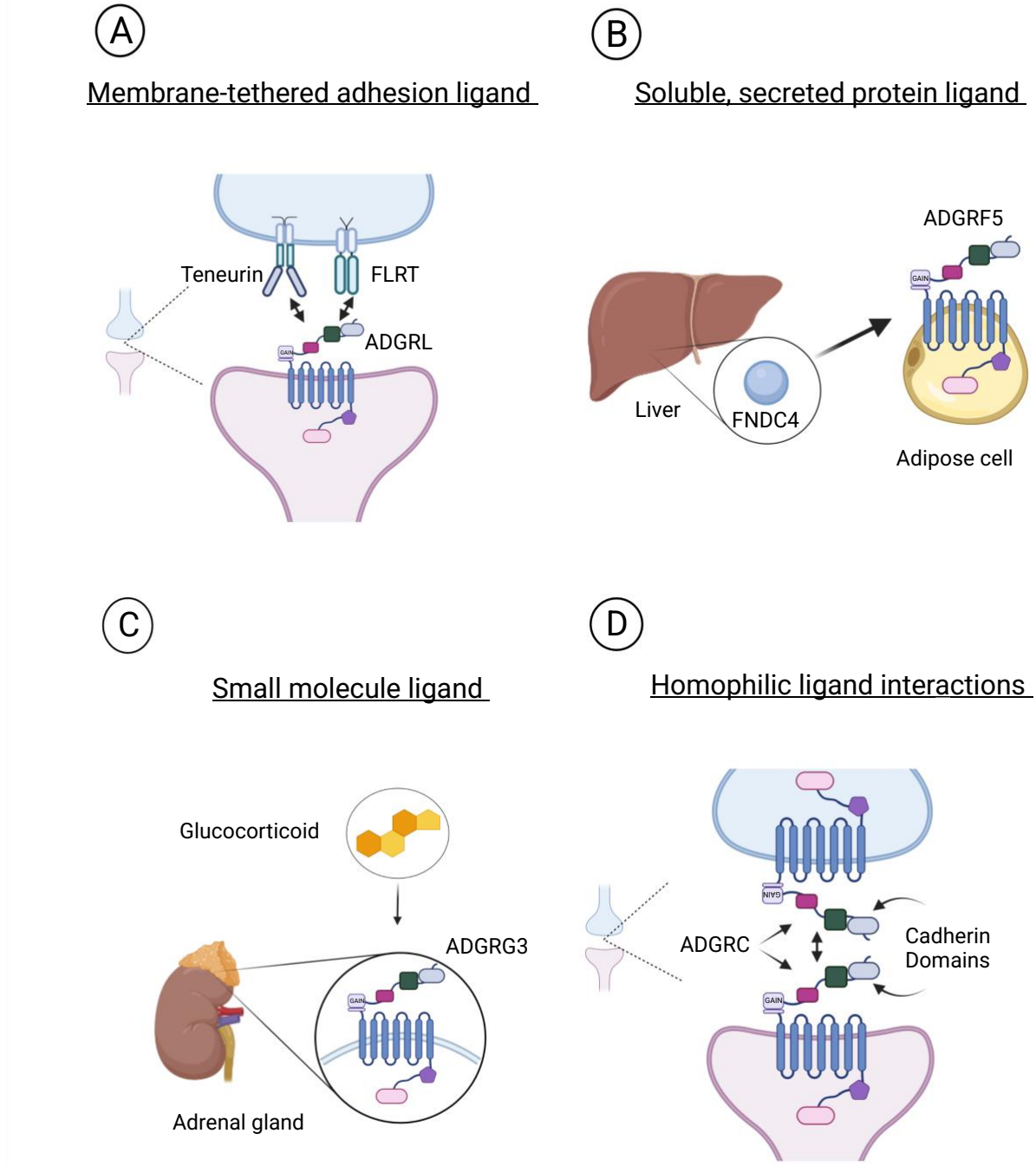

Figure 1.4. AGPCRs can engage with a diverse array of ligands to exert their physiological actions.

(A) Some AGPCR ligands are membrane-tethered adhesion ligands, for example the teneurins and FLRTs that interact across synapses with postsynaptic ADGRL1-3 to regulate synapse formation. (B) Other AGPCR ligands are soluble, secreted proteins, for example the hepatokine FNDC4, which can activate ADGRF5 to regulate adipose cell physiology. (C) Some AGPCRs bind to endogenous ligands that are small molecules, as in the case of glucocorticoids activating ADGRG3 to exert physiological effects in the adrenal cortex (Ping et al., 2021). (D) Certain AGPCRs can engage in homophilic or heterophilic interactions with other AGPCRs. For example, ADGRC receptors can interact with each other across cellular junctions via their cadherin-like domains.

TABLE 1.3: AGPCR-ligand binding and physiological significance

CLASS	RECEPTOR	ALT NAME	AGPCR LIGANDS	PHYSIOLOGICAL SIGNIFICANCE	REFERENCES
A	**ADGRA1**	**GPR123**			
	ADGRA2	**GPR124**	Integrin-αVβ3	Adhesion and migration during angiogenesis	(Vallon & Essler, 2006; Xiao et al., 2019)
			Glycosaminoglycans	CNS vascularization and BBB establishment	(Cullen et al., 2011; Vallon & Essler, 2006)
	ADGRA3	**GPR125**			
B	**ADGRB1**	**BAI1**	αVβ5 integrin	Endothelial cell proliferation	(Koh et al., 2004)
			Phosphatidylserine	Macrophage engulfment	(D. Park et al., 2007)
			Lipopolysaccharide	Macrophage engulfment	(Das et al., 2011)
			RTNR4	Neuronal development	(Chong, Ohnishi, Yusa, & Wright, 2018; J. Wang et al., 2021)
			CD36	Inhibition of angiogenesis	(Cork et al., 2012; Kaur et al., 2009)
	ADGRB2	**BAI2**			
	ADGRB3	**BAI3**	C1q11-C1q14, C1q-like-3	Synapse formation; myoblast fusion; insulin secretion	(Bolliger, Martinelli, & Sudhof, 2011; Gupta et al., 2018; Hamoud et al., 2018; Sigoillot et al., 2015; Sticco et al., 2021; C. Y. Wang, Liu, Ng, & Sudhof, 2020)
C	**ADGRC1**	**CELSR1**			
	ADGRC2	**CELSR2**	Homophilic interactions	Axon guidance; neurite growth	(Shima et al., 2007)

	ADGRC3	**CELSR3**	Homophilic interactions	Axon guidance; neurite growth	(Shima et al., 2007)
			Dystroglycan	Axon guidance; neurite growth	(Lindenmaier, Parmentier, Guo, Tissir, & Wright, 2019)
D	**ADGRD1**	**GPR133**			
	ADGRD2	**GPR144**			
E	**ADGRE1**	**EMR1**			
	ADGRE2	**EMR2**	Chondroitin sulfate	Adhesion	(Kwakkenbos et al., 2005; Stacey et al., 2003)
	ADGRE3	**EMR3**			
	ADGRE5	**CD97**	Chondroitin sulfate	T and B cell interaction	(Kwakkenbos et al., 2005; Stacey et al., 2003; T. Wang et al., 2005)
			Integrins- αVβ3, α5β1	Angiogenesis	(T. Wang et al., 2005)
			LPA Receptor	Tumor invasion	(Ward et al., 2011)
			CD90	Leukocyte trafficking to inflammatory sites	(Wandel, Saalbach, Sittig, Gebhardt, & Aust, 2012)
			CD55	T cell activation	(Capasso et al., 2006; Hamann, Vogel, van Schijndel, & van Lier, 1996; Hoek et al., 2010; Karpus et al., 2013; Niu et al., 2021; Qian, Haino, Kelly, & Song, 1999)
F	**ADGRF1**	**GPR110**	Synaptamide	Synaptogenesis	(Huang et al., 2020; J. W.

					Lee et al., 2016)
	ADGRF2	**GPR111**			
	ADGRF3	**GPR113**			
	ADGRF4	**GPR115**			
	ADGRF5	**GPR116**	FNDC4	Glucose homeostasis	(Georgiadi et al., 2021)
			Surfactant Protein-D	Pulmonary surfactant pool size regulation	(Bridges et al., 2013; Fukuzawa et al., 2013)
G	**ADGRG1**	**GPR56**	Collagen III	Cortical development and lamination; hemostatic plug formation	(Ackerman et al., 2018; Luo et al., 2011; Luo et al., 2014; Yeung et al., 2020; B. Zhu et al., 2019)
			Heparin	Cell adhesion and migration	(Chiang et al., 2016)
			Transglutaminase-2	Central nervous system myelination and melanoma progression	(Giera et al., 2018; Kitakaze et al., 2020; Salzman, Zhang, Fernandez, Arac, & Koide, 2020; L. Xu, Begum, Hearn, & Hynes, 2006; L. Yang, Friedland, Corson, & Xu, 2014)
			Progastrin	Colonic mucosal proliferation	(Jin et al., 2017)
			Phosphatidylserine	Synaptic pruning	(T. Li et al., 2020)
	ADGRG2	**GPR64**			
	ADGRG3	**GPR97**	Glucocorticoids	Adrenal cortex secretion	(Ping et al., 2021)
	ADGRG4	**GPR112**			
	ADGRG5	**GPR114**			

	ADGRG6	**GPR126**	Collagen IV	Peripheral nerve development	(Paavola, Sidik, Zuchero, Eckart, & Talbot, 2014)
			Laminin-211	Schwann cell development	(Petersen et al., 2015)
			Cellular Prion Protein	Schwann cell function	(Kuffer et al., 2016)
	ADGRG7	**GPR128**			
L	**ADGRL1**	**Lphn1/CIRL1**	α-latrotoxin	Toxin docking with cells	(Boucard et al., 2012; Ichtchenko et al., 1998; V. G. Krasnoperov et al., 1997; Lelianova et al., 1997; Rahman et al., 1999; Sugita et al., 1998; Tobaben, Sudhof, & Stahl, 2002)
			Teneurins	Neuronal pathfinding and synaptogenesis	(Boucard, Maxeiner, & Sudhof, 2014; Del Toro et al., 2020; Silva et al., 2011; Vysokov et al., 2018)
			Neurexins	Trans-synaptic connection formation	(Boucard et al., 2012)
			FLRT proteins	Synaptic development	(Del Toro et al., 2020; O'Sullivan et al., 2012)
	ADGRL2	**Lphn2/CIRL 2**	Teneurins	Axon guidance	(Boucard et al., 2014; Del Toro et al., 2020; Sando, Jiang, & Sudhof, 2019;

					Sando & Sudhof, 2021)
			FLRT proteins	Synaptic development	(Del Toro et al., 2020; O'Sullivan et al., 2012; Sando et al., 2019)
	ADGRL3	**Lphn3/CIRL 3**	Teneurins	Neuronal reshaping; synapse formation; axon guidance	(Boucard et al., 2014; J. Li et al., 2020; Sando et al., 2019; Sando & Sudhof, 2021)
			FLRT proteins	Synaptic development	(Jackson et al., 2016; J. Li et al., 2020; O'Sullivan et al., 2012; Sando et al., 2019)
	ADGRL4	**ETL**			
V	**ADGRV1**	**VLGR1**			

1.3.5.1 Mechanosensory signaling

The physical interaction of AGPCRs with extracellular adhesive ligands may, in many cases, not be enough to stimulate receptor signaling: the conveyance of mechanosensory force via these protein-protein associations may also be required. Indeed, over the past decade, multiple lines of evidence have emerged to suggest that detection of mechanosensitive stimuli is a primary physiological role of AGPCRs (Figure 1.5A). For example, the *Drosophila* ADGRL ortholog dCIRL is highly expressed in chordotonal neurons, the principal mechanosensory cells in flies, and genetic deletion of dCIRL results in sharply diminished touch sensitivity of the flies as well as greatly reduced physiological responses of the chordotonal neurons to mechanosensitive stimuli (Scholz et al., 2015). This mechanosensory action of dCIRL is dependent on the extracellular region of the receptor, tethered agonist sequence, and G-protein-dependent coupling to regulate cyclic AMP levels but is not dependent on autoproteolysis of the GAIN domain (Scholz et al., 2017). In addition to the expression of dCIRL in the chordotonal neurons, the receptor is also expressed in the flies' nociceptive neurons that respond to much higher intensities of mechanical stimulation; interestingly, while dCIRL sensitizes the responses of the chordotonal neurons to low-intensity mechanical stimuli, the receptor dampens high-intensity mechanosensitive activation of the nociceptive neurons, thereby revealing a differential role of the receptor in detecting low- vs. high-intensity mechanosensation (Dannhauser et al., 2020).

In addition to the body of work from studies in *Drosophila*, there is also evidence that vertebrate AGPCRs serve as mechanosensors. G protein-dependent signaling by ADGRG6 (Petersen et al., 2015) and ADGRG5 (GPR114, "G5") (Wilde et al., 2016) can be greatly enhanced by mechanically stressing cultured cells that express these receptors. Similarly, knockdown or deletion of ADGRV1 (VLGR1, or "V1") from certain cell types dramatically reduces cellular responses to mechanical

stretch, thereby providing evidence that V1 plays an important mechanosensory role (Kusuluri et al., 2021). In studies on cells expressing ADGRE5 (CD97), it was found that the application of mechanical force provokes phosphorylation of a key serine residue on the cytoplasmic C-terminus of the receptor, with this phosphorylation event disrupting the interaction of the receptor with the scaffold protein DLG1 and perturbing the ability of the receptor to mediate cellular adhesion (Hilbig et al., 2018). The recently-solved crystal structure of ADGRE5 in complex with its large extracellular ligand CD55 provides insight into how E5 can serve a mechanosensory role, as the anti-parallel binding of the E5/CD55 complex suggests a mechanism for the transmission of tensile force and the consequent force-dependent repositioning of the tethered agonist to modulate receptor activity (Niu et al., 2021).

1.3.5.2 Small molecule ligands

While the realization that AGPCRs can mediate mechanosensory signaling has been a surprising advance in recent years, an even more surprising insight has been that AGPCR signaling can be activated by small-molecule ligands (Figure 1.5B). Some of these ligands are putative endogenous ligands, for example the bioactive lipid synaptamide, which binds to the GAIN domain of ADGRF1 (GPR110) and agonizes receptor signaling (Huang et al., 2020; J. W. Lee et al., 2016). Additionally, steroid hormones such as glucocorticoids have been shown to bind to the 7TM region of ADGRG3 and promote coupling of the receptor to G proteins (Ping et al., 2021). These observations that AGPCRs can be activated by small-molecule ligands have led to a paradigm shift in the field, away from the view that the core function of AGPCRs is the mediation of adhesion and toward a more inclusive model in which AGPCRs serve as massive signaling platforms that are crucial for the integration of adhesive, mechanosensory, and chemical stimuli.

Beyond the putative endogenous ligands mentioned above, other small molecule ligands that have been recently identified for AGPCRs include drug-like compounds found in high-throughput screening campaigns. For example, beclomethasone was identified in high-throughput screens as an agonist for ADGRG3 (Gupte et al., 2012). Similarly, screens for ADGRG1 ligands identified 3-α-acetoxydihydrodeoxygedunin as an agonist (Stoveken et al., 2018; B. Zhu et al., 2019) and dihydromunduletone as an antagonist (Stoveken et al., 2016), whereas screens for ADGRG6 ligands identified apomorphine as an agonist (Bradley et al., 2019). Interestingly, beclomethasone, 3-α-acetoxydihydrodeoxygedunin, dihydromunduletone and apomorphine all exhibit four-ring structures that are reminiscent of steroid hormones. Thus, the aforementioned recent report that ADGRG3 is activated by glucocorticoids (Ping et al., 2021) may be a harbinger of more reports to come about AGPCR stimulation by steroid hormones. Indeed, there exists extensive literature on the rapid, "nongenomic" actions of steroid hormones that are not mediated by traditional nuclear steroid receptors (Losel et al., 2003; Schwartz, Verma, Bivens, Schwartz, & Boyan, 2016). In many cases, these mysterious steroid hormone effects are mediated by unidentified G protein-coupled receptors (Losel et al., 2003; Schwartz et al., 2016). Thus, given that the residues comprising the steroid hormone-binding pocket of ADGRG3 are highly conserved in many other AGPCRs (Ping et al., 2021), it is plausible that other AGPCRs may be activated by steroid hormones, with these steroid/AGPCR pairings accounting for some of the currently-unexplained rapid physiological actions of steroid hormones.

Figure 1.5. AGPCRs can integrate heterogeneous signals.

