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Jason Ryan Stephenson Date

Signaling, Regulation, and Synaptic Role of Adhesion G protein-coupled Receptor Brain-specific Angiogenesis Inhibitor-1

By

Jason Ryan Stephenson

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Program in Molecular and Systems Pharmacology

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Date

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Jason Ryan Stephenson

B.S., Illinois State University, 2005
M.S., Illinois State University, 2007

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 partial fulfillment of the requirements for the degree of

Doctor of Philosophy in

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Program in Molecular and Systems Pharmacology

Abstract

Signaling, Regulation, and Synaptic Role of Adhesion G protein-coupled Receptor Brain-specific Angiogenesis Inhibitor-1

By Jason Ryan Stephenson

G protein-coupled receptors (GPCRs) are one of the largest gene families in the human genome and major targets for therapeutics. GPCRs recognize a diverse array of extracellular stimuli and transduce intracellular signaling cascades via heterotrimeric G proteins and other intermediates, resulting in modifications to cellular physiology. Brain-specific angiogenesis inhibitor-1 (BAI1) is a member of the adhesion GPCR family that has been studied primarily for its anti-angiogenic and anti-tumorigenic properties, but it has been unknown how BAI1 exerts its effects on cellular physiology or whether this receptor can even couple to G proteins. We found that over-expression of BAI1 in HEK293T cells results in activation of the Rho pathway via a $G\alpha_{12/13}$-dependent mechanism, with truncation of the BAI1 N-terminus (NT) resulting in a dramatic enhancement in receptor signaling. This constitutive activity of the truncated BAI1 mutant also resulted in enhanced downstream phosphorylation of extracellular regulated kinase (ERK) as well as increased receptor association with β-arrestin2 and increased ubiquitination of the receptor. To gain insights into the regulation of BAI1 signaling, we screened the C-terminus (CT) of BAI1 against a proteomic array of PDZ domains to identify novel interacting partners. These screens revealed that the BAI1-CT interacts with a variety of PDZ domains from synaptic proteins, including MAGI-3. Removal of the BAI1 PDZ-binding motif resulted in attenuation of the receptor's signaling to Rho, but had no effect on ERK activation. Conversely, co-expression with MAGI-3 was found to potentiate signaling to ERK by constitutively-active BAI1 in a manner that was dependent on the receptor's PDZ-binding motif. Biochemical fractionation studies revealed that BAI1 is highly enriched in post-synaptic density (PSD) fractions, a finding consistent with our observations that BAI1 can interact with PDZ proteins known to be concentrated in the PSD. These findings demonstrate that BAI1 is a synaptic receptor that can activate both the Rho and ERK pathways, with the receptor's NT and CT regions playing key roles in the regulation of BAI1 signaling activity.

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2013

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List of Abbreviations

7-transmembrane region……7TM

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid…….AMPA

Apoptosis signaling kinase 1……..…….ASK1

Autism spectrum disorder……..ASD

Brain-specific angiogenesis inhibitor…….BAI

C-terminus……CT

Ca^{2+}/calmodulin-dependent protein kinase II……..CaMKII

Cadherin EGF LAG seven-pass G-type receptors……..…CELSR

Co-immunoprecipitation……..Co-IP

Cysteine-rich angiogenesis inducer 61…….cyr61

Dulbecco's modified eagle's medium……...…DMEM

Extracellular signal-regulated kinase…….ERK

Epidermal growth factor…….EGF

Epidermal growth factor receptor……....EGFR

Fetal bovine serum……..FBS

G protein-coupled receptor……..GPCR

GPCR-autoproteolysis inducing……..GAIN

GPCR proteolytic site…….GPS

G protein-coupled receptor kinase…….GRK

Gamma-Aminobutyric acid……GABA

Guanine-nucleotide exchange factor……..…GEF

Glutathione S-transferase……...GST

Hormone-binding domain…….HBD

Human embryonic kidney……..HEK

Human epididymal protein 6……..…HE6

Immunoglobulin…….Ig

Insulin receptor substrate p53……IRSp53

c-Jun N-terminal Kinase 3……JNK3

Knock-out…….KO

Latrotoxin……...…LTX

Mammalian Diaphanous formin……...……mDia

Membrane-associated guanylate kinase-like inverted…….MAGI

Methyl-CpG–binding domain protein 2…….……MBD2

MAP kinase kinase 4……..MKK4

Mitogen-activated protein kinase……...MAPK

Mitogen-activated protein kinase kinase 1……...….MEK-1

N-terminus……NT

N-Methyl-D-aspartate……NMDA

Phosphate buffered saline……...PBS

Phosphatidylinositol 3 kinase…….....PI3K

Polycystic kidney disease 1……PKD-1

Postsynaptic density protein 95…….PSD-95

Postsynaptic density protein, Drosophila disc large tumor suppressor, and zona occludens-1…….……PDZ

Proline-rich region……..PRR

Protease-activated receptor……PAR

Protein kinase B……PKB/AKT

Protein phosphatase 2A……..…..PP2A

Raf proto-oncogene serine/threonine protein kinase 1…….Raf-1

Regulators of G protein signaling……..RGS

Receptor tyrosine kinase……RTK

Rho-associated coiled-coil kinase 1/2…….ROCK

Rho-binding domain……..…..……..RBD

SRC homology 3……..……..…….SH3

Thrombospondin type 1 repeats…….……...…….TSR

Vascular endothelial growth factor…….……...…….VEGF

Wild-type……...…….WT

Very large G protein-coupled receptor-1….……..……..……..…..VL

Chapter I: Introduction

1.1 G protein-coupled receptors

The ability of cells to sense and respond to external cues underlies every organism's ability to survive and adapt. Cells express a diverse array of receptors on the cell surface, which detect a wide range of extracellular signals to elicit intracellular responses and corresponding changes in cellular physiology, a phenomenon known as signal transduction. G protein-coupled receptors (GPCRs) are a superfamily of 7-transmembrane (7TM) receptors that are one of the largest families of genes (Pierce et al., 2002), constituting over 1 percent of the human genome (Flower, 1999) and encoding approximately 800 different proteins (Bjarnadottir et al., 2006). GPCRs can recognize a diverse range of extracellular stimuli, including hormones, neurotransmitters, lipids, proteins/peptides, and even light, leading to transduction of intracellular signaling cascades and modifications to an assortment of physiological processes (Lefkowitz, 2007; Rosenbaum et al., 2009).

Following the cloning of the β2 adrenergic receptor in 1986, it was apparent that there was structural homology to rhodopsin, a visual receptor (Dixon et al., 1986). Further cloning of additional GPCRs including the adrenergic and serotonin receptor families provided further support for a large family of receptors with a conserved structure (Dohlman et al., 1991). The overall conserved structure of these receptors includes an extracellular N-terminal (NT) domain, a 7TM region and an intracellular C-terminal (CT) domain. GPCRs have been classically divided into six broad classes, A-F, with only classes A-C and F existing in humans (Attwood and Findlay, 1994). Class A contains the rhodopsin-like receptors and is the largest class, while Class B includes the

secretin receptors and also contains the adhesion receptors. The metabotropic glutamate receptors and taste receptors compromise Class C, and Class F includes the frizzled and smoothened receptors. The non-human classes D and E encompass the fungal pheromone receptors and the cAMP receptors, respectively. The main drawback to this classification system is the lack of sequence homology and high variability of receptor gene numbers between many mammalian and invertebrate GPCRs. More recently, a newer classification system has arisen for human GPCRs known as the GRAFS system, comprised of the glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin families (Fredriksson et al., 2003).

Within the GRAFS system of human GPCRs, the glutamate family contains 15 members, including eight metabotropic glutamate receptors, which are further divided into three groups based on physiological activity and structure. Also within this family are two metabotropic gamma-aminobutyric acid (GABA) receptors, also known as $GABA_B$ receptors, as opposed to ionotropic $GABA_A$ receptors, which are ligand-gated ion channels. The calcium sensing receptor and five taste receptors also comprise the glutamate family. The largest family in the GRAFS system is the Rhodopsin family, consisting of a total of 701 receptors, 241 of which are not olfaction-sensing receptors (Fredriksson et al., 2003). This family contains the classic biogenic amine receptors including the adrenergic, dopamine, serotonin, and muscarinic receptors. The adhesion receptor family is comprised of 33 receptors that have very long N-termini ranging from 200 to 2800 amino acids that contain adhesion-like domains and a GPCR proteolytic site (GPS) motif. The frizzled/taste 2 receptor family contains 11 frizzled receptors, which control cell proliferation and polarity during development, and 13 taste receptors that are

expressed in the tongue and are thought to function as bitter taste receptors (Chandrashekar et al., 2000). Finally, the secretin family contains 15 receptors characterized by binding to endogenous large peptide ligands such as glucagon, parathyroid hormone and corticotrophin-releasing hormone.

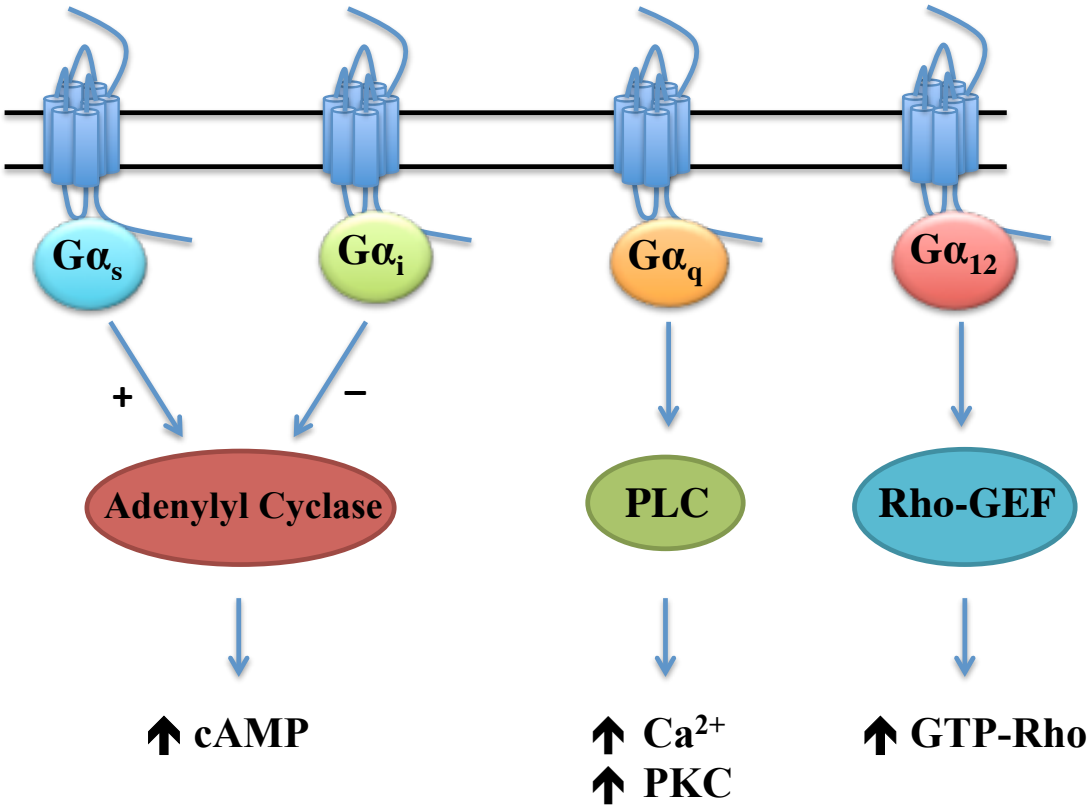
1.2 G protein-coupled receptor signaling