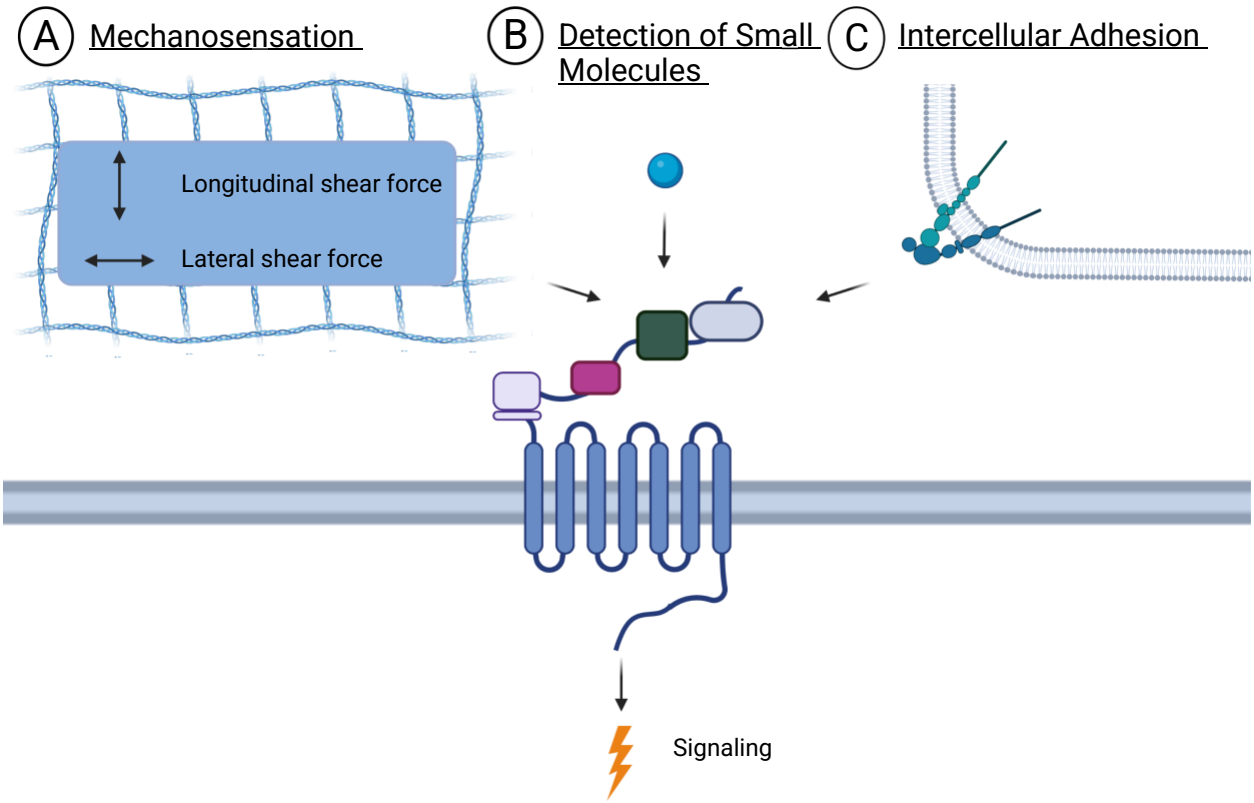

Figure 1.5. AGPCRs can integrate heterogeneous signals.

(A) AGPCRs can detect shear forces via the extracellular matrix and transduce these forces into intracellular signaling. (B) AGPCRs can also respond to secreted small molecules to induce signaling. (C) AGPCRs can mediate adhesion signaling by sensing intercellular interactions with ligands that are large proteins or membrane lipids. The integration of all of these heterogeneous signals may be a central function of AGPCRs.

1.3.6 BAI family of AGPCRs

The Brain-specific angiogenesis (BAI or ADGRB) family of AGPCRs consists of three members, ADGRB1-3, from here on referred to as B1-3. B1 was discovered first as a p53-inducible gene and originally implicated in angiogenesis inhibition and suppression of tumor formation, leading to the naming of this family (Nishimori et al., 1997). B2 and B3 were subsequently cloned and characterized as receptors with extensive homology to B1; however, B2 and B3 are not p53-inducible genes (Shiratsuchi, Nishimori, Ichise, Nakamura, & Tokino, 1997). Chromosomally, B1 is located at 8q24.3, B2 at 1p35.2 and B3 at 6q12-q13 (Lala & Hall, 2022; Shiratsuchi et al., 1997). B1-3 share 45% sequence homology at the amino acid level and contain similar structural domains: B1 is unique in that it has an N-terminal RGD motif which mediates integrin binding and has 5 thrombospondin repeats (TSRs) while the other two members have 4. All 3 members have a hormone-binding domain, juxtamembrane GAIN domain, canonical 7-TM domain and a C-terminal PDZ binding motif (PBM). B1 is also unique from the other two family members in that it contains a C-terminal proline-rich repeat, a domain known to mediate a variety of protein-protein interactions including binding to proteins with SH3 domains (Lala & Hall, 2022; Stephenson, Purcell, & Hall, 2014).

All members of this family are enriched in the CNS but also expressed in other cell types as explained in the subsequent section on BAI1 family physiology. The PBM present in all family members' CTF confers this family with the ability to engage with key synaptic proteins like PSD-95. Consistent with this, B1 plays a critical role in the regulation of synaptic plasticity and dendritic spine morphology, as discussed in the next section. Elucidation of the key protein-protein interactions and activation mechanisms of all family members will facilitate a better understanding of the

physiological roles of the receptors as well as the eventual targeting of these receptors with novel therapeutics.

1.3.6.1 BAI family physiology

The subsequent three subsections describe what is presently known about the three family members in different organ systems, focusing on the immune system, nervous system and muscular system. Finally, this section ends with a discussion on this family's known links to pathophysiology.

1.3.6.1.1. Immune System

ADGRB1 (BAI1, "B1") has been shown to play a key role in macrophage engulfment of apoptotic cells (Das et al., 2014; D. Park et al., 2007) (Figure 1.6). B1 possesses multiple N-terminal thrombospondin-like repeats, which can bind to externalized phosphatidylserine, a key signal of apoptosis (Das et al., 2014; D. Park et al., 2007). In further work, the B1 NTF was also shown to recognize surface lipopolysaccharides on Gram-negative bacteria, with this association allowing for macrophage engulfment of the bacteria (Das et al., 2011). The role of B1 in engulfment is facilitated by the interaction of the B1 C-terminus with ELMO and DOCK proteins, which allow B1 to stimulate Rac pathways crucial for engulfment (Das et al., 2011; D. Park et al., 2007). Interestingly, ADGRB1 also plays an important role in promoting the production of reactive oxygen species by macrophages (Billings et al., 2016), demonstrating that the receptor assists macrophages in their battle against bacteria both by enhancing macrophage microbicidal activity as well as by promoting engulfment. In addition to mediating macrophage antibacterial activity, B1 can also promote antiviral actions by macrophages (Bolyard et al., 2017). Recent work has shown that B1 can be difficult to detect in monocyte-derived macrophages (Hsiao, van der Poel, van Ham, & Hamann, 2019), suggesting that the expression of the receptor in these cells may be regulated in ways that are not yet defined.

Figure 1.6. B1-mediated engulfment of apoptotic cells.

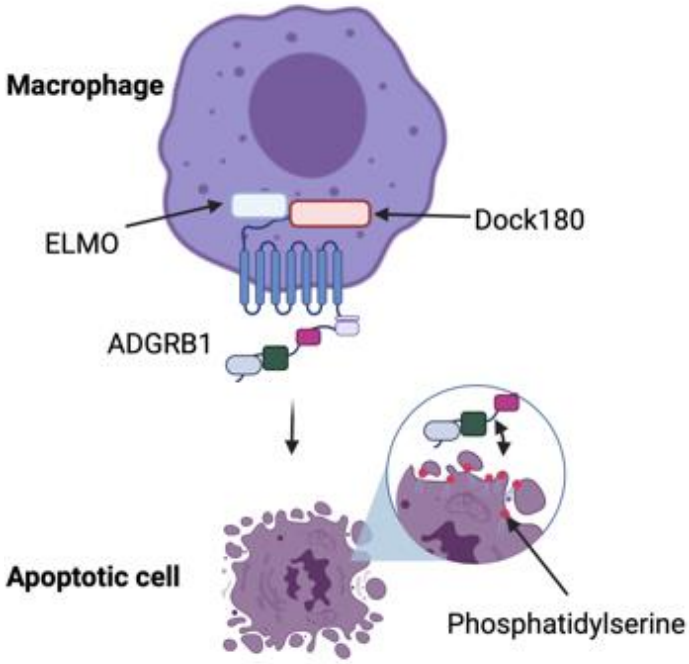

Figure 1.6. B1-mediated engulfment of apoptotic cells.

B1 in macrophages can interact via its thrombospondin repeats with exposed phosphatidylserine on apoptotic cells to induce engulfment.

1.3.6.1.2 Nervous System

B1-3 exert profound effects on synaptic function. Early work demonstrated that ADGRB1 is concentrated in the post-synaptic density (Duman et al., 2013; Stephenson et al., 2013) and that knockdown of B1 both in cultured neurons (Duman et al., 2013) and *in vivo* (Tu, Duman, & Tolias, 2018) reduces dendritic spine formation in a manner dependent on the ability of the B1 C-terminus to interact with Tiam1 to regulate Rac. ADGRB1 has also been shown to regulate dendritic arborization via association with the RhoGEF Bcr to control Rho signaling (Duman et al., 2019). These findings demonstrate that different signaling pathways emanating from a single AGPCR can exert highly distinct physiological actions (Figure 1.7). Consistent with the idea that B1 is a key regulator of excitatory synapses in the brain, knockout mice lacking B1 were found to exhibit perturbations in post-synaptic density structure in addition to profound defects in synaptic plasticity and spatial learning (D. Zhu et al., 2015).

Studies on B2 and B3 suggest that these receptors play roles similar to B1 in the nervous system, albeit at different populations of synapses. Mice lacking B2 have been shown to exhibit enhanced hippocampal neurogenesis and resistance to depressive phenotypes in mouse models of depression (Okajima, Kudo, & Yokota, 2011). B3 has been found to control dendritic arborization and branching both *in vitro* in cultured neurons and *in vivo* in Purkinje cells of the cerebellum (Lanoue et al., 2013). Further work in this area will likely clarify the synapse-specific actions of the various members of the ADGRB sub-family.

The striking effects of ADGRB1-3 in controlling dendritic growth and synaptic function are dependent on interactions of the NTF regions of the receptors with various extracellular binding partners. For example, B1 has been shown to interact via its N-terminal thrombospondin-like

repeats with reticulon-4 receptors (RTN4Rs) (Chong et al., 2018; J. Wang et al., 2021) in a manner that regulates dendritic arborization and synapse formation (J. Wang et al., 2021). Conversely, the thrombospondin-like repeats of ADRGB3 have been found to associate with the complement-like proteins C1ql1-4, with this association influencing synapse formation in cultured neurons (Bolliger et al., 2011). *In vivo*, C1ql1 promotes dendritic spine formation in Purkinje cells in a manner that depends upon the presence of B3 (Sigoillot et al., 2015). Similarly, at a specific synaptic connection in the olfactory bulb, deletion of either C1ql3 or B3 results in a very similar phenotype (suppressed acquisition of the social transmission of food preference), thereby providing further evidence for the importance of the B3/C1ql interaction *in vivo* (C. Y. Wang et al., 2020). Interactions of ADGRB3 with different members of the C1ql family have distinct effects on physiology, as for example binding of B3 to C1ql4 inhibits secretion from pancreatic β-cells (Gupta et al., 2018). The C1ql proteins most likely exert their effects on nervous system physiology by linking the ADGRB receptors to other key synaptic proteins, an idea advanced by recent work identifying the neuronal pentraxins NPTX1 and NPTXR as components of cell-cell adhesion complexes with C1ql3 and B3 (Sticco et al., 2021).

Figure 1.7. B1 can couple to multiple signaling pathways with differential effects on physiology.

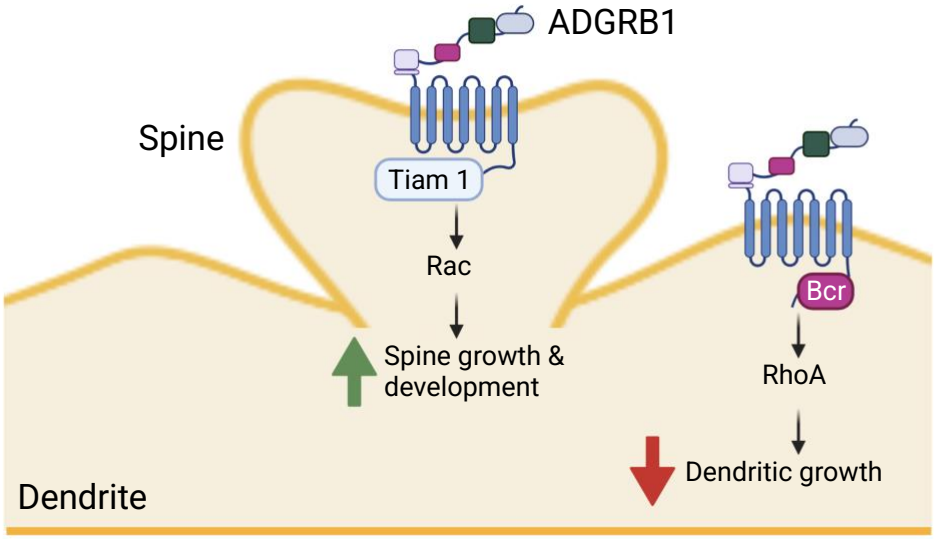

Figure 1.7. B1 can couple to multiple signaling pathways with differential effects on physiology.

B1 is known to associate with Tiam1 to stimulate Rac in a G protein-independent manner to promote dendritic spine growth and development. Conversely, B1 can also couple of Bcr to stimulate RhoA signaling via a completely distinct G protein-independent mechanism to inhibit dendritic growth. Thus, B1 provides an example of how a given AGPCR can engage not only in multiple G protein-dependent pathways but also in multiple G protein-independent pathways that can exert differential effects on physiology.

1.3.6.1.3 Muscular System

In addition to expression in the immune and nervous systems, B1 (Hochreiter-Hufford et al., 2013) and B3 (Hamoud et al., 2018; Hamoud et al., 2014) are also expressed in muscle tissue and promote myoblast fusion. B1 is especially highly expressed in cells of the Myo/Nog lineage, which are defined by co-expression of the skeletal muscle-specific transcription factor MyoD and the secreted protein Noggin (Gerhart et al., 2020). The regulation of myoblast fusion by B1 and B3, similar to some of the aforementioned actions of these receptors in the immune and nervous systems, is dependent on the interaction of the CTF regions of the receptor with ELMO/DOCK proteins (Hamoud et al., 2014; Hochreiter-Hufford et al., 2013). Interestingly, B1 and B3 are both essential for normal myoblast fusion and cannot functionally substitute for each other (Hamoud et al., 2014; Hochreiter-Hufford et al., 2013) revealing that the receptors exert unique and non-redundant effects on myoblast physiology.

1.3.6.2 BAI family pathophysiology

The clinical relevance of adhesion GPCRs is clear, given that mutation and/or dysfunction of many members of this family have been shown to underlie human disease. The frequent involvement of AGPCRs in pathophysiology contributes to the attractiveness of these receptors as therapeutic targets for treating disease and enhancing human health.

In particular, consistent with the aforementioned important roles of the ADGRB sub-family in the brain (Duman, Tu, & Tolias, 2016; Stephenson et al., 2014), genetic variation ADGRB1-3 has been linked to various psychiatric and neurological disorders. For example, B3 variants or changes in copy number have been linked in genetic studies to intellectual disability, cerebellar atrophy, and schizophrenia (DeRosse et al., 2008; Liao et al., 2012; Lips et al., 2012; Scuderi et al., 2019).

Similarly, a B2 variant that encodes a receptor with increased constitutive signaling activity has been linked in genetic analyses to a rare neurodegenerative condition marked by severe spinal cord atrophy (Purcell et al., 2017).

ADGRB receptors have also been implicated in human cancers. In glioblastoma, B1 acts as a tumor suppressor (Kaur, Brat, Devi, & Van Meir, 2005; Kaur et al., 2009), and its expression is lost during glioblastoma progression (Kaur, Brat, Calkins, & Van Meir, 2003) due to epigenetic silencing (D. Zhu, Hunter, Vertino, & Van Meir, 2011). Similarly, in medulloblastoma, B1 also exerts a tumor suppressor action and again its expression is lost due to epigenetic silencing during cancer progression (Cork et al., 2012; H. Zhang et al., 2020; D. Zhu et al., 2018).

1.4 Fluid mosaic model and lipid asymmetry

As mentioned in the preceding section, B1 has been shown to bind via its TSRs to externalized phosphatidylserine (Das et al., 2014; D. Park et al., 2007). To appreciate the physiological importance of this receptor/lipid interaction, it is important to provide further background about lipid bilayers in general and the plasma membrane in particular.