The activation of a G protein-coupled receptor is typically initiated by the binding of a ligand to extracellular or transmembrane regions of the GPCR to induce a conformational change. The activated GPCR can then interact with cytoplasmic heterotrimeric guanine-nucleotide protein (G protein) complexes comprised of three subunits: α, β, and γ. Recent advancements in protein crystallography have provided evidence for various conformations of active vs. inactive GPCRs. The details of the various conformational shifts are still being determined and appear to be specific to each GPCR. Generally speaking, though, concerted movements of the transmembrane helices open up a G protein-binding pocket (Preininger et al., 2013; Rasmussen et al., 2011). Upon interaction of the GDP-bound G protein with the activated GPCR, a conformational change in the C-terminus of the Gα protein occurs, opening up the binding pocket for GDP, thus allowing for the exchange of GDP for GTP (Oldham et al., 2006; Preininger et al., 2013). In this regard, the activated GPCR acts as a guanine-nucleotide exchange factor (GEF), promoting dissociation of GDP and association of GTP within the G protein. The activated GTP-bound Gα subunit then dissociates from the Gβγ subunit, and

the Gα and Gβγ are released from the GPCR to activate downstream effectors to elicit cellular responses and changes in cellular physiology.

The requirement of GTP for the hormonal activation of adenylyl cyclase was the first indication of the involvement of G proteins in transmembrane signaling (Gilman, 1987; Rodbell et al., 1971). Since then, a variety of G protein subunits regulating a multitude of physiological responses have been discovered. Despite the overwhelmingly large and diverse superfamily of GPCRs, the number of G proteins utilized for cellular responses is quite small. In total, the human genome encodes 16 genes for a total of 21 Gα subunits, 6 Gβ subunits encoded by 5 genes, and 12 Gγ subunits (Oldham and Hamm, 2008). The heterotrimeric G protein complexes can be divided into four main groups based on sequence similarity of the Gα subunit (Simon et al., 1991). These groups are comprised of $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ and are shown in Figure I-1 (Kristiansen, 2004). The $G\alpha_s$ proteins stimulate adenylyl cyclase, which increase cytosolic cAMP from ATP and $G\alpha_i$ inhibits adenylyl cyclase causing inhibiting the production of cAMP. $G\alpha_q$ activates phospholipase C (PLC), which converts phosphatidylinositol 4,5-bisphosphate (PIP2) in diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which causes increases of intracellular Ca^{2+} activation of PKC. The $G\alpha_{12}$ protein regulates RhoGEF, which increase the activation of Rho by enhancing the exchange of GDP for GTP, which modulates the actin cytoskeleton (Kristiansen, 2004; Neves et al., 2002). G proteins regulate multiple downstream effectors from metabolic enzymes to ion channels in turn controlling a broad range of physiological responses including development, cardiac function, learning and memory (Neves et al., 2002)

Figure I-1: Representation of various G protein-mediated signaling pathways. Schematic representation of GPCR signaling via $G\alpha_s$ which stimulates adenylyl cyclase to increase cytosolic cAMP, $G\alpha_i$ which inhibits adenylyl cyclase to decrease cytosolic cAMP, $G\alpha_q$ which activates phospholipase C (PLC) which increases intracellular Ca^{2+} and activation of PKC, and $G\alpha_{12}$ which activates RhoGEF increasing GTP-Rho (Kristiansen, 2004).


Gαs
Gαi
Gαq
Gα12
+
–
Adenylyl Cyclase
PLC
Rho-GEF
↑ cAMP
↑ Ca2+
↑ PKC
↑ GTP-Rho

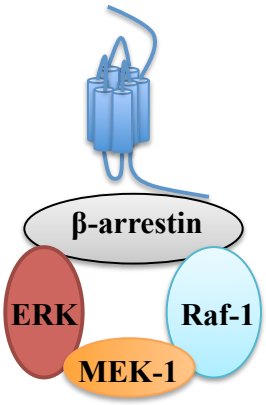
The role of the Gβγ subunit has evolved over the years from simply targeting the Gα subunit to the plasma membrane (Sternweis, 1986) to an actual signaling molecule in and of itself (Smrcka, 2008). Upon GPCR activation of the Gα subunit and dissociation of the Gβγ subunit, the Gβγ subunit can elicit multiple physiological responses on its own. The first example of this was the ability of the Gβγ subunit to associate with inward rectifying K^+ channels in cardiac myocytes and thus regulating channel activity (Logothetis et al., 1987). Another key interaction of the Gβγ subunit is the G protein-coupled receptor kinase 2 (GRK2). Gβγ can directly interact with GRK2, recruiting it to the plasma membrane and modulating its enzymatic activity, enhancing the ability of GRK2 to phosphorylate activated GPCRs (Pitcher et al., 1995). In all, Gβγ signaling is becoming a known regulator of a variety of physiological systems including cardiac function and inflammatory responses (Smrcka, 2008).

Aside from the classical G protein-mediated signaling of G protein-coupled receptors, there are also a number of G protein-independent signaling mechanisms utilized by GPCRs. One well-studied G protein-independent GPCR signaling pathway is mediated via the receptors' interactions with β-arrestins. Classically, activated GPCRs are phosphorylated on their C-terminus by GRKs (a family that includes GRK2 and 6 other kinases), which allows binding of β-arrestins to the activated GPCR and termination of receptor signaling. β-arrestins also recruit machinery involved in clathrin-mediated endocytosis, subsequently leading to the endocytosis of the GPCRs (Luttrell and Lefkowitz, 2002). However, β-arrestins can also recruit c-Src to the β2-adrenergic receptor to promote phosphorylation of the extracellular signal-regulated kinase (ERK), a protein in the mitogen-activated protein kinase pathway (MAPK) (Luttrell et al., 1999).

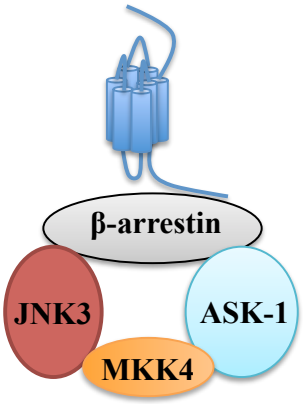
Furthrmore, using the angiotensin 1A receptor, it was shown that β-arrestin2 can promote the formation of a Raf proto-oncogene serine/threonine protein kinase 1 (Raf-1), mitogen-activated protein kinase kinase 1 (MEK-1), and ERK signaling complex (Luttrell et al., 2001). In a similar fashion, β-arrestins have been shown to signal to a separate MAPK signaling protein, c-Jun N-terminal Kinase 3 (JNK3) via recruitment of the upstream kinases MAP kinase kinase 4 (MKK4) and apoptosis signaling kinase 1 (ASK1) into a complex with JNK3, thereby increasing the phosphorylation state of JNK3 (McDonald et al., 2000). More signaling pathways mediated by β-arrestins have been uncovered, including activation of p38, phosphatidylinositol 3 kinase (PI3K), and protein kinase B (PKB/AKT) (DeWire et al., 2007). A schematic representation of various signaling pathways mediated by β-arrestins is shown in Figure I-2. The ability of GPCRs to signal through various G proteins, including both the Gα and Gβγ subunits, as well as through β-arrestins and other mechanisms adds a layer of complexity with regards to the signaling pathways activated by GPCRs and the downstream physiological responses elicited following receptor activation.

Figure I-2: Representation of various signaling pathways mediated by β-arrestins. (A) Schematic representation of the β-arrestin scaffold for the activation of ERK. ERK and Raf-1 bind directly to β-arrestin and MEK-1 binds via ERK and Raf-1. (B) Schematic representation of the β-arrestin scaffold for the activation of JNK. JNK and ASK1 bind directly to β-arrestin and MKK4 binds to JNK and ASK1. (C) Schematic representation of the β-arrestin scaffold for the activation of Akt. A signaling complex of β-arrestin2, Akt, and protein phosphatase 2A (PP2A), a negative regulator of Akt, was shown for the dopamine receptors upon stimulation with dopamine (Beaulieu et al., 2005). Image modified from DeWire SM, Ahn S, Lefkowitz RJ and Shenoy SK (2007) Beta-arrestins and cell signaling. *Annual review of physiology* **69**: 483-510 (DeWire et al., 2007).

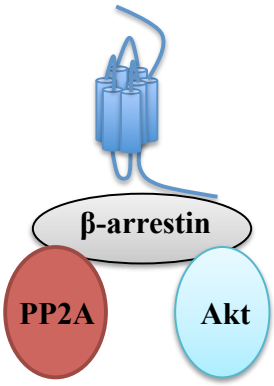
A


β-arrestin
ERK
Raf-1
MEK-1

B


β-arrestin
JNK3
ASK-1
MKK4

C


β-arrestin
PP2A
Akt

1.3 Clinical relevance of G protein-coupled receptors

G protein-coupled receptors are one of the most highly studied groups of proteins, due in part to the fact that they are important targets for therapeutics. Since GPCRs are involved in a vast array of cellular physiological responses, and each cell can express at least a few dozen GPCRs, they have become attractive targets for therapeutic intervention (Civelli et al., 2006). Currently, GPCRs represent approximately 50 percent of current drug targets and are one of the main targets of drug discovery (Drews, 2000; Lagerstrom and Schioth, 2008). As previously mentioned, the GPCR superfamily constitutes over 1 percent of the human genome (Flower, 1999), encoding approximately 800 different proteins (Bjarnadottir et al., 2006). Given the large number of GPCRs encoded by the human genome and the fact that they play roles in almost every physiological response, it is easy to understand why these receptors are such attractive targets for drugs. In addition to the large number of receptors and the vast physiological roles of GPCRs, the members of the GPCR superfamily also possess inherent characteristics that make them attractive therapeutic targets for drug intervention.

One of the most attractive characteristics of G protein-coupled receptors for drug discovery is their surface expression. Interestingly, 60 percent of all drug targets are localized to the cell surface, while a much smaller fraction (22 percent) of total proteins expressed are trafficked to the plasma membrane (Overington et al., 2006). Expression of a drug target on the cell surface is very desirable and circumvents a variety of problems associated with targeting cytoplasmic or nuclear proteins. One such problem is that many drugs are not membrane-permeable and have difficulty crossing the membrane

bilayer, mainly due to the hydrophilic nature of many small molecule drugs. The ability of GPCRs to detect extracellular signals and induce intracellular responses is a primary reason why GPCRs are such outstanding drug targets.

Aside from their expression at the plasma membrane, GPCRs are also excellent therapeutic targets because of their ability to initiate intracellular second messenger signaling cascades. Binding of a ligand to a GPCR classically activates a G protein, which then acts on effector molecules to induce the formation of many second messengers. This signal amplification is another important feature of GPCRs that makes these receptors attractive drug targets, since the binding of a single ligand can cause a greatly amplified response inside the cell. A classic example of signal amplification can be found in the well-characterized rhodopsin GPCR in the visual system. In this system, a single photon of light is absorbed by the rhodopsin-associated chromophore 11-*cis* retinal causing isomerization to all-*trans* retinal inducing a conformational change and subsequent activation of rhodopsin, further activating hundreds of molecules of transducin, a heterotrimeric G protein found in the visual system. Activated transducin can then activate cGMP phosphodiesterase, which hydrolyzes hundreds of molecules of cGMP per second, resulting in closure of hundreds of cGMP-gated Na^{2+} channels and cell membrane hyperpolarization (Burns and Arshavsky, 2005). This is just one example of a single ligand-receptor interaction (photon-rhodopsin) that can cause a dramatic change in intracellular signaling resulting in modification to overall cellular physiology (membrane hyperpolarization). Compensatory mechanisms are in place to reverse these changes including phosphorylation of receptors by GRKs, subsequent arrestin binding to

inactivate G protein coupling to the receptor, and cGMP restoration by guanylate cyclase (Burns and Arshavsky, 2005).