The lipid bilayer is described as a fluid mosaic model comprised of amphipathic phospholipids, proteins and carbohydrates attached to an intracellular actin cytoskeleton and an extracellular matrix. This structure is flexible and offers fluidity and elasticity to the plasma membrane. Lipid mobility between the outer leaflet and the inner leaflet of the plasma membrane is important for membrane curvature (P. Xu, Baldridge, Chi, Burd, & Graham, 2013). This property is especially important to support the fusing of vesicles and is the way that presynaptic neurons release neurotransmitters onto postsynaptic neurons in the brain. This 30-angstrom-wide plasma membrane is also protective and

prevents the lateral diffusion of membrane components and separates the intracellular contents of a cell from its surroundings (van Meer, Voelker, & Feigenson, 2008). The receptors in the plasma membrane mediate the ability of a cell to engage with its extracellular environment. Cell-cell signaling, cell division, vesicular release and apoptosis are all possible because of the dynamic regulation of the plasma membrane.

While receptors are the integral signal transducers of the plasma membrane, an often-overlooked component, the phospholipids, also play a critical role in cell health and normal physiology. Approximately 5% of eukaryotic genes are dedicated to lipid synthesis, and lipidomics approaches have led to predictions that there may be up to 100,000 distinct lipid species in eukaryotes (Lydic & Goo, 2018; van Meer et al., 2008). The brain has the second-highest lipid content after adipose tissue and 50% of the brain's weight is brain lipids (Bruce, Zsombok, & Eckel, 2017). Eukaryotic membranes are made of structural lipids: glycerophospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid; sphingolipids are another structural lipid group. With the heterogeneity of phospholipids and the significant genomic real estate dedicated to regulating their processing, phospholipids must serve a larger purpose than spontaneously forming membrane structures. In fact, lipids have at least three major functions: (1) spontaneous membrane formation, (2) energy storage and (3) signal transduction. Lipids can act as first or second messengers and contribute to signal transduction. Hydrolysis of glycerolipids and sphingolipids can also produce messenger lipids (van Meer et al., 2008). Clearly, lipids are incredibly useful important for proper cell physiology.

The plasma membrane of a cell is a vast structure with varied topography. In the case of the brain, specialized neurons in the hippocampus have dendritic spines that extend off the soma and exist as

subcellular compartments that facilitate the encoding of memories. The neuron is an extreme example of the importance of cellular polarity. In more simple cells, polarity is established by both receptor microdomains and lipid asymmetry. Regulators of polarity in epithelial cells bind phospholipids and regulate localization of proteins. The cellular polarity is both conferred by and regulated by phospholipids (Krahn, 2020).

In addition to the role phospholipids play in cell polarity along the plasma membrane, the lipids themselves maintain asymmetry along the membrane in a purposeful manner. In the 1970s, examination of the human erythrocyte membrane demonstrated lipid asymmetry with PC and sphingomyelin in the outer leaflet and PS, PE and PI in the inner leaflet (Bretscher, 1972; Verkleij et al., 1973). Different cell types in different species maintain different lipid asymmetries. For example, freeze-fracture replica labeling electron microscopy has revealed that PC exists in equal amounts in both leaflets in cells other than erythrocytes (Iyoshi et al., 2014). While some lipids remain exclusively in one leaflet of the membrane, ATP-dependent aminophospholipid transporters exist to move lipids like PS and PE from one side of the membrane to the other (van Meer et al., 2008). The P4 subfamily of P-type ATPases move lipids against their concentration gradient and are leveraged in the experiments described in Chapter 2.

1.4.1 Significance of phosphatidylserine

Phosphatidylserine is traditionally known as an "eat-me" signal externalized by dying cells to trigger efferocytosis, or the engulfment of and degradation of apoptotic cells. Apoptotic mechanisms like caspase activation can trigger flippase-mediated extrusion of PS to the outer leaflet of the plasma membrane of a dying cell (Segawa & Nagata, 2015). There exist heterogeneous structural and expression profiles of PS-binding recognition receptors which, when bound to PS, can trigger

various signaling cascades downstream and activate anti-inflammatory processes in different cell types in the body (Naeini, Bianconi, Pirro, & Sahebkar, 2020). Secreted soluble proteins like growth arrest-specific gene 6, protein S and milk-fat globule epidermal growth factor 8 and type I membrane proteins like CD300 can recognize PS. Other PS receptors expressed on phagocytes include receptor for advanced glycosylation end products (RAGE), stabilin-2, CD36 and CD14 (Nagata, 2018). B1, of course, is among the PS-binding receptors, as Ravichandran and colleagues demonstrated its ability to bind PS and clear apoptotic cells (D. Park et al., 2007). Clearly, there exists great diversity in the phagocytic recognition of PS in the body.

Moreover, outside of the canonical PS-binding mechanism, PS-binding by other types of PS-recognition receptors mediates processes independent of efferocytosis, further expanding the diversity of PS-induced signaling in the body (Shlomovitz, Speir, & Gerlic, 2019). P4-ATPase-mediated movement of PS enhances membrane curvature during vesicle-mediated transport between organelles of the secretory and endocytic pathways (P. Xu et al., 2013). PS also serves as a scaffold in platelets to facilitate blood clotting (Lentz, 2003; Reddy & Rand, 2020). PS has even been implicated in contributing to the thromboinflammation associated with COVID-19 (Lind, 2021). PS also plays a crucial role in axon fusion to restore an injured neuron (Abay et al., 2017). PS can trigger a variety of signaling processes depending on the receptor and cell type detecting this lipid in the body. Further study of PS may reveal its involvement in even more physiological processes. The pharmacological impact of PS detection by B1 is the focus of Chapter 2.

1.4.3 PS exposure modulation

As mentioned, lipid asymmetry is critical to normal cell physiology, and is maintained by different proteins called “flippases”, “floppases” and “scramblases”. Flippases move lipids from the outer

leaflet of the plasma membrane to the inner leaflet, floppases do the opposite, and scramblases move lipids bidirectionally to either leaflet of the plasma membrane (Figure 8). Flippases include the P4-ATPases mentioned earlier. Scramblases include the TMEM family. Flippases are ATP-dependent, while scramblases are energy-independent and often can be calcium-activated (Andersen et al., 2016).

1.4.3.1 Flippases

P4-ATPases use energy from ATP hydrolysis to mobilize lipids across their concentration gradient to maintain lipid asymmetry (Coleman, Quazi, & Molday, 2013; Lopez-Marques et al., 2015). These proteins specifically move lipids from the outer or exoplasmic leaflet to the cytoplasmic leaflet of the membrane (Coleman, Kwok, & Molday, 2009; Tang, Halleck, Schlegel, & Williamson, 1996; X. Zhou & Graham, 2009). P4-ATPases exist only in eukaryotes, and the human genome encodes 14 such proteins classified into six classes (Andersen et al., 2016). Flippases are made of four domains, including 3 cytoplasmic domains required for the ATPase catalytic cycle wherein a motif goes through a transient phosphorylation intermediate. The fourth domain serves as the pathway through which the lipid substrate moves across the plasma membrane (Andersen et al., 2016). A schematic diagram of flippase structure is shown in Figure 1.8A.

Flippases can be localized to the endoplasmic reticulum or are trafficked to the plasma membrane with chaperones or accessory proteins that are referred to as β-subunits. These subunits belong to the CDC50 family of proteins expressed in all eukaryotes (Katoh & Katoh, 2004; Saito et al., 2004). CDC50A serves as the β-subunit for most P4-ATPases (Takatsu et al., 2011). These subunits also participate in ATP-dependent phospholipid transport (Lenoir, Williamson, Puts, & Holthuis, 2009).

Flippases are leveraged in the experiments described in Chapter 2 to decrease the level of exposed PS in a cell type we reveal to have a high baseline level of PS exposed. The specific flippase utilized, ATP11A, is described in greater detail in the next chapter.

1.4.3.2 Scramblases

Scramblases include the Anocatmin or TMEM16 family of proteins that mediate ion transport, phospholipid scrambling and regulation of other membrane proteins (Medrano-Soto et al., 2018). There are 10 such anoctamin family members in eukaryotes, most of which have been identified as calcium-activated chloride channels. Of the family members, Ano6 or TMEM16F has also been shown to act as a lipid scramblase (Pang et al., 2014) (Figure 1.8B). The scramblase Ano6 is utilized in some of the studies described in Chapter 2.

Figure 1.8. Flippases and Scramblases regulate phospholipid asymmetry in the plasma membrane.

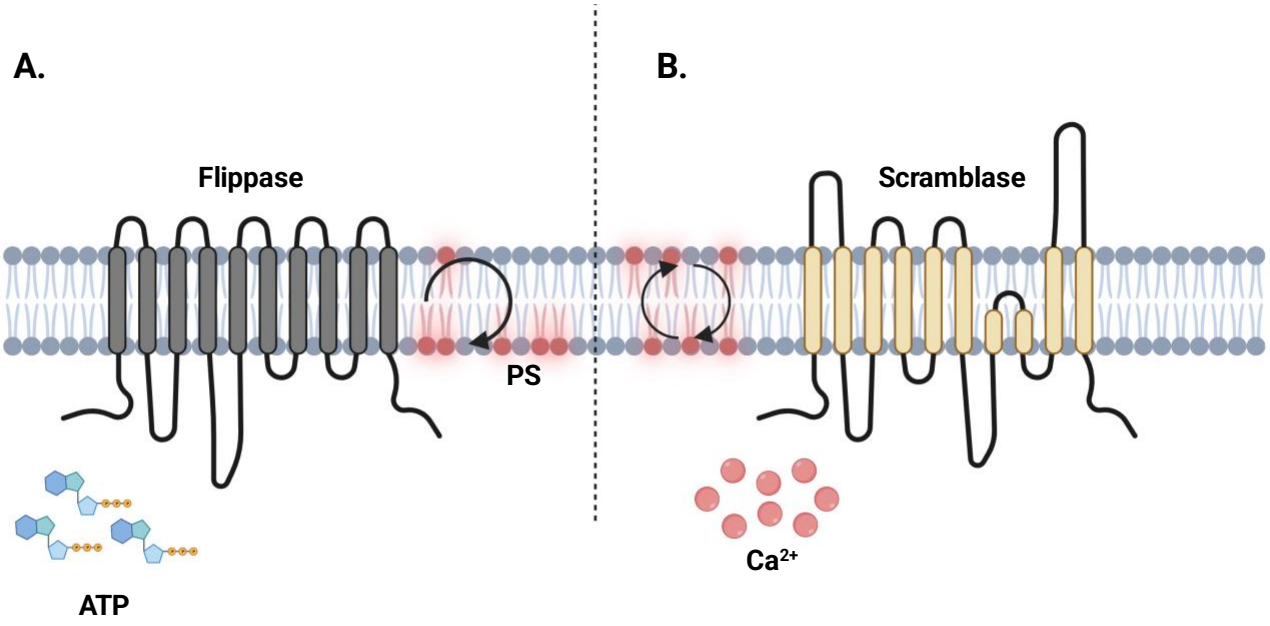

Figure 1.8. Flippases and Scramblases regulate phospholipid asymmetry in the plasma membrane.

(A) Flippases utilize ATP to drive phospholipids from the outer leaflet to the inner leaflet of the plasma membrane, against the concentration gradient. The flippase depicted here is ATP11A. (B) Scramblases function independent of ATP to bidirectionally move phospholipids across the membrane. The scramblase depicted here is Anoctamin 6 or TMEM16F, which is a calcium-sensitive chloride channel in addition to its lipid scrambling abilities.

1.5 Research aims

The great therapeutic potential of AGPCRs suggests that studying the pharmacology of these receptors can lead to insights that may provide the foundation for the development of future therapeutics. The AGPCR B1 presents a particularly appealing target given its role in the regulation of learning, memory and dendritic spine morphology. Given that this receptor is an orphan receptor with no known activation method, investigations into the endogenous cellular ligands that may activate this receptor are highly important.

The work described here builds upon previously published work indicating that B1 binds PS (D. Park et al., 2007). Given that prior studies from our laboratory demonstrated that B1 can signal via Gα12/13 and the Rho pathway (Kishore et al., 2016; Stephenson et al., 2013), we sought to investigate whether there might be a link between PS engagement by B1 and the subsequent G protein-dependent signaling of the receptor. To alter PS exposure in cells expressing B1, we assessed B1 signaling in cells co-expressing either the flippase ATP11A or the scramblase Ano6. The detailed methods and results of these studies are described in the next chapter.

Chapter 2: Modulation of Phosphatidylserine Exposure Regulates B1 Signaling Activity

This chapter is adapted from Lala et al. JBC, 2022 (in submission).

2.1 Phosphatidylserine Exposure Modulates Adhesion GPCR BAI1 (ADGRB1) Signaling Activity

2.1.1 Introduction

G protein-coupled receptors (GPCRs) are a diverse superfamily of receptors characterized by a conserved seven-transmembrane-domain architecture. Given that over 500 FDA-approved drugs and almost 100 drug candidates in clinical trials target GPCRs, there is great interest in the elucidation of the pharmacology of orphan GPCRs that lack well-defined ligands (D. Yang et al., 2021). Adhesion GPCRs (AGPCRs) are one of five major GPCR families, and most receptors in this family are still considered to be orphans (Lala & Hall, 2022; Morgan et al., 2019; Vizurraga et al., 2020). Members of the AGPCR family play crucial roles in a myriad of physiological processes, and several clinical disorders are associated with the dysfunction of this receptor type (Lala & Hall, 2022; Morgan et al., 2019; Vizurraga et al., 2020). Thus, pharmacological modulation of these receptors has the potential to provide powerful new therapeutics.

AGPCRs derive their name from the adhesive properties of the receptor class, conferred by their large extracellular N-terminal fragments (NTFs). Most members of this receptor family undergo autoproteolytic cleavage via a conserved GPCR autoproteolysis-inducing (GAIN) domain, which cleaves the NTF from the C-terminal fragment (CTF) that contains the seven-transmembrane region (Arac, Boucard, et al., 2012). Following GAIN-mediated cleavage, the resultant NTF and CTF remain non-covalently associated for some period of time, with this interaction inhibiting downstream signaling by the CTF. The engagement of the NTF by extracellular ligands may either

remove the NTF from the CTF or cause conformational changes in the CTF that activate downstream signaling (Lala & Hall, 2022; Morgan et al., 2019; Vizurraga et al., 2020).

The AGPCR known as Brain-specific Angiogenesis Inhibitor 1 (BAI1; also known as ADGRB1 or B1) was originally discovered as a thrombospondin repeat (TSR)-containing receptor enriched in the brain and capable of modulating angiogenesis when over-expressed (Nishimori et al., 1997). While B1 remains an orphan receptor, a seminal paper by Ravichandran and colleagues revealed that the TSRs of B1 can bind to phosphatidylserine (PS) to facilitate the engulfment of apoptotic cells by macrophages (D. Park et al., 2007). Subsequently, other physiological roles for B1 in macrophages have also been elucidated, such as the binding of Gram-negative bacteria to facilitate their engulfment (Billings et al., 2016; Bolyard et al., 2017; Das et al., 2011; Das et al., 2014).

Parallel to this work in macrophages, there exists a completely separate literature of studies by multiple groups on B1 regulation of brain physiology. B1 is enriched in the postsynaptic density (PSD) and regulates the morphology of dendritic spines in cultured neurons (Duman et al., 2019; Duman et al., 2013; Stephenson et al., 2013; Tu et al., 2018). Mice lacking B1 exhibit reduced PSD thickness, disrupted synaptic plasticity, impaired spatial learning, and social deficits (Shiu et al., 2022; D. Zhu et al., 2015). B1 has been shown to stimulate RhoA signaling via coupling to both Gα12/13 (Kishore et al., 2016; Stephenson et al., 2013) and Bcr (Duman et al., 2019) and to additionally promote Rac1 signaling via coupling to Tiam1 (Duman et al., 2013; Tu et al., 2018) in transfected cells and cultured neurons. However, it is unknown whether B1 activation of any of these signaling pathways is influenced by B1 binding to PS.

We sought to connect the work done on PS-binding by B1 in macrophages with the literature on B1 signaling in the nervous system. PS is normally found in the inner leaflet of the plasma membrane, and the asymmetric distribution of PS is maintained by a class of enzymes known as flippases, which are P4-ATPases that actively translocate PS from the outer leaflet of the plasma membrane to the inner leaflet (H. W. Shin & Takatsu, 2020). Cellular stress can promote PS externalization to the outer leaflet of the plasma membrane by inhibiting flippases and/or activating transporters known as floppases and scramblases that move lipids in the opposite direction to the flippases (H. W. Shin & Takatsu, 2020). Externalization of PS is known to occur during apoptosis, but it is also now well-appreciated that PS externalization can occur under normal physiological conditions and serve as an important cellular signal in the nervous system (and other systems) that leads to pleiotropic effects depending on how the signal is decoded by various PS-binding receptors (Bevers & Williamson, 2016; Kay & Grinstein, 2013; Naeini et al., 2020).

We leveraged recent advances in the understanding of PS biology to create cellular conditions in which B1 would encounter differing levels of PS exposure in the outer leaflet. We hypothesized that B1 interaction with externalized PS, embedded in the plasma membrane, might induce conformational changes in the B1 NTF and thereby modulate B1 signaling. This hypothesis was tested in the experiments described below.

2.1.2 Results

2.1.2.1 HEK293T cells exhibit a baseline level of exposed PS that can be modulated by the PS flippase ATP11A

To assess whether exposure of PS can modulate B1 signaling, we developed a cell culture model in which levels of exposed PS could be reproducibly manipulated. B1 signaling has previously been

studied in HEK293T cells (Kishore et al., 2016; Stephenson et al., 2013), and HEK293T cells are known to express an endogenous scramblase, Anoctamin 6 (ANO6/TMEM16F) (Schenk, Schulze, Henke, Weide, & Pavenstadt, 2016), which results in a measurable population of HEK293T cells in culture being PS+ (meaning that a quantifiable amount of PS in the outer leaflet of the plasma membrane can be measured via Annexin V binding) under most growth conditions (Schenk et al., 2016). HEK293T cells also express endogenous CDC50A (Coleman & Molday, 2011; Munoz-Martinez, Torres, Castanys, & Gamarro, 2010), a chaperone protein that is required for the function of ATP11-family of flippases (Takatsu et al., 2014). Thus, given that HEK293T cells exhibit a basal level of PS exposure and also express the machinery needed for PS flippase function, they represented an attractive model for our studies.

We investigated PS exposure in HEK293T cells using flow cytometry to assess whether overexpression of ATP11A, a phospholipid flippase, could modulate the levels of PS exposed on the outer leaflet in these cells. Figure 2.1A is a schematic diagram of ATP11A, which is a large 10-transmembrane protein with both its N- and C-terminal regions in the cytoplasm. We treated HEK293T cells with 10μM of the calcium ionophore A23187 to strongly activate endogenous scramblases such as ANO6 and found that an Annexin V probe detected very high levels of exposed PS in the A23187-treated HEK293T cells (Figure 2.1C). This signal was probe-dependent, as no measurable signal could be detected in the absence of the probe (Figure 2.1B). We next measured baseline levels of PS exposure in HEK293T cells. As expected based on previous reports (Schenk et al., 2016), the HEK293T cells at baseline exhibited a measurable level of PS exposure. Moreover, we found that the levels of externalized PS could be reduced via overexpression of ATP11A. Figure 2.1D overlays baseline PS exposure in wild-type cells versus ATP11A-transfected cells. These studies revealed that HEK293T cells exhibit a quantifiable amount of externalized PS at baseline,

and that overexpression of the PS flippase ATP11A in this cell type dramatically reduces PS exposure (total reduction = 58%; quantification shown in Figure 2.1E).

Figure 2.1. Evaluation of phosphatidylserine exposure in HEK293T cells at baseline and when overexpressing ATP11A.

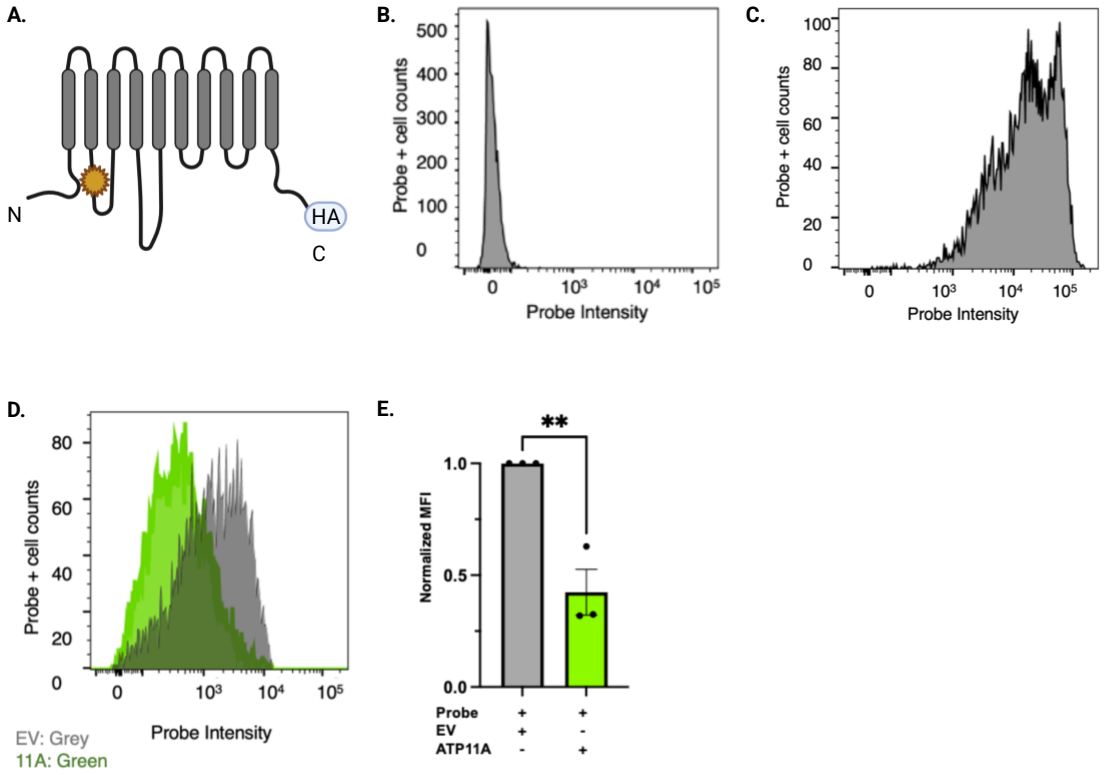

Figure 2.1. Evaluation of phosphatidylserine exposure in HEK293T cells at baseline and when overexpressing ATP11A.

Flow histograms of Annexin V binding (in relative units) vs. cell counts are shown; 20k cells counted. A) *ATP11A schematic:* Depiction of the PS flippase, ATP11A. Star indicates wild-type flippase. HA tag location shown. B) *Negative control:* Flow histogram of mock-transfected HEK293T cells (-Annexin V) demonstrates little to no non-specific probe binding. C) *Positive control:* Flow histogram of mock-transfected HEK293T cells (+Annexin V) treated with 10μM A23187 for 20 minutes to induce scramblase activity, resulting in higher levels of PS exposed. D) *ATP11A-induced reduction in PS:* Flow histogram of mock-transfected (grey) HEK293T cells (+Annexin V) overlaid with ATP11A-transfected (green) HEK293T cells (+Annexin V) demonstrates that these cells exhibits baseline exposure of PS that can be reduced via overexpression of ATP11A. E) *Quantification of ATP11A-induced reduction in PS exposure:* Mean fluorescence intensity (MFI) shown with ATP11A+ condition normalized to mock-transfected condition. ATP11A overexpression in HEK293T cells results in a 58% reduction in PS exposure (mean ± SEM shown, unpaired t-test, $p=0.0049$, $n=3$)

2.1.2.2 Coexpression of ATP11A with B1 reduces the constitutive signaling activity of B1

We next investigated whether coexpression of ATP11A with B1 might modulate the G protein-dependent signaling of the receptor. It has previously been shown that full-length B1 expressed in HEK293T cells exhibits high constitutive signaling activity (Kishore et al., 2016; Stephenson et al., 2013). It is plausible that at least some portion of this activity may be dependent on stimulation of B1 signaling by baseline levels of PS exposure. Thus, we performed SRF-luciferase assays to assess full-length B1 (B1FL) coupling to Gα12/13 in control cells and cells co-expressing ATP11A. To investigate whether B1 signaling activity might be sensitive to PS exposure in a TSR-dependent manner, we also tested B1ΔNT, which lacks the NTF of the receptor and therefore lacks the TSRs that bind to PS (Figure 2.2A).

Coexpression of B1FL with ATP11A reduced G protein-dependent signaling activity by 52% (Figure 2.2B, one-way ANOVA with Tukey's multiple comparisons test, $p<0.0001$). In comparison, the G protein-dependent signaling of B1ΔNT was not significantly altered by coexpression with ATP11A (Figure 2.2C), suggesting that the NTF of B1 is required for the impact of ATP11A on the signaling activity of the receptor. To determine whether the effect of ATP11A on B1 stimulation of SRF-luciferase was due to an overall reduction in the expression of the receptor, we measured B1 expression in the absence and presence of ATP11A and found that ATP11A does not alter the total protein levels of either B1FL (Figure 2.2D) or B1ΔNT (Figure 2.2E). We also assessed B1 surface expression and found that ATP11A had no significant effect on trafficking of B1FL to the plasma membrane (Fig. 2.2F). These findings demonstrated that the presence of ATP11A reduces B1 signaling activity but not the total or surface expression of the receptor.

Figure 2.2. ATP11A coexpression reduces B1 signaling activity.

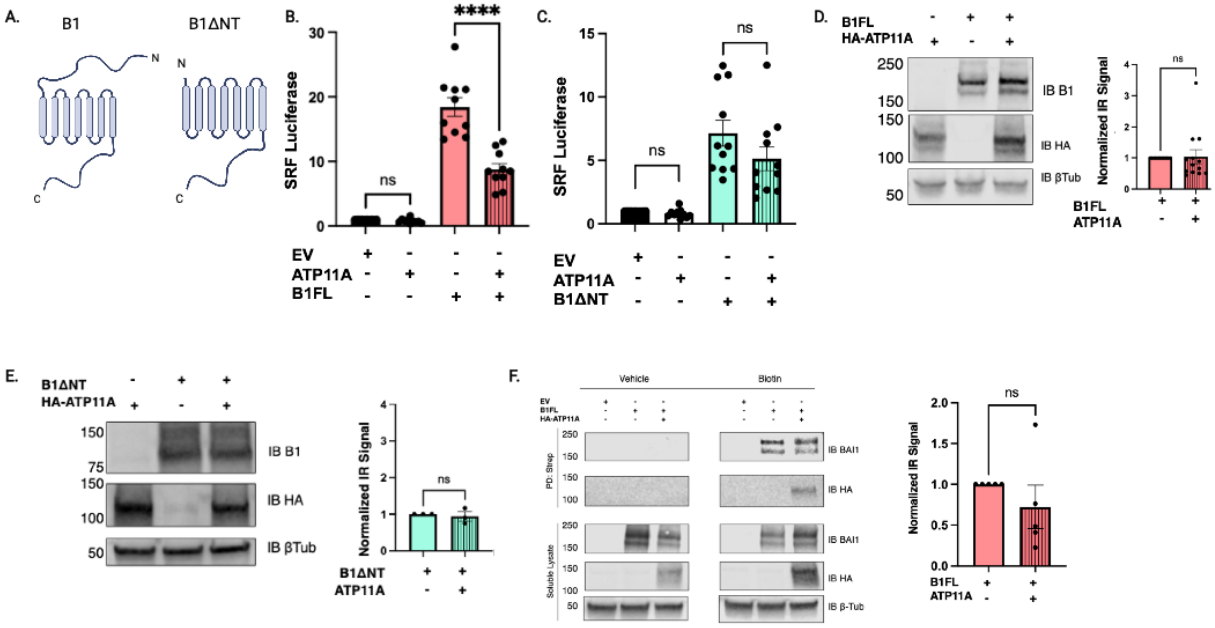

Figure 2.2. ATP11A coexpression reduces B1 signaling activity.

A) *B1 schematics:* Depiction of B1FL on left, shown with full NTF, and B1ΔNT on right, lacking NTF up to site of predicted GAIN domain cleavage. B) Coexpression with B1FL in HEK293T cells resulted in 52% reduction in B1FL activation of SRF-luciferase (mean ± SEM shown, ordinary one-way ANOVA with Tukey's multiple comparisons test, $p<0.0001$, n=10, ROUT method used at 10% to remove 2 outliers). C) ATP11A coexpression with B1ΔNT resulted in no significant change in receptor activation of SRF-luciferase (mean ± SEM shown, ordinary one-way ANOVA with Tukey's multiple comparisons test, n=11, ROUT method used at 10% to remove 1 outlier). D) ATP11A coexpression with B1FL did not significantly alter total cell lysate expression levels of receptor. Representative Western blot shown on left with quantification on right (normalized mean ± SEM shown, unpaired t-test, n=13). E) ATP11A coexpression with B1ΔNT did not significantly alter total cell lysate expression levels of receptor. Representative Western blot shown on left with quantification on right (normalized mean ± SEM shown, unpaired t-test, n=3). F) ATP11A coexpression with B1FL did not significantly alter receptor surface expression. Representative Western blot shown on left with quantification on right (normalized mean ± SEM shown, unpaired t-test, n=5).

2.1.2.3 The flippase activity of ATP11A is required for modulation of B1 signaling

To dissect the mechanism of ATP11A-mediated regulation of B1 signaling activity, we next investigated whether the flippase function of ATP11A was required for the impact on B1FL signaling. For these studies, we utilized a mutant version of ATP11A that has a glutamate residue changed to glutamine at position 186 (E186Q). This mutation abolishes ATP11A flippase activity but does not affect the protein's localization in the plasma membrane (Naito et al., 2015; Roland et al., 2019; Takatsu et al., 2014; Tone, Nakayama, Takatsu, & Shin, 2020). Figure 2.3A depicts the position of the ATP11A-E186Q mutation with an "X".

Using flow cytometry, we first confirmed that this flippase-null mutant was indeed unable to significantly alter PS levels in HEK293T cells (Figure 2.3B). Next, we performed SRF-luciferase signaling assays like those described above, but in this case observed no significant impact of ATP11A-E186Q coexpression on the signaling activity of either B1FL or B1ΔNT (Figure 2.3C-D). The E186Q mutant also did not have any impact on the total expression of B1 (Figure 2.3E-F). These findings showed that the PS flippase function of ATP11A is required for modulation of B1 signaling activity.

2.1.2.4 Increased cell density does not promote B1 signaling

The dependence on the flippase function of ATP11A for the protein's effect on B1FL suggested that B1 detection of PS was stimulating receptor signaling. However, it was unclear whether the B1/PS interactions promoting signaling occurred on the same cell (*cis*) or between cells (*trans*). This question is important in understanding how PS might modulate B1 signaling physiologically. If B1 was predominantly detecting PS in *trans* on neighboring cells, then increasing cell density should magnify the effect and promote B1 signaling. Thus, we developed a co-culture assay in which the

number of B1/SRF-luciferase-transfected cells was held constant but additional untransfected HEK293T cells were added to some of the wells to determine whether cell density could modulate B1 signaling. As shown in Figure 2.3G, we observed that B1FL signaling activity was largely unaffected by co-culturing the B1/SRF-luciferase-transfected cells with additional untransfected cells. These observations suggest that B1FL does not predominantly detect PS in *trans* in this system, but rather likely detects PS in the same cell, at least under the conditions of these experiments.

Figure 2.3. Flippase-null mutant ATP11A (E186Q) does not alter B1 signaling activity.

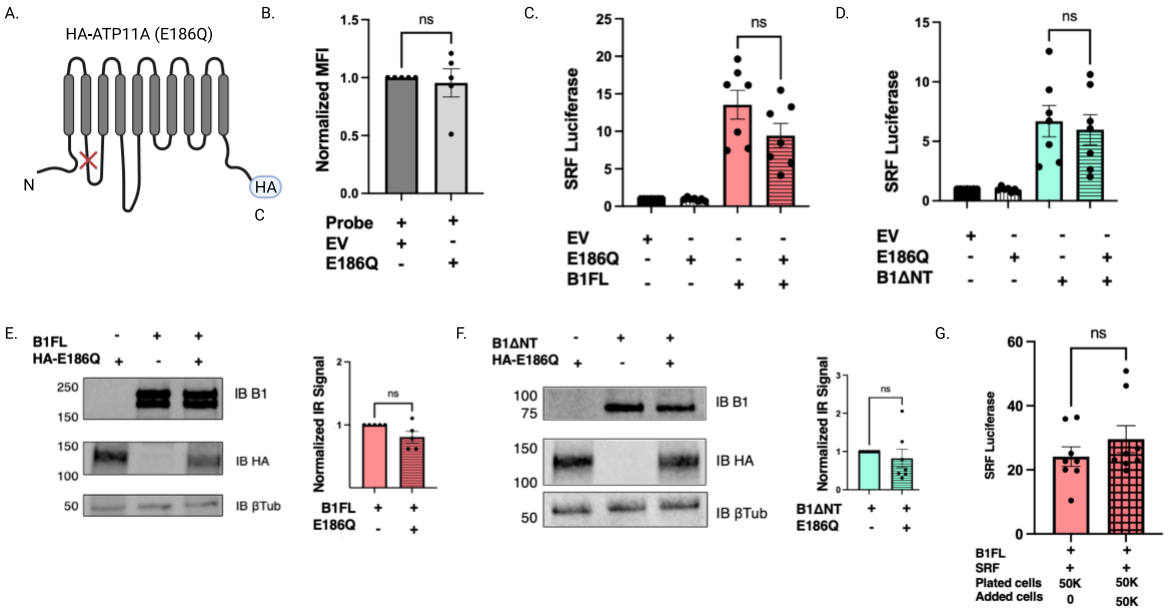

Figure 2.3. Flippase-null mutant ATP11A (E186Q) does not alter B1 signaling activity.