Another attribute of GPCRs that makes them attractive drug targets is their selective tissue and cell type expression (Insel et al., 2007). The pattern of distribution exhibited by most GPCRs is beneficial with regards to reducing side effects by allowing GPCR-targeted therapeutics to act on discrete tissues or organs while bypassing off-target areas. In addition to tissue specificity, GPCRs also have numerous subtypes that are even more localized and can therefore allow for even more exquisite subtype-specific targeting. One classic example of this phenomenon is the drug cimetidine, which is a specific histamine H_2-receptor antagonist used in the treatment of heartburn and peptic ulcers. There are four histamine receptors that have a broad range of tissue distribution and affect a wide variety of physiological responses (Seifert et al., 2013). The histamine H_2-receptor is localized in the parietal cells of the stomach and specific antagonists to this GPCR block the secretion of stomach acid without the unwanted side effects of targeting any of the other histamine receptors in other tissues or cell types. The blockade of the histamine H_2-receptor with cimetidine provided the first treatment for stomach ulcers and acid reflux disease, and the tremendous utility of cimetidine led to the development of the longer-acting histamine H_2-receptor antagonists that are in wide use today (Seifert et al., 2013).

Until recently, the crystal structures of G protein-coupled receptors have remained elusive. With recent advances in protein engineering for stabilization of membrane-bound protein crystallography, the initial crystal structures solved were for rhodopsin (Palczewski et al., 2000) and the β2 and β1 adrenergic receptors (Rasmussen et al., 2007;

Warne et al., 2008). Many structures of GPCRs have since been solved, including the dopamine D3 (Chien et al., 2010), adenosine A2A receptor (Jaakola et al., 2008), histamine H1 receptor (Shimamura et al., 2011), M2 and M3 muscarinic receptors (Haga et al., 2012; Kruse et al., 2012), CXCR4 receptor (Wu et al., 2010), sphingosine 1 phosphate receptor (Hanson et al., 2012), mu opioid receptor (Manglik et al., 2012), corticotropin-releasing factor 1 receptor (Hollenstein et al., 2013) and a handful of other GPCRs. Moreover, the agonist-bound β2 adrenergic receptor was recently crystallized in complex with $G\alpha_s$, providing the first structure of agonist-bound receptor in complex with a G protein (Rasmussen et al., 2011). These crystal structures will allow for enhanced drug screening efforts involving structure-based drug design facilitated by increased understanding of GPCR binding pockets. Indeed, the advances in GPCR crystallography have already led to the creation of new drugs through structure-based drug design and *in silico* docking (Shoichet and Kobilka, 2012).

Lastly, not only are GPCRs excellent targets for therapeutic intervention, the pathways utilized by these receptors are also of interest with regards to drug design. As previously mentioned, GPCRs signal through the α and βγ subunits of G proteins to induce a signal transduction cascade. Since GPCRs regulate a wide range of physiological responses, the downstream effectors of the signaling pathways have also generated therapeutic interest. Many GPCRs are overexpressed in cancer, and mutations to G proteins themselves have been found to play a role in cancer progression (Smrcka, 2013). For example, over 80% of ocular melanomas have point mutations in either $G\alpha_q$ or $G\alpha_{11}$ that cause a constitutively GTP-bound hyperactive state (Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2010). In accordance with this, small molecule inhibitors of

G proteins have shown efficacy in the treatment of certain animal models of cancer. For example, BIM-46174, a pan Gα GDP release inhibitor, inhibits the growth of multiple human cancer cell lines *in vitro* and human lung and pancreatic tumor xenographs in nude mice *in vivo* (Prevost et al., 2006). The Gβγ subunit inhibitor M119K has also been studied for its ability to inhibit the growth rate of MDA-MB-231 breast cancer cell line *in vitro* (Tang et al., 2011). Another example of downstream regulators of GPCR signaling that show therapeutic promise are the regulators of G protein signaling (RGS) class of proteins. These proteins promote G protein hydrolysis of GTP to GDP, thereby attenuating the duration of the signal cascade (McCoy and Hepler, 2009; Neitzel and Hepler, 2006). Since GPCRs regulate diverse physiological roles, it is not surprising that RGS proteins have also been implicated in multiple pathophysiological processes, including diseases of the immune, cardiovascular, and central nervous systems (Sjogren et al., 2010). Indeed, small molecule modulation of RGS proteins could prove beneficial in treating hypertension, breast cancer migration, and even opioid dependence (Sjogren et al., 2010).

Finally, postsynaptic density protein, Drosophila disc large tumor suppressor, and zona occludens-1 (PDZ) domain containing proteins regulate a variety of GPCR functions including signaling, regulation, and trafficking, and represent additional GPCR regulators that might be targeted therapeutically (Ritter and Hall, 2009). Indeed, drugs that block interactions between GPCRs and PDZ domain-containing proteins are already in development. For example, the nonsteroidal anti-inflammatory drug sulindac suppresses Wnt-mediated β-catenin signaling in breast, lung, and colon cancer cell lines (Han et al., 2008). Dishevelled is a PDZ domain-containing protein that is necessary for

Wnt signaling to β-catenin, and it has been shown that sulindac inhibits Wnt signaling to β-catenin by binding to the PDZ domain on Dishevelled and blocking its interaction with the PDZ-binding motif on Wnt (Lee et al., 2009). Thus, downstream GPCR effectors and regulators of GPCR signaling comprise a set of attractive therapeutic targets for future drug development efforts.