A) *ATP11A-E186Q schematic:* Depiction of mutant ATP11A at point 186 from E to Q that abolishes the flippase function. X shows general location of mutation. HA tag is also shown. B) *Quantification of PS exposure in HEK293T cells overexpressing E186Q mutant:* Mean fluorescence intensity (MFI) shown with E186Q+ condition normalized to mock-transfected condition. E186Q overexpression in HEK293T cells resulted in no significant reduction in PS exposure (mean ± SEM shown, unpaired t-test, n=3). C) ATP11A-E186Q coexpression with B1FL in HEK293T cells resulted in no change in B1FL activation of SRF-luciferase (mean ± SEM shown, ordinary one-way ANOVA with Tukey's multiple comparisons test, n=7). D) ATP11A-E186Q coexpression with B1ΔNT also resulted in no significant change in receptor activation of SRF-luciferase (mean ± SEM shown, ordinary one-way ANOVA with Tukey's multiple comparisons test, n=7). E) ATP11A-E186Q coexpression with B1FL did not significantly alter total cell lysate expression levels of receptor. Representative Western blot shown on left with quantification on right (normalized mean ± SEM shown, unpaired t-test, n=5). F) ATP11A-E186Q coexpression with B1ΔNT also did not significantly alter total cell lysate expression levels of receptor. Representative Western blot shown on left with quantification on right (normalized mean ± SEM shown, unpaired t-test, n=7). G) In comparison to baseline signaling of B1FL (pink bar on left), addition of 50k additional mock-transfected HEK293T cells did not significantly alter B1FL activation of SRF-luciferase (pink and checkered bar on right; mean ± SEM shown, unpaired t-test, n=8).

2.1.2.5 B1 multimerizes via its transmembrane domains in a PS-independent manner

PS has been proposed to modulate receptor function in some cases by promoting receptor clustering and multimerization (Birge et al., 2016; Lemke, 2017). Multimerization has been well-documented for certain GPCRs (Milligan, Ward, & Marsango, 2019; Prinster, Hague, & Hall, 2005), but nothing is known about the potential multimerization of B1. To determine whether B1 forms multimers and whether this process might be influenced by PS, we leveraged several modified versions of B1 as shown in Figure 2.4A. When untagged B1FL and HA-tagged B1ΔNT were co-expressed in HEK293T cells, we observed robust co-immunoprecipitation (co-IP) of these two receptors, suggesting that B1 forms multimers in a manner that does not require the NTF (Figure 2.4B). In parallel experiments, B1FL was co-transfected with B1-NTF, but no co-IP was observed, consistent with the idea that the NTF of B1 does not participate in B1 multimer formation (Figure 2.4C). We also co-expressed B1FL with myc/His-tagged B1ΔCT, which lacks the majority of the B1 CTF and observed robust co-IP of these two receptors (Figure 2.4D). Taken together, these co-IP studies suggest that B1 forms multimers via its transmembrane regions.

We next investigated whether B1 multimerization might be modulated by PS. Co-IP of B1FL and myc/His-tagged B1ΔCT was assessed in the absence and presence of ATP11A, which was shown earlier to reduce the levels of externalized PS. However, the presence of ATP11A was found to have no effect on B1 multimerization (Figure 2.4D, with quantification in Figure 2.4E). These findings suggested that B1 multimerization is independent of PS engagement by B1. Surprisingly, these experiments also yielded the observation that ATP11A itself robustly associates with myc/His-B1ΔCT (Figure 2.4D). This interaction is intriguing because it could allow ATP11A to locally modulate PS exposure levels in close proximity to B1.

2.1.2.6 B1 interacts via its NTF region with ATP11A

To elucidate the structural determinants of the novel interaction that was serendipitously observed between B1 and ATP11A, we utilized the panel of truncated constructs described earlier and performed a series of co-IP assays. Given that both B1 and ATP11A contain numerous transmembrane domains, and that B1 multimerization is dependent on these transmembrane regions, we hypothesized that the B1/ATP11A interaction was most likely mediated via transmembrane domain interactions. However, experiments assessing co-IP between B1-NTF and ATP11A unexpectedly revealed complex formation between ATP11A and B1-NTF, a truncated version of B1 that completely lacks the transmembrane domains of the receptor (Figure 2.4F). Reciprocally, we coexpressed ATP11A with B1ΔNT, which *does* contain the transmembrane domains of the receptor, and could not detect any co-IP of a B1ΔNT/ATP11A complex (Figure 2.4G). This series of co-IP experiments indicated that the B1 association with ATP11A is mediated by the B1 NTF region.

Figure 2.4. B1 forms PS-independent multimers and also interacts with ATP11A.

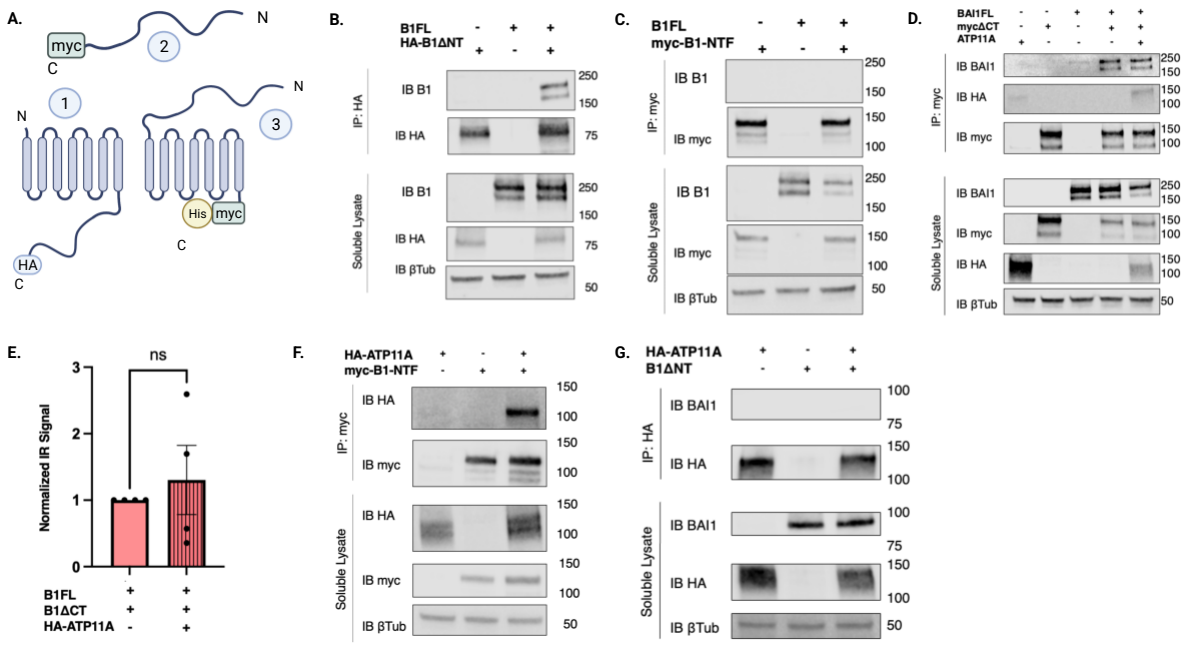

Figure 2.4. B1 forms PS-independent multimers and also interacts with ATP11A.

A) *B1 schematics*: B1 constructs used to evaluate multimer formation included (1) HA-tagged B1ΔNT, (2) myc-tagged B1-NTF, (3) His/myc-tagged B1ΔCT. B) Immunoprecipitation (IP) of HA-B1ΔNT resulted in co-IP of B1FL (n=3). C) IP of myc-tagged B1-NTF did not result in any co-IP of B1FL (n=3). D) IP of His/myc-B1ΔCT resulted in co-IP of B1FL in a manner that was not affected by co-expression with ATP11A (n=4). Intriguingly, ATP11A itself also co-immunoprecipitated with His/myc-B1ΔCT. E) *Quantification of effect of ATP11A on B1FL-His/myc-B1⊿CT dimer formation:* HA-ATP11A coexpression with B1FL and His/myc-B1ΔCT did not disrupt the ability of B1 to form multimers (normalized mean ± SEM shown, unpaired t-test, n=4). F) *HA-ATP11A interacts with myc-tagged B1-NTF*: IP of myc-tagged B1-NTF resulted in co-IP of HA-ATP11A (n=3). G) *HA-ATP11A does not interact with untagged B1⊿NT:* IP of HA-ATP11A did not result in any detectable co-IP with B1ΔNT (n=4).

2.1.2.7 B1 signaling is reduced in cells lacking the scramblase ANO6

To further test the idea that B1 signaling activity is enhanced by receptor binding to externalized PS, we sought to assess B1 signaling under conditions where PS exposure was manipulated in a manner independent of ATP11A. As mentioned earlier, ANO6 is a lipid scramblase known to be endogenously expressed in HEK293T cells (Schenk et al., 2016). Thus, we assessed B1 signaling in an *ANO6* knockout (KO) cell line derived from HEK293T cells, which has been previously described (Le, Jia, et al., 2019; Le, Le, & Yang, 2019; Liang & Yang, 2021; Y. Zhang et al., 2020). We first confirmed via Western blot that these cells lack ANO6 expression (Figure 2.5A) and also confirmed via flow cytometry that the *ANO6*KO cells exhibit significantly reduced PS exposure in comparison to wild-type HEK293T cells (Figure 2.5B). Quantification of these flow data demonstrated that the *ANO6*KO cells exhibited a 26% reduction in PS exposure in comparison to wild-type HEK293T cells (Figure 13C, unpaired t-test, $p<0.0001$).

With the *ANO6*KO cell line confirmed as having low basal PS exposure, B1FL G protein-dependent signaling to SRF luciferase was measured (Figure 2.5D). In comparison to B1FL signaling in HEK293T cells in matched experiments, B1FL G protein-dependent signaling in *ANO6*KO cells was lower by 67% (one-way ANOVA with Tukey's multiple comparisons test, $p<0.0001$). These findings provide further evidence, utilizing an independent method of altering PS exposure, that externalized PS promotes B1 signaling activity.

Figure 2.5. B1 signaling activity is reduced in cells lacking ANO6.

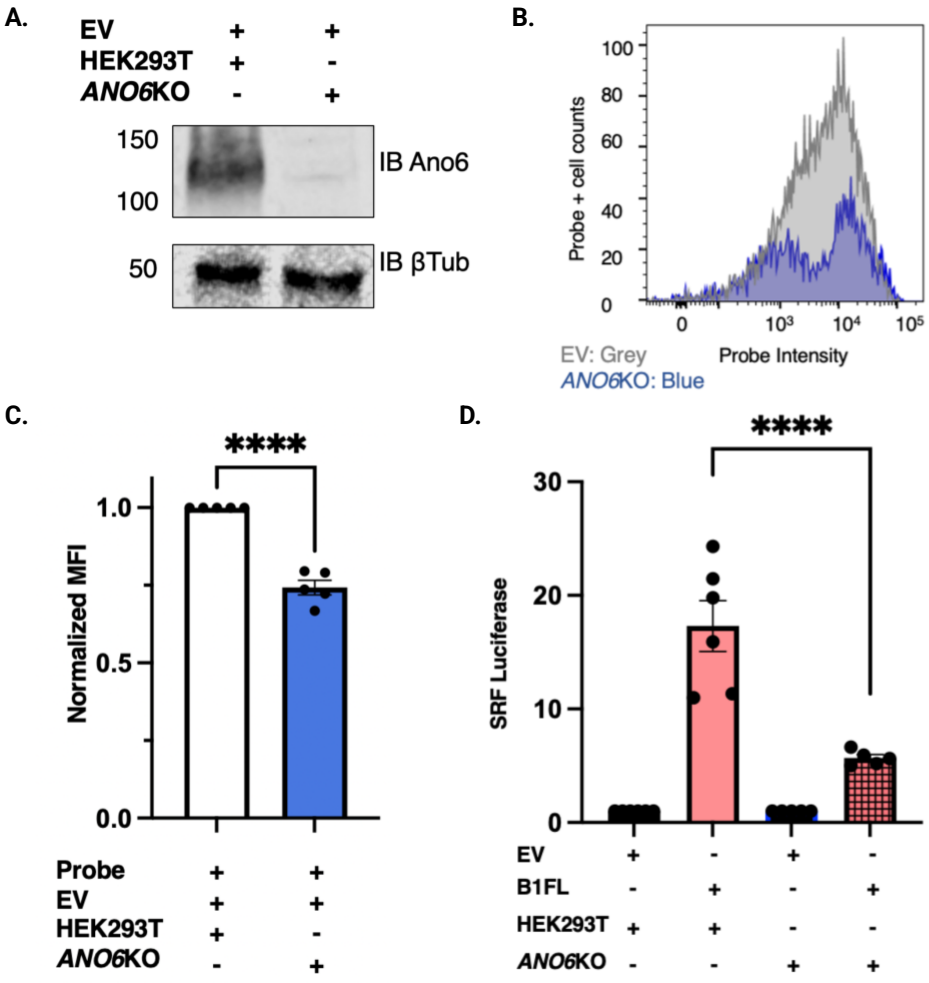

Figure 2.5. B1 signaling activity is reduced in cells lacking ANO6.

A) Confirmation of *ANO6* KO in HEK293T cell line: On left, Western blot of wild-type HEK293T lysates vs. lysates from *ANO6*KO cell line immunoblotted for Ano6 and β-Tubulin. B) Flow histogram of mock-transfected *ANO6*KO mutant line (+Annexin V). Lower probe positive cells were observed in the ANO6KO cells (n=3). C) Quantification of PS exposure in *ANO6*KO mutant cell line compared to WT HEK293T cells: Mean fluorescence intensity (MFI) shown with *ANO6*KO condition normalized to mock-transfected condition. PS exposure was reduced by 26% in the *ANO6*KO cells (mean ± SEM shown, unpaired t-test, $p<0.0001$, n=3). D) B1FL activation of SRF-luciferase in *ANO6*KO cells was reduced by 67% relative to matched wild-type HEK293T cells (normalized to cell line mean ± SEM shown, ordinary one-way ANOVA with Tukey's multiple comparisons test, $p<0.0001$, n=5, ROUT method used at 10% to remove 1 outlier).

2.1.3 Discussion

B1 has long been known to bind PS in the context of macrophage-mediated engulfment of apoptotic cells (D. Park et al., 2007), but the relationship of PS binding to B1 signaling activity has not been explored. The work described here demonstrates that B1 binding to externalized PS promotes the G protein-dependent signaling activity of the receptor. B1 has previously been reported to exhibit high constitutive activity in HEK293T cells (Kishore et al., 2016; Stephenson et al., 2013), and the findings shown here reveal that at least a portion of this high constitutive activity is due to stimulation of B1 signaling by the basal level of exposed PS found in HEK293T cells).

While HEK293T cells represent a useful model for studies on modulation of B1 signaling by PS, it is ultimately more physiologically important to understand whether (and how) PS might influence B1 signaling *in vivo*. PS exposure is increasingly appreciated as not just a marker of programmed cell death but also an important cellular signal that can exert distinct physiological effects depending on how it is decoded by a diverse array of PS-binding receptors (Bevers & Williamson, 2016; Kay & Grinstein, 2013; Naeini et al., 2020). For example, in the central nervous system (CNS), where B1 is most abundantly expressed, PS exposure is known to play a critical role in marking dendritic spines for pruning by phagocytic cells such as microglia and astrocytes (G. C. Brown & Neher, 2014; T. Li et al., 2020; Nonaka & Nakanishi, 2019; Scott-Hewitt et al., 2020; Vilalta & Brown, 2018).