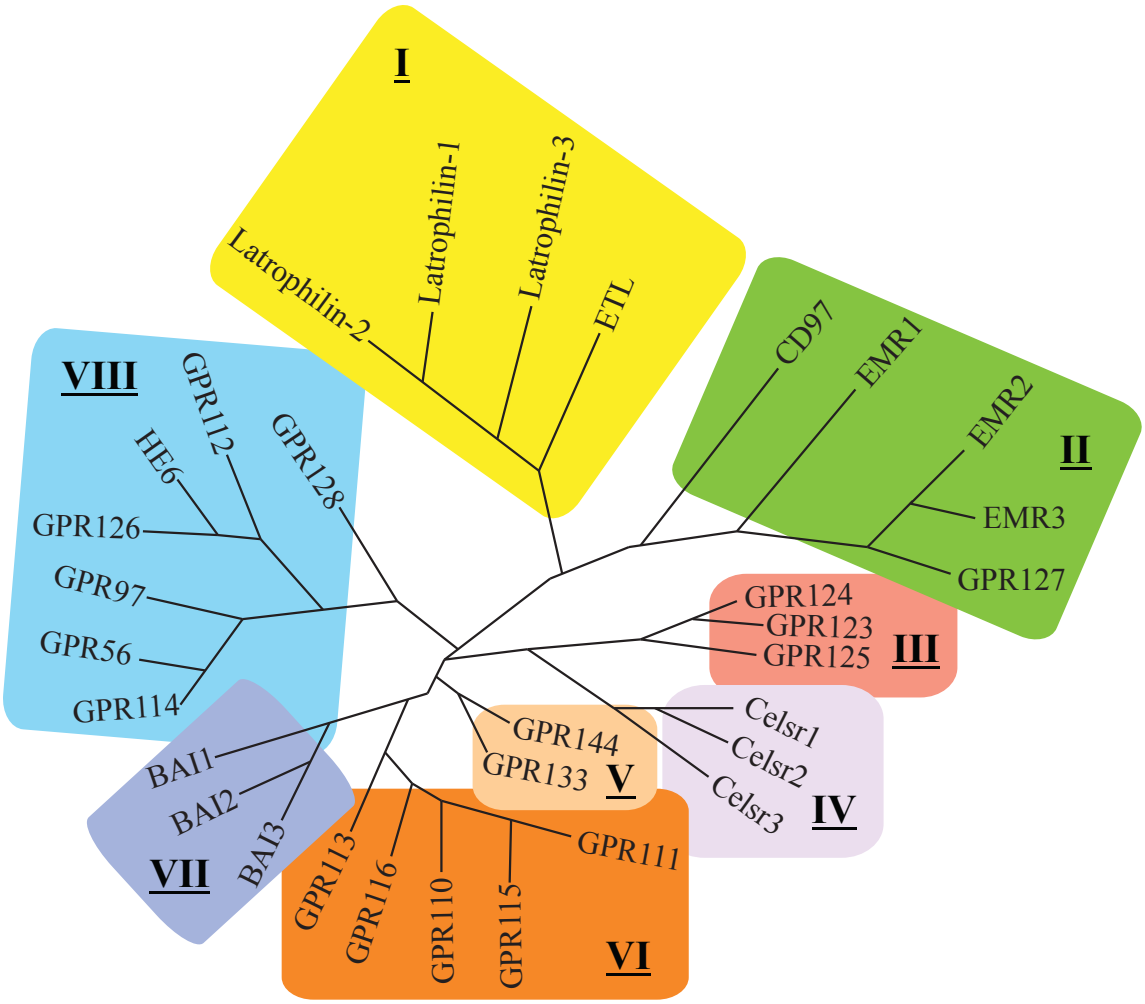
1.4 Adhesion G protein-coupled receptors

The adhesion family of GPCRs includes several dozen orphan receptors that are considered class B GPCRs in the classical system but comprise their own family in the newer GRAFS system (Bjarnadottir et al., 2007; Paavola and Hall, 2012; Yona et al., 2008b). In the past, the adhesion GPCRs have been referred to by various nomenclatures, including LN-TM7 referring to the 7-transmembrane region associated with an extremely long N-terminus ("LN"). These receptors have also been called EGF-TM7 to reflect the presence of epidermal growth factor domains on the N-terminus of some receptors within the family (McKnight and Gordon, 1998), and B2/LNB-7TM indicating a homology to the secretin receptors (Harmar, 2001). More recently, the International Union of Basic and Clinical Pharmacology (IUPHAR) has initiated talks with the Adhesion-GPCR Consortium to unify the nomenclature within the adhesion GPCR field, a process that is currently underway (unpublished communication). There are 33 members of the adhesion G protein-coupled receptors encoded by the human genome (Bjarnadottir et al., 2004), although others species have considerably more, including the *Strongylocentrotus purpuratus* (sea urchin) genome, which contains over

90 adhesion GPCRs (Whittaker et al., 2006). Interestingly, adhesion GPCRs are also found in Fungi, and are believed to have evolved from the Class E cAMP receptor proteins (Krishnan et al., 2012).

The adhesion GPCRs can be further divided into 8 subfamilies (I-VIII), shown in Figure I-3, based on homology of the 7TM regions and functional domains located on the N-termini (Bjarnadottir et al., 2004). These families include the latrophilins (I), the EGF-containing receptors (II), the cadherin EGF LAG seven-pass G-type receptors (Celsr) family (IV) and the brain-specfic angiogenesis inhibitor (BAI) family (VII), among others. The several dozen members of the adhesion GPCR family are expressed in very different patterns throughout the body, with some individual receptors being highly localized to specific tissues. For example, Celsr1-3 are predominantly expressed in the central nervous system (Tissir et al., 2002), whereas, the human epididymal protein 6 (HE6) is specifically expressed in the epididymis of the testis (Osterhoff et al., 1997). As previously mentioned, selective tissue expression of GPCRs is one feature that makes GPCRs excellent targets for therapeutic intervention, and thus the highly discrete tissue distributions of certain adhesion GPCRs makes these receptors especially attractive as potential drug targets.