If indeed B1 acts as a PS sensor in the CNS, then the function of the receptor *in vivo* may be fairly complex, given that B1 is known to be expressed in neurons (Cahoy et al., 2008; Duman et al., 2013; Mori et al., 2002; Sokolowski et al., 2011; Stephenson et al., 2013), astrocytes (Cahoy et al., 2008; Sokolowski et al., 2011) and microglia (Cahoy et al., 2008; Mazaheri et al., 2014). Conceivably, the actions of B1 as a PS sensor in these distinct cell types may be very different or even opposing. For

example, studies by Tolias and colleagues have provided evidence (based on viral transduction of B1 into neurons *in vivo*) that neuronal B1 expression promotes the stability/maintenance of dendritic spines (Tu et al., 2018). Conversely, B1 expression in microglia has been shown to promote microglial engulfment of apoptotic cells (Mazaheri et al., 2014) and may also plausibly mediate engulfment of dendritic spines (or entire synapses) that are marked for elimination due to externalization of PS (G. C. Brown & Neher, 2014; T. Li et al., 2020; Nonaka & Nakanishi, 2019; Scott-Hewitt et al., 2020; Vilalta & Brown, 2018). Similarly, astrocytes are also known to prune synaptic elements (Bosworth & Allen, 2017), and thus astrocytic B1 may play a role in detecting externalized PS on postsynaptic spines and initiating pruning of those spines. Indeed, these opposing actions of B1 in distinct cell types may help to explain the mystery of why B1 knockdown in neurons decreases the numbers of dendritic spines (Duman et al., 2013; Tu et al., 2018) yet mice lacking B1 globally in all cell types exhibit no discernible changes in spine density (D. Zhu et al., 2015). Conceivably, B1 in neurons may detect PS as a stress signal and act to protect dendritic spines, whereas B1 found in glia (microglia and astrocytes) may detect PS as an engulfment signal and thereby facilitate spine pruning. These ideas may be tested in future studies in which B1 is deleted *in vivo* in a cell-specific manner.

While B1 was the first AGPCR identified as a PS-binding receptor, another AGPCR, GPR56 (ADGRG1), was also recently shown by Piao and colleagues to bind PS in a manner that facilitates synaptic refinement by microglia (T. Li et al., 2020). Interestingly, there are several notable differences between B1 and GPR56 as PS sensors. First, B1 binds PS via its TSR repeats (D. Park et al., 2007) whereas GPR56 binds PS via its GAIN domain (T. Li et al., 2020). Most AGPCRs possess GAIN domains (Lala & Hall, 2022; Morgan et al., 2019; Vizurraga et al., 2020), but at this point, it is unknown whether PS-binding is a common property of GAIN domains or whether this property is

unique to the GAIN domain of GPR56. Second, B1 and GPR56 exhibit distinct cellular distributions within the CNS: as described above, B1 is expressed in neurons, astrocytes and microglia, whereas GPR56 is not expressed at all in mature neurons but rather is expressed in oligodendrocytes, microglia, astrocytes and neuronal precursor cells (Bennett et al., 2016; Cahoy et al., 2008; Giera et al., 2015). Third, the findings presented above in our Results section demonstrate that B1 engagement of externalized PS stimulates the signaling activity of the receptor, whereas the effect of PS on GPR56 signaling has not yet been explored. Thus, it will be interesting in future studies to dissect the differential roles in PS detection played by B1 vs. GPR56 and also assess whether other AGPCRs have the ability to act as PS sensors.

2.1.4 Experimental Procedures

2.1.3.1 Constructs

Human B1 full-length (FL) (1-1584), B1 ΔNT (927-1584) (and HA-B1 ΔNT), B1 myc-ΔCT (1-1200), B1-NTF (1-927; also known as "Vstat120") constructs have been described previously (Kishore et al., 2016; Stephenson et al., 2013; D. Zhu et al., 2018). The latter two constructs were kindly provided by Erwin Van Meir (University of Alabama at Birmingham). Human HA-ATP11A and HA-ATP11A (E186Q) have also been described previously (Naito et al., 2015; Okamoto et al., 2020; Segawa, Kurata, & Nagata, 2016; Takada et al., 2018; Takada, Takatsu, Miyano, Nakayama, & Shin, 2015; Takatsu et al., 2011; Takatsu et al., 2017; Takatsu et al., 2014; Tone et al., 2020; J. Wang et al., 2018; J. Wang et al., 2017).

2.1.3.2 Cell culture

HEK293T cells were acquired from ATCC and maintained in DMEM (Thermo Fisher) supplemented with 10% fetal bovine serum (Rockland) and 1% penicillin/streptomycin (VWR) in a

humid, 5% CO_2, 37°C incubator. Cells were transfected with PEI or Mirus TransIT-LT1 (Mirus Bio) according to the manufacturer's protocol. The *ANO6*KO cell line was kindly provided by Huanghe Yang (Duke University) and was developed as described (Le, Jia, et al., 2019; Le, Le, et al., 2019; Liang & Yang, 2021; Y. Zhang et al., 2020).

2.1.3.3 Luciferase reporter assay

HEK293T cells were seeded into 96-well plates (Corning) at 50,000 cells per well 20-24 hours prior to transfection. Each well was transfected with 50 ng SRF-luciferase (a reporter of RhoA signaling via Gα12/13, pGL4.34, Promega), 1ng *Renilla-luciferase* and 50ng receptor or empty vector (EV) DNA, as previously described (17).

At 48hr after transfection, Dual-Glo luciferase assay was performed according to manufacturer's protocol by adding luciferase reagent (Promega) to cells for 10min in the dark at room temperature and read on FLUOstar Omega (BMG Labtech). Next stop-and-glo reagent (Promega) was added to stop the reaction for the *Renilla* luciferase read after another 10min incubation in the dark and at room temperature (also read on the same plate reader). Results were calculated for each assay by determining the luminescence ratio of firefly: Renilla luciferase counts, normalized to EV-transfected wells.

2.1.3.4 Co-culture experiments

HEK293T cells were seeded into 96-well plates (Corning) at 50,000 cells per well 20-24 hours prior to transfection using Mirus or PEI. Concurrently, a 10cm dish was also plated and transfected with EV DNA. In the 96-well dish, each well was then transfected with reporter (SRF-luciferase), *Renilla* and receptor or EV DNA. At 24hr after transfection, 50,000 cells were collected and counted from the 10cm dish and plated onto half of the wells in the 96-well plate to observe whether the

additional cells altered the signaling of the B1FL-positive cells. Signaling was then measured 48 hours after transfection.

2.1.3.5 Western blot

Protein samples were reduced and denatured in Laemmli buffer, loaded into 4-20%, Tris-Glycine gels (Bio-Rad) for SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad). Blots were blocked with EveryBlot blocking buffer (BioRad) and incubated while shaking with primary antibodies (specific antibodies listed below, all used at 1:1000) overnight at 4°C. Goat anti-rabbit IgG or goat anti-mouse IgG secondary antibody (IRDye 800CW, 1:5000, Licor) was then used to amplify signal (1-hour incubation, shaking at room temperature) and blots were imaged on a Licor Fc machine. ImageStudio was used for quantification of bands on the resultant Western blots. The primary antibodies used were: anti-BAI1 (Thermo Fisher PA1-46465, host: rabbit), anti-HA (Cell Signaling Technology C29F4, host: rabbit), anti-Myc (Cell Signaling Technology 9B11, host: mouse), anti-β-Tubulin (Cell Signaling Technology 2146S, host: rabbit), and anti-ANO6 (Invitrogen PA5-58610, host: rabbit).

2.1.3.6 Co-immunoprecipitation

At 48hrs after transfection, 10cm plates containing HEK293T cells were washed with cold PBS+ 0.9mM Ca^{2+} and solubilized in 1 ml harvest buffer (150mM NaCl, 25mM HEPES, pH 7.2, 1mM EDTA, 1% Triton X-100, 1X HALT protease/phosphatase inhibitors) overnight at 4°C, end-over-end. Next, unsolubilized material was cleared by centrifugation (15 min at 13,500 rpm, 4°C) and 90ul supernatant was collected for blotting (mixed 1:1 with 2X Laemmli buffer; BioRad) while the remainder (~910 ul) was mixed with washed beads (either anti-HA or anti-Myc agarose beads from Pierce). The lysate-bead mixture was rotated end-over-end overnight at 4°C. Next, beads were

briefly centrifuged in a table-top centrifuge, washed 3x in harvest buffer, and eluted in Laemmli loading buffer before loading on 4-20% Tris-glycine gels for SDS-PAGE and Western blotting. Western blot bands were quantified using Image Studio software (Licor).

2.1.3.7 Cell surface biotinylation

The Pierce Cell Surface Biotinylation and Isolation kit (Thermo, A44390) was used according to the manufacturer's protocol to evaluate receptor presence in the plasma membrane. Briefly, 48hr after transfection, 10cm plates of transfected HEK293T cells were placed on ice and washed with ice-cold PBS before being incubated with membrane-impermeant Sulfo-NHS-SS-Biotin in PBS for 10 min to biotinylate the surface. Cells were then washed in TBS three times, lysed in kit-provided buffer and cleared by centrifugation. The lysates were incubated with NeutrAvidin Agarose beads. Beads were then washed three times in the manufacturer's wash buffer and resuspended in Laemmli buffer. Biotinylated proteins were detected via Western blotting.

2.1.3.8 Flow cytometry

The Cell Meter Phosphatidylserine Apoptosis Assay Kit (Green Fluorescence Optimized for Flow Cytometry; from AAT Bioquest; 22824), which utilizes Annexin V to detect externalized PS, was used according to the manufacturer's protocol. Briefly, HEK293T cells or *ANO6*KO cells were plated in 10cm dishes and transfected using PEI with 4ug EV, ATP11A or ATP11A (E186Q). 48hr later, cells were collected into DMEM containing 10% fetal bovine serum (Rockland) and 1% penicillin/streptomycin (VWR), triturated and counted. Note: no trypsin was used when collecting the transfected cells in this step to avoid protease-mediated cleavage of B1. Instead, mechanical dissociation was used in complete media to obtain single-cell suspension of cultured cells. Next, $5x10^5$ cells were aliquoted per experimental condition, spun down, and resuspended in kit-provided

proprietary assay buffer with Annexin V probe and incubated for a minimum of 30 min in the dark before analysis by flow cytometry on a FACSymphony A3 5-Laser Cell Analyzer. Single-cell populations of cells were identified using side scatter (SSC)-width and SSC-height. To gate for saturation of signal, A23187 was used to induce PS exposure and served as positive control in parallel with same gates used in all experiments run (Millipore Sigma). Negative control was gated for lack of signal using cells without probe. Once positive and negative control were used to establish voltage and gating, these same settings were used for all experiments described. A total of 20,000 events were recorded for each sample.

2.1.3.9 Quantification and data analysis

GraphPad Prism was used to analyze data. Ordinary one-way ANOVA with Tukey's multiple comparisons test or unpaired t-tests were used to determine statistically significant differences among experimental conditions. ROUT method was used at 10% to identify any outliers in signaling assays (and indicated in legend where outliers were appropriately removed). Sample sizes are reported in the Results section; n values refer to the number of biological replicates for each set of experiments.

Chapter 3: Discussion and Future Directions

Parts of this chapter are adapted from Lala, T., & Hall, R. A. (2022). Adhesion G protein-coupled receptors: structure, signaling, physiology and pathophysiology. *Physiol Rev,* 102(4), 1587-1624. doi:10.115/physrev.00027.2021 and from Lala et al. JBC, 2022 (in submission).

3.1 Summary of Advances

Advances in the AGPCR field have revealed that this receptor class can bind to a diverse array of ligands, expanding their functional relevance to more than simply adhesive properties. Investigation of AGPCRs has revealed that its members' signaling can be activated by mechanosensory forces and chemical stimuli as well. Cryo-electron microscopy and electron microscopy techniques have also facilitated a better understanding of AGPCR ligand engagement and have revealed that some members of this receptor class can even bind steroid hormones (An et al., 2022; Ping et al., 2021) or bioactive lipids (J. W. Lee et al., 2016). AGPCR signaling is controlled by dynamic changes in the association between the receptors' NTF and CTF regions, with mechanical forces on the NTF changing the position of the stalk region (also known as the "tethered agonist" or "stachel") to modulate receptor signaling activity (Lala & Hall, 2022; Morgan et al., 2019; Vizurraga et al., 2020).

Adhesion GPCRs may now be regarded as massive signaling platforms that integrate heterogeneous signaling modalities (Lala & Hall, 2022). The work presented here builds upon previous work investigating B1 as a PS-binding receptor via its NTF (D. Park et al., 2007) and on work demonstrating B1 to be a regulator of dendritic morphology via Rho (Duman et al., 2019) and Rac (Duman et al., 2013) signaling. We have shown that B1 signaling is activated via engagement of externalized PS, revealing PS as one of the ligands that B1 can sense and integrate with other stimuli. These findings suggest that B1 may serve *in vivo* as a PS sensor and play an important role in PS-sensing at synapses and in other systems.

The studies described here utilized the PS flippase ATP11A as a tool to manipulate PS exposure in HEK293T cells. Intriguingly, far from just being a tool, ATP11A and related flippases are well-known to be localized to dendritic spines *in vivo* and to regulate synaptic plasticity. For example, knockout of the PS flippase ATP11B, a close relative of ATP11A, results in striking perturbations to dendritic spine morphology and hippocampal synaptic plasticity (J. Wang et al., 2019). Similarly, knockdown of CDC50A, a chaperone protein required for proper trafficking of ATP11-family phospholipid flippases (Takatsu et al., 2014), was shown to increase PS exposure at synapses and regulate aberrant pruning by microglia (T. Li et al., 2021). Moreover, the synaptic function of the ATP11-family of flippases is clinically relevant, given that a *de novo* heterozygous ATP11A point mutation was recently found to result in severe developmental delays and neurological deterioration (Segawa et al., 2021). The well-established synaptic actions of the ATP11-family of flippases, in conjunction with our data shown here that ATP11A modulates B1 activity and that ATP11A and B1 can robustly associate, suggest the possibility that B1 and ATP11 flippases form complexes at synapses that may act synergistically to sense and regulate PS levels in dendritic spines (Figure 3.1C).

Figure 3.1: Proposed mechanism for B1 interaction with PS and subsequent impact of ATP11A on B1 signaling.

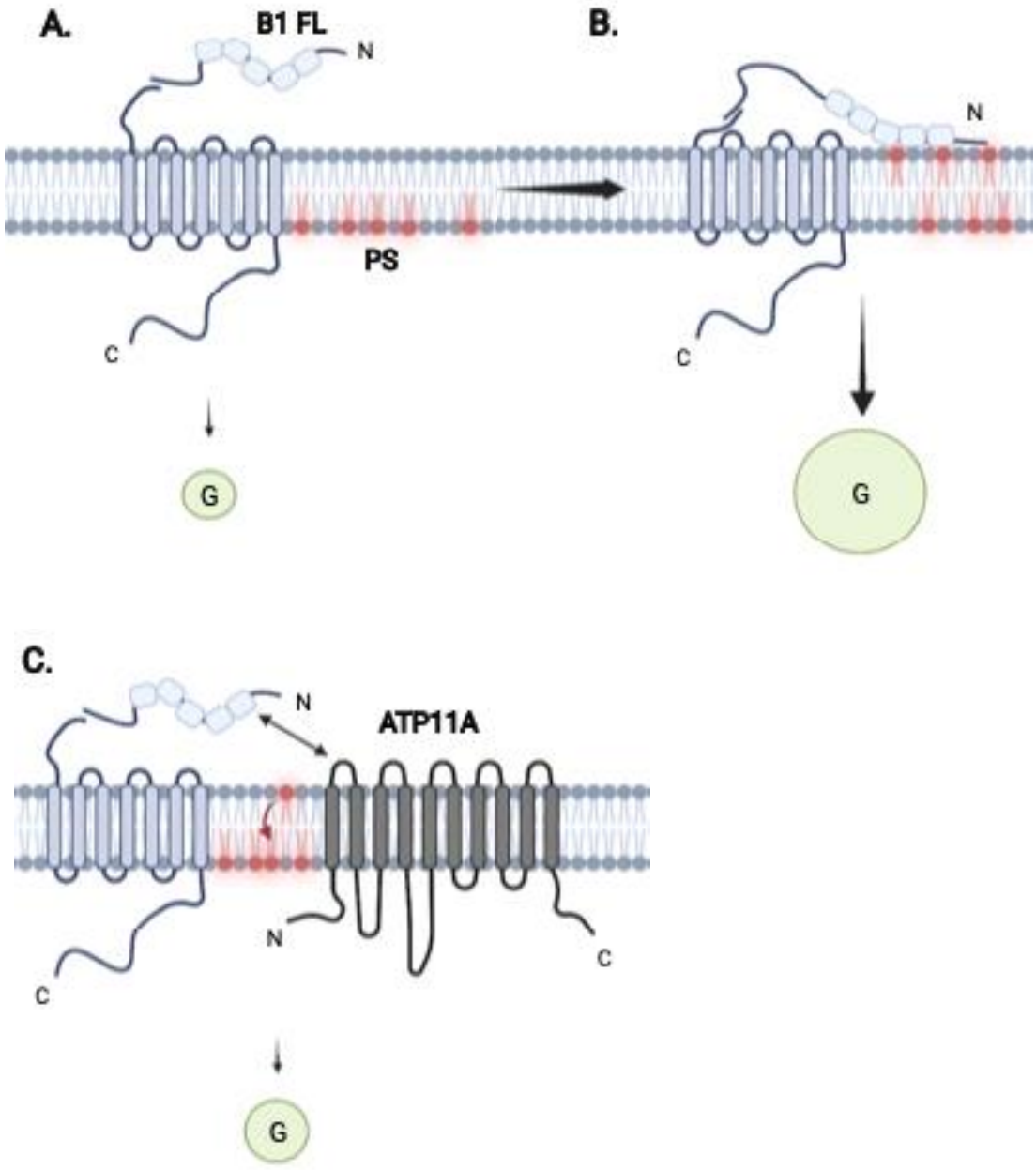

Figure 3.1. Proposed mechanism for B1 interaction with PS and subsequent impact of ATP11A on B1 signaling.

A) Without PS engagement of the B1 NTF, B1 exhibits minimal G protein-dependent signaling. B) B1FL binds to externalized PS, which triggers a conformational change in the NTF and results in enhanced G protein-dependent signaling. C) The flippase ATP11A binds to B1 and reduces PS exposure, lowering B1 signaling activity when the flippase is active.

3.2 Is PS the Only Ligand for B1?

Although B1 may serve as a PS sensor at synapses and in other cellular contexts, this by no means suggests that PS is the sole ligand for B1. Most AGPCRs possess massive extracellular NTF regions featuring numerous modular domains, and it seems highly likely that these multiple domains engage with multiple ligands (Lala & Hall, 2022; Morgan et al., 2019; Vizurraga et al., 2020). As was discussed in Chapter 1 Table 3, AGPCRs are documented to have multiple different ligands that mediate varying receptor physiological functions. Different ligand-receptor interactions can preferentially bind to specific effector proteins, referred to as biased agonism (Wootten, Christopoulos, Marti-Solano, Babu, & Sexton, 2018).

In the case of B1, it has been shown that the B1 NTF binds not just to PS but also binds with high-affinity to reticulon-4 receptors (RTN4Rs) to regulate dendritic arborization and synapse formation (Chong et al., 2018; J. Wang et al., 2021). RTNR4 also happens to bind at least one TSR of B1, so it would be interesting in future studies to investigate the interplay of PS and RTNR4 binding on B1 signaling. The TSR of B1 also bind to the integrin αvβ5 in the context of endothelial cell proliferation (Koh et al., 2004), and additionally mediates the interaction of the receptor with lipopolysaccharides in the context of macrophage-mediated engulfment of gram-negative bacteria (Das et al., 2011). The TSRs of B1 also binds to CD36 to regulate angiogenesis and this interaction is believed to play a role in cancer pathogenesis in the brain (Cork et al., 2012; Kaur et al., 2009). B1 has furthermore been shown to bind to the postsynaptic cell adhesion molecule Neuroligin-1 (NL-1) via its NTF to mediate NL-1-dependent spine growth and synapse development (Tu et al., 2018). Finally, the complement protein C1q is yet another protein that has been shown to bind to the TSRs of B1, although the physiological effects of this interaction have not yet been elucidated (Benavente et al., 2020).

The ability of different ligands to bind the NTF of B1 to mediate different processes underscores the need for further study of B1 signaling, including the possibility of biased agonism. It also remains unknown whether these multiple ligands can bind B1 at the same time, or if their interactions are either mutually exclusive or synergistic with one another. Furthermore, aside from the impact of PS binding on B1 G protein-dependent signaling, which was the focus of the studies described in Chapter 2, it remains unknown whether these other B1 ligands modulate receptor signaling. The NTF of B1 is massive and probably capable of engaging numerous ligands in different cellular contexts. It seems likely that there are additional ligands for the B1 NTF that are yet be discovered, which will only add to the complexity of the situation. The presently-known ligands of B1 are shown in a schematic in Figure 15.

In comparison to traditional ligand-receptor complexes, wherein it is quite clear where and how ligand binding to a GPCR can trigger signal transduction, AGPCR activation at this point is more nebulous due to the ability of the large NTF to bind so many different molecules. For example, it is very well known how and where epinephrine binds to β-adrenergic receptors to subsequently trigger signaling (Weis & Kobilka, 2018). Epinephrine is a classical and "true" ligand, or orthosteric agonist, for its cognate GPCRs. When comparing this interaction to the ability of PS to trigger B1 signaling, it is unclear whether this lipid or any of the other B1 ligands mentioned above are traditional agonists or allosteric modulators of receptor signaling. For other AGPCRs, like G3, the case seems more clear: G3 binds glucocorticoids in a transmembrane pocket in the receptor to trigger G_o signaling (Ping et al., 2021). In this case, as in the case of the adrenergic receptors, there is a specific binding pocket where a small molecule ligand binds to initiate signal transduction. For B1, in

contrast, it is not clear at present what pharmacological terms should be used to describe the various ligands that bind to the B1 NTF.

Given the complexity of AGPCR signaling and the emerging view that these receptors are massive platforms that integrate a variety of signals, it may be that traditional pharmacological terms like "orthosteric agonist" or "allosteric modulator" are simply not appropriate to describe the multi-ligand-binding nature of this receptor class. This classical terminology may not fully capture the complexity of AGPCR signaling, in that one NTF region can bind to multiple ligands to trigger different receptor responses in different cell types. Moreover, there is the question of whether the post-cleavage stalk (also known as the "tethered agonist" or stachel peptide) represents the true "orthosteric agonist" for most AGPCRs, or conversely whether the stalk might more accurately be described as a region of the receptor that is essential to the receptor activation mechanism. Thus, the pharmacology of AGPCR signaling remains murky at present, and determining a single "orthosteric agonist" for the members of this receptor class may not be possible. Perhaps instead it would be preferable to refer to modulators of AGPCR signaling simply as "ligands" at this point and not try to become more specific until the interplay of the various ligands can be evaluated and high-resolution structures can shed more light on the active conformation that can be achieved by the members of this receptor class.

Figure 3.2: B1 ligands that bind the receptor NTF are varied and trigger diverse processes in different cellular contexts.

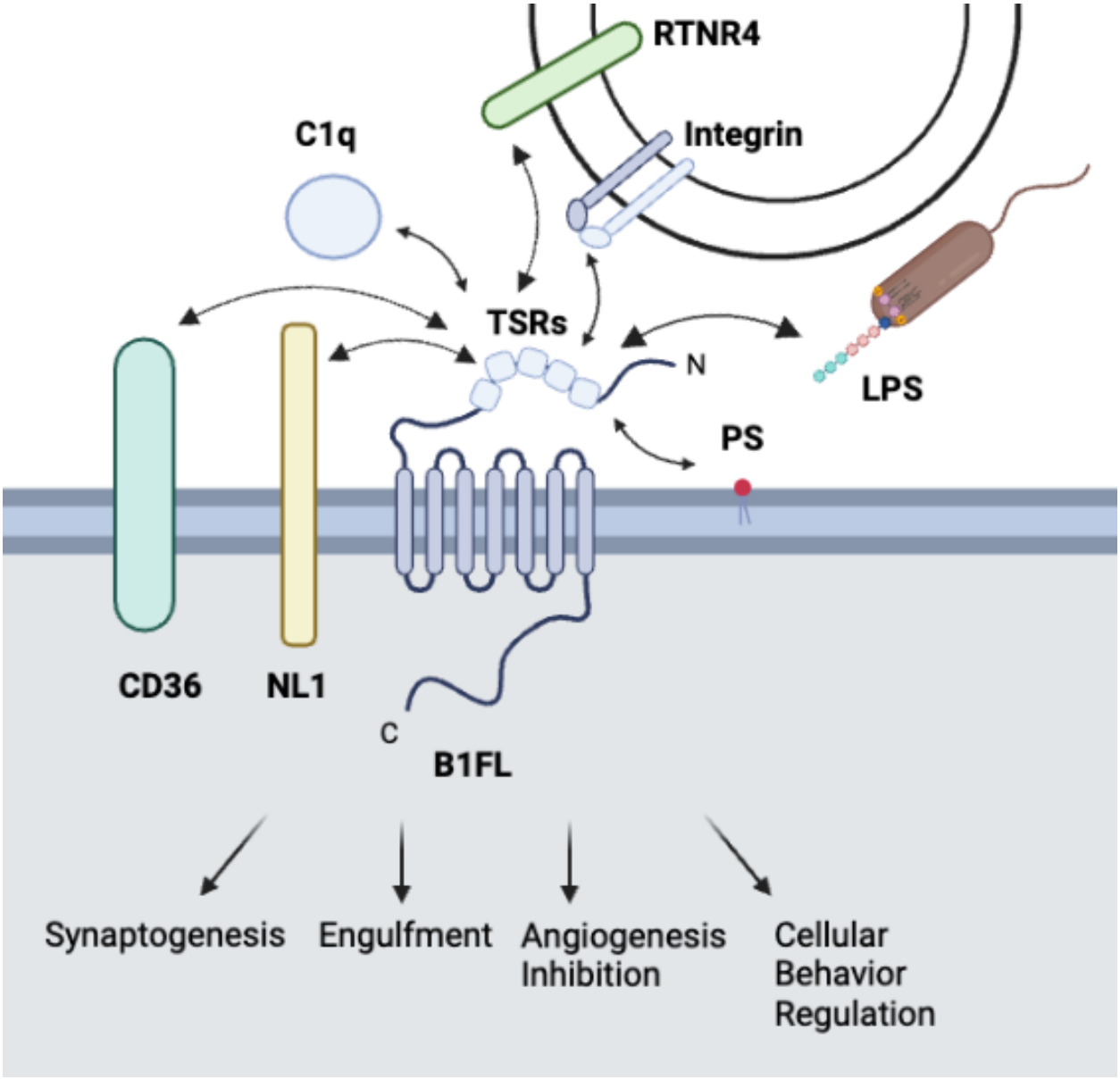

Figure 3.2: B1 ligands that bind the receptor NTF are varied and trigger diverse processes in different cellular contexts.

B1, like most AGPCRs, is a massive signaling platform that can integrate heterogeneous signals via its N-terminal fragment (NTF) to mediate diverse cellular processes. B1 engages with PS or LPS in the context of macrophage-mediated engulfment and the data presented in the previous chapter demonstrate that PS engagement also modulates the G protein-dependent signaling of the receptor. B1 engagement of NL1 and RTN4R can mediate synaptogenesis. B1 binds CD36 in the context of angiogenesis regulation and can also engage C1q.

3.3 Limitations

The work here is a pharmacological study of B1 detection of PS and builds upon previous investigations of this receptor. Notwithstanding, there are limitations to the studies described that must be considered. While the heterologous cell line HEK293T cells and protein overexpression were used to evaluate B1 sensitivity to PS, as well as the ability of the receptor to recognize trans elements and to multimerize, the scope of these studies is limited by the fact that HEK293T cells are an artificial environment for B1, which is natively expressed in the CNS in neurons (Cahoy et al., 2008; Duman et al., 2013; Mori et al., 2002; Sokolowski et al., 2011; Stephenson et al., 2013), astrocytes (Cahoy et al., 2008; Sokolowski et al., 2011) and microglia (Cahoy et al., 2008; Mazaheri et al., 2014), in addition to its expression in macrophages (Das et al., 2011) and muscle cells (Hochreiter-Hufford et al., 2013; Weng et al., 2019). B1 physiology in HEK293T cells may not fully recapitulate the receptor's physiology in these native cell types, due not only to differences in expression level but also due to the inherent differences in plasma membrane lipid composition and intracellular signaling machinery. It is an assumption implicit in these studies that the responses of B1 to externalized PS as a ligand will be consistent among different cell types (Desai & Miller, 2018).

However, a major advantage of using the HEK293T cell system is that these cells do not endogenously express B1. Moreover, these cells express detectable levels of externalized PS (Schenk et al., 2016) and the signaling of B1 has been well-defined in this cell type (Kishore et al., 2016; Stephenson et al., 2013), which is why HEK293T cells represented an attractive system for our studies. While moving experiments on PS stimulation of B1 signaling into primary cell culture and native tissues may be challenging, such future directions will be necessary to better evaluate the physiology of this receptor *in vivo*.

Another limitation of the studies presented here relates to the approaches we utilized to modulate levels of externalized PS. Specifically, there was a discrepancy in the PS exposure reduction vs. B1 signaling reduction induced by ATP11A overexpression versus *ANO6* knockout. ATP11A overexpression resulted in a 58% reduction in PS exposure in comparison to mock-transfected HEK293T cells. This reduced B1 signaling by 52%, which is roughly a one-to-one ratio of PS-exposure reduction to B1 signaling reduction. In comparison, *Ano6*KO reduced PS exposure by 26% in comparison to mock-transfected WT HEK293T cells, which reduced B1 signaling by 67% in comparison to its signaling in WT HEK293T cells. Although we and others have evaluated that the Ano6 expression is negligible in this KO cell line, it has not been characterized for its PS exposure levels in comparison to HEK293T cells until now. While *ANO6* was knocked out in this cell line, it is possible other lipid scramblases are expressed. Moreover, in comparison to ATP11A transient transfection, it is possible the knockout of *ANO6* results in other, heretofore unmeasured impacts on the lipid composition of the cell line that transient transfection could not accomplish. It is also important to recall that ATP11A itself can interact with B1, while the possibility of direct interaction with B1 was not assessed for Ano6. It is possible that ATP11A was able to exert an impact on B1 signaling that is roughly 1:1 because it binds to B1 and regulates local PS levels around the receptor in a way that is not recapitulated upon *ANO6*KO. It is also possible that *ANO6*KO results in more stable reductions in PS, or a different distribution of externalized PS in microdomains of the plasma membrane, relative to transient overexpression of ATP11A.

3.3 Future Directions

The field of AGPCR research will no doubt continue to expand in breadth and depth as breakthroughs are made regarding the various family members' pharmacology and roles in pathophysiology. Understanding modulators of AGPCR signaling will most likely facilitate the

generation of therapeutics, as many AGPCRs are implicated in human disease. While the work here elucidates the nature of B1-PS engagement for this receptor's G protein-dependent signaling, it also prompts many further questions regarding the physiology of this receptor.

The work here demonstrates that B1 binds PS, and that this lipid engagement alters the receptor's signaling (Figure 3.1). It would be of interest to understand whether B1 itself regulates PS externalization, such that B1 might serve as a PS sensor that provides real-time feedback to regulate exposure of PS. One potential way to measure this would involve the measurement of PS exposure in WT primary hippocampal neurons via Annexin V staining in comparison to B1KO primary neurons. If KO of B1 disrupts the normal regulation of PS exposure, this would suggest that B1 not only signals via PS but also provides feedback to control PS exposure levels. To further investigate this, it would also be interesting to measure whether B1 signaling is required for any effects of B1 on PS externalization. Thus, future studies might compare the effects of WT B1 vs. signaling-deficient B1 mutants, such as the B1NTF construct described in Chapter 2, in terms of their ability to regulate exposure of PS.

Initial coculture experiments described in the previous chapter demonstrate that B1 G protein-dependent signaling does not appear to respond to added cells. However, there was a trend showing an increase in signaling that was not statistically significant. While flow cytometry experiments revealed that HEK293T cells do have PS exposed at baseline, to determine whether B1 is responsive to PS in trans, more robust experiments may be needed to measure B1 sensitivity to elements in trans. Perhaps coculture conditions in which further exposure of PS is achieved in added cells could be used to more conclusively determine whether B1 signaling is modulated by exposed PS in trans. Such enhanced PS externalization in the cocultured cells could be achieved by treating cells with

A23187 to induce scramblase-dependent PS exposure and then coculturing these cells onto B1+, luciferase+ cells and measuring if B1 is responsive to these cocultured cells. Related experiments could be performed expressing B1 in the *ANO6*KO cells, where its signaling is very low at baseline, and coculturing with A23187-treated cells to measure B1 responsiveness to these PS+ cells. This may improve the signal-to-noise ratio of B1 signaling as opposed to measuring B1 signaling in WT HEK293T cells, where its constitutive signaling is fairly high already.

Further experimentation should focus on B1 recognition of PS in CNS cell types such as neurons and measuring the impact of B1-mediated downstream Rho and Rac signaling on dendritic morphology. As B1 is also documented to be present in astrocytes (Cahoy et al., 2008; Sokolowski et al., 2011) and microglia (Cahoy et al., 2008; Mazaheri et al., 2014), it would also be interesting to assess the importance of B1 PS recognition for B1 signaling in these cell types. Along these lines, it would be exciting to generate CNS organoids with WT vs. B1KO neurons, astrocytes and microglia configured in 3D space to study the developmental interplay among these cell types in the absence vs. presence of B1.