Figure I-3: Phylogenic tree of the adhesion GPCR family. Representative phylogenic analysis of the 8 subfamilies of the adhesion GPCR family is shown, image modified from Bjarnadottir TK, Fredriksson R, Hoglund PJ, Gloriam DE, Lagerstrom MC and Schioth HB (2004) The human and mouse repertoire of the adhesion family of G-protein-coupled receptors. *Genomics* **84**(1): 23-33 (Bjarnadottir et al., 2004). VLGR1 is not shown.


I
Latrophilin-2
Latrophilin-1
Latrophilin-3
ETL
II
CD97
EMR1
EMR2
EMR3
GPR127
III
GPR124
GPR123
GPR125
IV
Celsr1
Celsr2
Celsr3
V
GPR144
GPR133
VI
GPR111
GPR113
GPR116
GPR110
GPR115
VII
BAI1
BAI2
BAI3
VIII
GPR112
HE6
GPR128
GPR126
GPR97
GPR56
GPR114

1.5 Adhesion G protein-coupled receptor structure

Adhesion GPCRs possess a unique architecture compared to most classical GPCRs, and are characterized by extremely long N-termini including a conserved GPCR proteolysis site (GPS motif) which results in autoproteolytic cleavage of the N-terminus, separating it from the 7TM region (Langenhan et al., 2013). The N-termini of adhesion GPCRs are heavily glycosylated and contain a wide variety of adhesion domains that are known to play roles in cellular adhesion, including EGF-like repeats, lectin-like repeats, cadherins, immunoglobulin (Ig), and thrombospondin repeats (Langenhan et al., 2013; Yona et al., 2008b). One of the defining characteristics of adhesion GPCRs is the presence of a GPCR-autoproteolysis inducing (GAIN) domain, which is an evolutionarily highly conserved region that contains within it an integral GPS motif (Arac et al., 2012b; Promel et al., 2013). The GAIN domains functions as the site of autoproteolysis within the adhesion GPCRs, separating the N-termini of the receptors from the 7TM regions. The GPS motif is an approximately 40 amino acid sequence within the much larger GAIN domain, which encompasses approximately 320 residues.

The GPS motif includes a conserved catalytic serine/threonine residue, which performs a nucleophilic attack on the carbonyl carbon of the peptide backbone, resulting in autoproteolytic cleavage (Deyev and Petrenko, 2010; Promel et al., 2013; Wei et al., 2007). The conserved consensus sequence for the cleavage site within the GPS is a His/Arg at the -2 position, followed by a Leu/Met/Ile at -1 and a Thr/Ser at the +1 position, with proteolysis occurring between the -1 and +1 residues. The catalytic mechanism of autoproteolytic cleavage at the GPS of adhesion GPCRs was first

determined in EMR2 (Lin et al., 2004) and was confirmed in the GPS-containing polycystic kidney disease 1 (PKD-1), which is not a GPCR (Wei et al., 2007). It was also shown for EMR2 that the proteolytic event occurs in the endoplasmic reticulum and the NT and 7TM regions of the receptor remain non-covalently associated and traffic to the plasma membrane as a heterodimer (Lin et al., 2004). The ability of the N-terminus to remain with the 7TM regions has been experimentally shown for a handful of other adhesion GPCRs including CD97 (Gray et al., 1996), CIRL (Krasnoperov et al., 2002; Volynski et al., 2004), GPR56 (Paavola et al., 2011) and BAI2 (Okajima et al., 2010).

Mutations within the GPS motif inhibit the ability of the adhesion receptors to undergo autoproteolytic cleavage, which for certain receptors can cause aberrations in protein folding and trafficking (Jin et al., 2007). It should also be noted that mutations outside of the conserved GPS motif can also inhibit autoproteolysis, likely due to improper folding, indicating a certain conformation is necessary for proper autoproteolytic cleavage (Chang et al., 2003). As will be discussed later, the idea that autoproteolytic cleavage at the GPS motif is necessary for proper trafficking to the plasma membrane does not appear to apply to the entire family of adhesion GPCRs.

Although a number of studies have shown an association of adhesion GPCR N-termini with their cognate 7TM regions following autoproteolytic cleavage, the biological effects of this association remain mysterious for the most part. Questions that are currently being addressed for various adhesion GPCRs include whether the NT and 7TM regions function independently from each other and whether the dynamic interplay between the two receptor fragments regulate each other. Using EMR2 as an example, the NT and 7TM regions can exist independently of each other at the plasma membrane and

remain inactive when isolated; however, when these regions interact, they translocate to lipid raft microdomains and form a functionally active receptor (Huang et al., 2012). Conversely, it has been shown for GPR56 (Paavola et al., 2011), CD97 (Ward et al., 2011) and BAI2 (Okajima et al., 2010) that following complete removal of the N-terminus, the signaling capabilities of the 7TM region are dramatically enhanced. This suggests a model wherein the 7TM region is in an inhibited state when associated with the NT, but separation of the two regions results in an active conformation of the 7TM and subsequent receptor signaling. In one study, the 7TM region of GPR56 co-immunoprecipitated with the NT region of latrophilin from homogenized rat brains, indicating cross-reactivity between N-terminal and 7TM regions of different adhesion receptors (Silva et al., 2009). This functional cross-reactivity between two distinct halves of adhesion GPCRs adds yet another complex layer of possible regulatory interactions that may contribute to the signaling and physiological effects of this receptor family. Understanding the complex nature of these receptors regarding the intricate relationship between the N-termini and the 7TM regions is crucial in deciphering the signaling capabilities and overall physiological roles of the adhesion GPCRs.