The studies described here revealed that PS-engagement by B1 alters the receptor's G protein-dependent signaling. It would be interesting to measure whether this PS engagement also alters the receptor's ability to engage in other key protein-protein interactions. For example, in addition to coupling to G proteins, B1 is also known to bind intracellularly to beta-arrestins (Kishore et al., 2016; Stephenson et al., 2013), IRSp53 (Oda et al., 1999), MDM2 (D. Zhu et al., 2015; D. Zhu et al., 2018), MAGI-3 (Stephenson et al., 2013), Tiam-1 (Duman et al., 2019; Duman et al., 2013; Tolias, Duman, & Um, 2011; Tu et al., 2018), Bcr (Duman et al., 2019) and PSD-95 (Stephenson et al., 2013; D. Zhu et al., 2015). What is the effect of B1 PS engagement on B1 interactions with these

various cytoplasmic binding partners? Does PS binding by B1 lead only to enhanced G protein-dependent signaling, such that PS serves as a "biased ligand" (Rankovic, Brust, & Bohn, 2016; Reiter et al., 2012; Southern et al., 2013), or does PS equally promote all signaling pathways downstream of B1?

To build on our observation that B1 forms multimers and extend this work to other AGPCRs, it is of great importance to evaluate the signaling properties of B1 multimers. Does multimerization facilitate signaling as it does for other GPCR multimers or does it abrogate normal receptor pharmacology (Gonzalez-Maeso, 2011; Prinster et al., 2005)? GPCR multimerization has therapeutic relevance as well. For example, the Wnt secreted glycoproteins play roles in cell fate and proliferation via signaling through the Frizzled GPCR family. Mutated Frizzled receptors in the autosomal dominant form of familial exudative vitreoretinopathy manifest as eventual retinal degeneration and importantly this mutated receptor, when homodimerized with the wildtype form of the receptor, leads to endoplasmic reticulum retention of these receptors (Robitaille et al., 2002). It is believed that the ability of GPCRs to form dimers alters the functional capabilities of receptors, which ultimately leads to clinical consequences (Prinster et al., 2005). Elucidation of AGPCR multimer formation in disease pathophysiology may provide insights that will lead to future therapeutic advances.

The techniques that have been most useful in evaluating receptor multimer formation in recent years include fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), and computational molecular dynamic (MD) simulations (Gahbauer & Bockmann, 2016). The temporal resolution offered by FRET and BRET, which use energy transfer between two closely spaced probes as a readout, are used to measure signaling, protein-protein interactions or

conformational changes in a protein (Lohse, Bunemann, Hoffmann, Vilardaga, & Nikolaev, 2007). These techniques investigate protein associations but have inherent limitations due to the impact of multimerization on signaling efficiency and subsequent data interpretation. MD provides complementary atomic resolution to receptor pharmacodynamics but presents its own limitations, as simulations are limited in their timescale and cost. However, applying these techniques to the study of AGPCRs will facilitate an understanding of the physiological significance and potential therapeutic value of AGPCR multimerization.

FRET and BRET could be useful in evaluating the signaling capability of the B1 multimers identified in the previous chapter. Furthermore, comparison of mutant versions of B1 that do not form multimers, perhaps through replacement of the TM domains that mediate B1 multimer formation, to wild-type B1 multimers will be useful in understanding the functional differences among B1 multimers vs. monomers. Efforts to this end have already been made, with Beliu and colleagues using FRET to demonstrate that AGPCR heterodimers of B3, E2, E5, G1 and L1 exist at the cell surface with their respective stachel peptides exposed in a manner predicted to facilitate their signaling activity (Beliu et al., 2021). It is likely that further investigation into AGPCR multimerization and its impact on signaling properties will provide new information regarding the physiological and pharmacological properties of AGPCRs.

Given our observation that B1 can form complexes with the flippase ATP11A, it would be interesting to evaluate the ability of B1 to form complexes with other flippases, especially those enriched in the brain like ATP11B (J. Wang et al., 2019) and ATP8A1 (Levano et al., 2012). It would be very interesting if B1 and flippases like ATP11A work synergistically, especially as mutations in ATP11A have been linked to neurological disease (Segawa et al., 2021). Segawa and colleagues

discovered a point mutation in ATP11A that changed the lipid-specificity of ATP11A resulting in severe neurological deterioration in a patient (Segawa et al., 2021). Perhaps therapeutics could be developed by further investigating the interaction of ATP11A and B1. ATP11A is known to be inhibited by caspase-mediated cleavage in the presence of calcium (Segawa et al., 2016). Thus, it would also be interesting to measure whether release of calcium stores can manipulate the ATP11A-B1 complex. Experiments of this type may help elucidate whether ATP11A and B1 work synergistically in the context of the brain. Similarly, it would be of interest to determine whether B1 interacts with scramblases like Ano6 (TMEM16F). Anoctamins are expressed throughout the body and in the brain, with several scramblases of this family implicated in neurological diseases like Alzheimer's Disease (Satoh et al., 2014) and Chronic Traumatic Encephalopathy (Cherry et al., 2018).

As mentioned at the end of the previous chapter, ADGRG1 (GPR56) is another AGPCR that was recently shown to bind PS, albeit in a different location than B1. G1 was observed to bind PS via its GAIN domain (T. Li et al., 2020). Thus, it would be of interest to measure whether PS engagement by G1 via its GAIN domain alters its G protein-dependent signaling, as this has not been evaluated. Since most AGPCRs contain a GAIN domain, it would also be of interest to measure whether other AGPCRs can bind PS via the same domain. For example, it remains a question of interest whether B1 can bind to PS via its GAIN domain in addition to the previously documented B1 binding of PS via the receptor's thrombospondin-like repeats (D. Park et al., 2007).

3.4 PS regulation of synaptic pruning

The significance of PS signaling extends beyond B1-mediated PS signaling and there has been considerable investigation into the role this lipid plays in the developing brain (Scott-Hewitt et al.,

2020). The establishment of proper neuronal circuitry requires not only synapse formation and maintenance but also synapse elimination or pruning. The balance of these opposing forces maintains homeostasis in the brain, without which disruption of brain development and neurological dysfunction can occur (Ikegami, Haruwaka, & Wake, 2019). Disruption of synaptic pruning has been linked to neurodevelopmental disorders like schizophrenia and autism, considered together as diseases of the spine or "spinopathies" (Hansel, 2019; Phillips & Pozzo-Miller, 2015).

The resident immune cell of the brain, microglia, is a major mediator of synaptic pruning. This cell forms processes with neuronal synapses to engulf immature synapses through varying mechanisms and it is believed that microglia recognize synapses in a spatially and temporally specific manner. Scott-Hewitt and colleagues demonstrated that the microglial receptor TREM2 is critical for synaptic pruning. This receptor senses PS exposed on specific synapses to developmentally regulate neuronal circuitry (Scott-Hewitt et al., 2020). Transient and localized exposure of PS is detected by this receptor to facilitate targeted synapse elimination. Thus, PS appears to be an important signal to direct microglia. Further investigation is required to elucidate exactly what triggers non-apoptotic PS exposure in the brain and will help the field understand more about neuronal circuitry regulation.

Synaptic pruning may go awry in neurodegenerative disorders and abnormal synaptic regulation can facilitate disease progression (Peet, Bennett, & Bennett, 2020). Abnormal glial function can be an active inducer of disease induction and progression (E. Lee & Chung, 2019). *Trem2* is an Alzheimer's risk gene in addition to being a microglial receptor that detects PS (E. Lee & Chung, 2019). The CNS AGPCR ADGRG1/GPR56 has also been demonstrated to control appropriate synapse numbers; its expression in microglia mediates PS detection on presynaptic elements, and knockout of G1 results in increased synapses due to reduced microglial engulfment (T. Li et al., 2020).

Additionally, knockout of *Cdc50a*, the chaperone protein required by flippases, was demonstrated to result in increased PS exposure in mature neuron somas and loss of inhibitory post-synapses, without impact on other neurons. This KO resulted in abnormal excitability and audiogenic seizures (J. Park et al., 2021). These examples demonstrate the importance of normal PS regulation in the brain and the danger of abnormal PS exposure in disease. PS detection in the brain by microglia is a crucial process to normal development and prevention of neurological disorders.

B1 being a PS-detecting receptor on neurons may also play a homeostatic role in developing spines that may have transiently exposed PS due to the stressful nature of spine dynamics. B1 on a neuron may sense and reduce or control PS to protect the spine in question before PS reaches amounts detectable by microglia via TREM2 or G1. Regulation of B1 may protect spines and may be a tractable area for therapeutic development in the future. For example, it would be interesting to know if B1 in spines can prevent engulfment by microglia. If B1 loss coincided with PS exposure and microglial engulfment, it may suggest that the presence of this receptor is required to maintain normal PS levels. When a spine is unhealthy, B1 could be downregulated and PS exposure could be dysregulated such that microglia can engulf the spine. Further investigation of the regulation of PS exposure and the consequences of PS-mediated signaling in the brain by different CNS cell types is needed.

Figure 3.3: B1 presence in spines is protective and without it, PS is dysregulated and makes the spine susceptible to microglial engulfment.

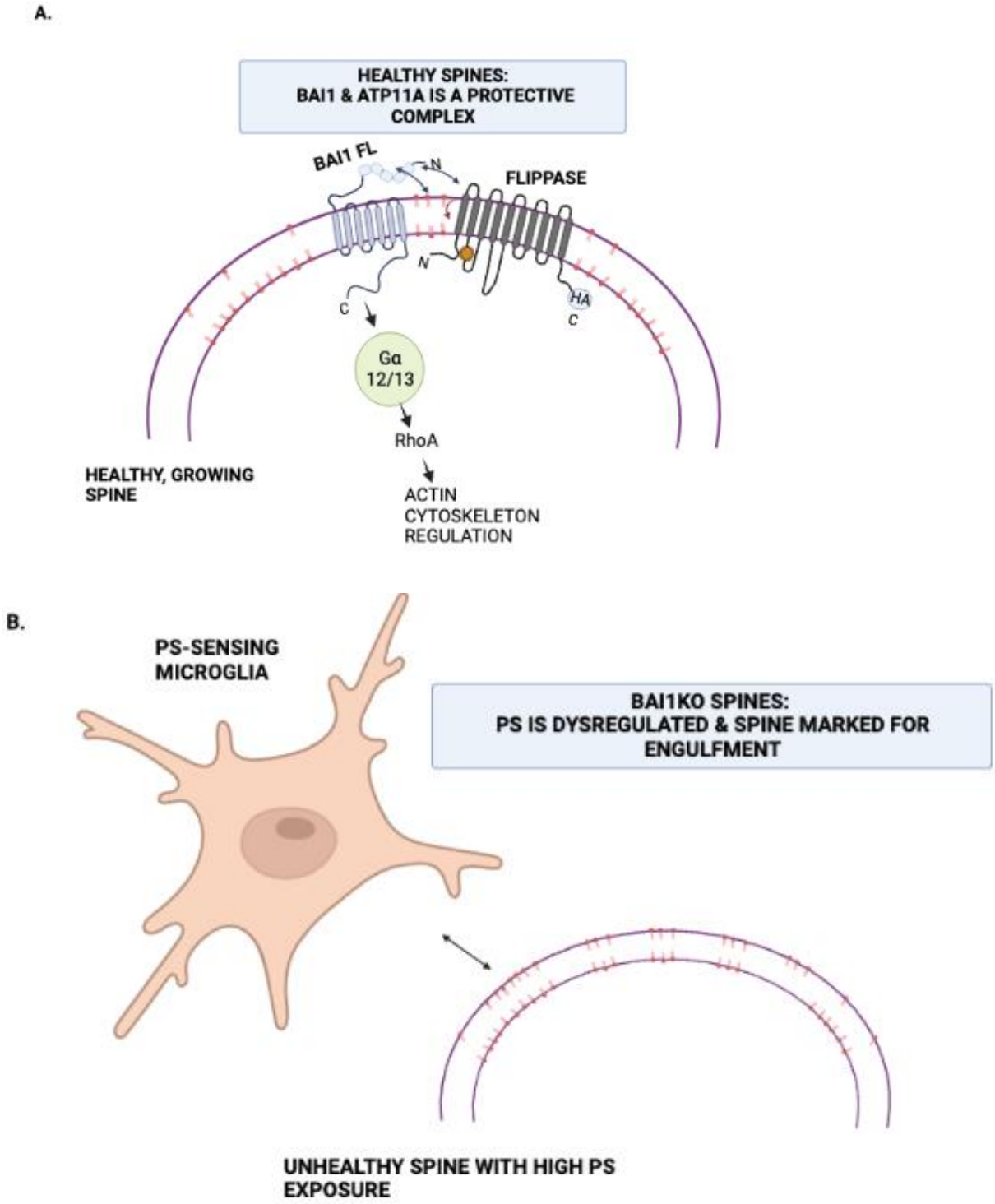

Figure 3.3: B1 presence in spines is protective and without it, PS is dysregulated and makes the spine susceptible to microglial engulfment.

A) B1, in concert with a flippase like ATP11A, may function in normal spines to detect PS via B1 and reduce PS exposure via ATP11A. Further investigation is required to determine whether B1 can regulate ATP11A or other flippases, but we hypothesize that this complex may prevent B1+ spines from having high levels of PS exposure. B) Contrastingly, in spines lacking B1, flippases may be dysregulated, leading to higher-than-normal levels of PS exposed and making the spine susceptible to microglial detection and subsequent engulfment.

3.5 Conclusion

The adhesion GPCRs are a diverse and fascinating family of receptors that play crucial roles in many different physiological processes. The importance of these receptors in human physiology is highlighted by the many clinical disorders associated with AGPCR dysfunction. The past decade has seen an explosion of interest in AGPCRs as well as a wide expansion in their perceived function. Early work on AGPCRs focused mainly on the ability of these receptors to mediate adhesion, but the emerging view is that AGPCRs serve as large-scale signaling platforms that integrate and interpret multiple types of stimuli, including adhesive, mechanosensory, and chemical signals.

This paradigm shift raises many interesting questions. For example, in the case of AGPCRs that can be activated both by small-molecule ligands and mechanosensory forces, how exactly are these signals integrated to determine receptor activation state? Should one of these signals be viewed as the "orthosteric" agonist controlling receptor activity, with the other(s) considered as "allosteric" modulation? Or will a completely different model be required to understand the pleiotropic nature of AGPCR signaling?

Another set of mysteries driving future research in this area relates to the AGPCR GAIN domains. What is the physiological significance of GAIN domain autoproteolysis? Despite a decade of intense research in this area, including crystal structures of multiple GAIN domains and numerous studies on AGPCRs harboring mutant GAIN domains deficient in self-cleavage, there is still no definitive answer to this question. Do the NTF and CTF fragments of AGPCRs usually dissociate during receptor activation, or is it more typical for ligands (and/or mechanosensory forces) to merely alter the conformations of the two associated fragments to initiate receptor signaling? Future studies, including the elucidation of cryo-EM structures of active vs. inactive AGPCRs, will likely provide

critical insights into the structural changes that underlie AGPCR activation and the importance of GAIN domain autoproteolysis for this process.

GPCRs remain major drug targets, and although no existing drugs target AGPCRs (Roth & Kroeze, 2015), many AGPCRs have been implicated in human disease. The challenge with this class of largely orphan receptors is the difficulty in modulating receptor activity. The work here has demonstrated that PS can modulate B1 signaling and may inform therapeutic development in the future. In the context of the brain, the novel interaction of B1 and ATP11A may present a protective protein-protein complex (Figure 3.3). Dendritic spines are plastic in nature and can alter their morphology in response to neuronal activity. The size and shape of spines reflect in part their activity state, and dendritic spines are believed to represent loci for memory storage through mechanisms of potentiation and depression of synaptic activity (Pchitskaya & Bezprozvanny, 2020). The dynamic changes occurring in spines may trigger PS exposure that can be detected and pruned by neighboring microglia (Scott-Hewitt et al., 2020).

Given the extensive body of work demonstrating the importance of flippases in the brain and for synaptic plasticity in the hippocampus (Levano et al., 2012; K. Sun et al., 2020; J. Wang et al., 2019; J. Wang et al., 2018), wherein knockout of various flippases results in severe hippocampal-dependent learning deficiencies that are similar in some respects to the consequences of knockout of B1, it may be the case that flippases and B1 work synergistically to protect dendritic spines from pruning. Further investigation will be required to test this idea and also evaluate whether B1 can regulate flippase function.

AGPCR investigation will undoubtedly reveal many additional physiological roles for this receptor family and also lead to new therapeutic approaches for targeting these receptors. The relatively limited number of small molecule ligands currently known for AGPCRs (Bradley et al., 2019; Gupte et al., 2012; Huang et al., 2020; Ping et al., 2021; Stoveken et al., 2016; Stoveken et al., 2018; B. Zhu et al., 2019) seems destined to expand dramatically in the coming years into a much more extensive armamentarium, which will create novel tools to facilitate research in this area while also providing exciting new avenues for the treatment of human disease.

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