1.6 Adhesion G protein-coupled receptor signaling

Although the adhesion receptors belong to the GPCR superfamily, only a few adhesion GPCRs have actually been shown to couple to G proteins. The lack of known endogenous ligands for this receptor family has made uncovering the signaling pathways a difficult endeavor. Several methods have been utilized to circumvent this problem,

including the use of activating toxins and antibodies. For example, latrotoxin (LTX) is the main component in the venom of the black wider spider and was shown to exert its effects in part via interaction with the adhesion GPCR latrophilin-1 (Krasnoperov et al., 1997; Lelianova et al., 1997). Latrophilin-1 was shown to couple to both $G\alpha_q$ and $G\alpha_o$ upon interaction with LTX (Lelianova et al., 1997; Rahman et al., 1999). One caveat of this interaction is that LTX can also form a pore by integrating itself into the plasma membrane, complicating some of the interpretations. However, G protein coupling was still observed for latrophilin-1 using a mutant version of LTX that can still bind to the receptor but not insert itself into the plasma membrane to form a pore (Capogna et al., 2003; Volynski et al., 2003). Similarly, an antibody directed against the N-terminus of GPR56 was shown to enhance receptor coupling to $G\alpha_{12/13}$ leading to activation of the small GTPase Rho (Iguchi et al., 2008). Further studies on GPR56 revealed that removing the N-terminus also resulted in constitutive activation of the Rho pathway via $G\alpha_{12/13}$ (Paavola et al., 2011). Receptors that couple to $G\alpha_{12/13}$ can also typically couple to $G\alpha_{q/11}$ or other G proteins (Riobo and Manning, 2005), and this phenomenon is also seen in the adhesion family, as GPR56 is capable of interacting with $G\alpha_{q/11}$ via co-immunoprecipitation (Little et al., 2004). However, the ability of GPR56 to elicit activation of this pathway remains to be determined. Similarly CD97 activates the Rho pathways via coupling to $G\alpha_{12/13}$ (Ward et al., 2011) and both GPR133 (Bohnekamp and Schoneberg, 2011) and GPR114 (Gupte et al., 2012) have been shown to couple to $G\alpha_s$ to increase cAMP production. Likewise, GPR126 was also shown to increase cAMP levels likely via $G\alpha_s$, and this signaling is crucial for myelination induced by Schwann cells (Monk et al., 2009). GPR97 has the ability to couple to a chimeric $G\alpha_o$ and signal

constitutively when overexpressed in HEK293 cells (Gupte et al., 2012). In studies utilizing the promiscuous G protein, $G\alpha_{16}$, BAI2 was able to promote enhanced signaling to NFAT, indicating an ability to couple to G proteins (Okajima et al., 2010). Two other members of the BAI1 family were shown to signal in G protein-independent mechanisms. BAI1 and BAI3 have been shown to activate the Rac pathway via C-terminal interaction with ELMO/Dock (Lanoue et al., 2013; Park et al., 2007) and BAI1 was also shown to activate Rac through PDZ interactions with Tiam1 (Duman et al., 2013). The signaling pathways utilized by the BAI family members of the adhesion GPCRs will be discussed in more detail later.

The growing evidence for the ability of adhesion GPCRs to signal via G protein-dependent and independent mechanisms is compelling, although much work is still necessary to elucidate these pathways. The continued progress towards understanding the signaling mechanisms utilized by this receptor family should help to further uncover the physiological roles they are performing. Likewise, advances in understanding adhesion GPCR signaling will help advance the search for endogenous ligands and synthetic agonists for these receptors.

1.7 Physiological roles of adhesion G protein-coupled receptors

The adhesion G protein-coupled receptors have been shown to play roles in a variety of physiological processes including immune function, development, angiogenesis, and sensory function. For example, the members of the EMR sub-family of adhesion GPCRs are heavily involved in the development and activation of white

blood cells (Kwakkenbos et al., 2005; Stacey et al., 2003; Yona et al., 2008a). EMR2 specifically has been shown to induce proinflammatory cytokine production by monocytes (Huang et al., 2012) and play a major role in neutrophil migration (Yona et al., 2008a). Moreover, CD97, another member of the EMR family, can bind to CD55 and stimulate T-cell proliferation and production of IL-10, an inflammatory cytokine (Capasso et al., 2006).

Various adhesion GPCRs have also been implicated in development. For example, GPR126 is critical for myelination mediated by Schwann cells in zebrafish, and genetic knockout of GPR126 results in incomplete myelination of the peripheral nervous system along with peripheral nerve defects (Monk et al., 2009; Monk et al., 2011). Furthermore, the members of the Celsr family of adhesion GPCRs have emerged as regulators of planar cell polarity and brain development (Tissir et al., 2005; Tissir et al., 2010). Double mutation of Celsr2 and Celsr3 in mice results in abnormal ciliation of ependymal cells and a hydrocephalic phenotype (Tissir et al., 2010). GPR56 also plays a crucial role in brain development, as mutations of this receptor result in the human disease bilateral frontoparietal polymicrogyria, which presents with mental retardation, motor deficits and seizures (Piao et al., 2005; Piao et al., 2004). Mice lacking GPR56 exhibit developmental abnormalities of the pial membrane resulting in a cobblestone-like phenotype of the cortex due to overmigration of neuronal progenitor cells (Li et al., 2008). GPR56 is present on neuronal progenitor cells and activation of this receptor results in inhibition of migration via activation of the Rho pathway (Iguchi et al., 2008). Finally, the adhesion G protein-coupled receptor very large G protein-coupled receptor -1 (VLGR1) is important in the physiology of both the inner ear and the retina and

mutations to this receptor underlie Usher's Type II disease (Maerker et al., 2008; McGee et al., 2006; Weston et al., 2004). Usher's syndrome is the most common form of combined deafness-blindness and up to 10 genes have been associated with three subtypes of Ushers syndrome, with one of those genes being VLGR1 (Weston et al., 2004).

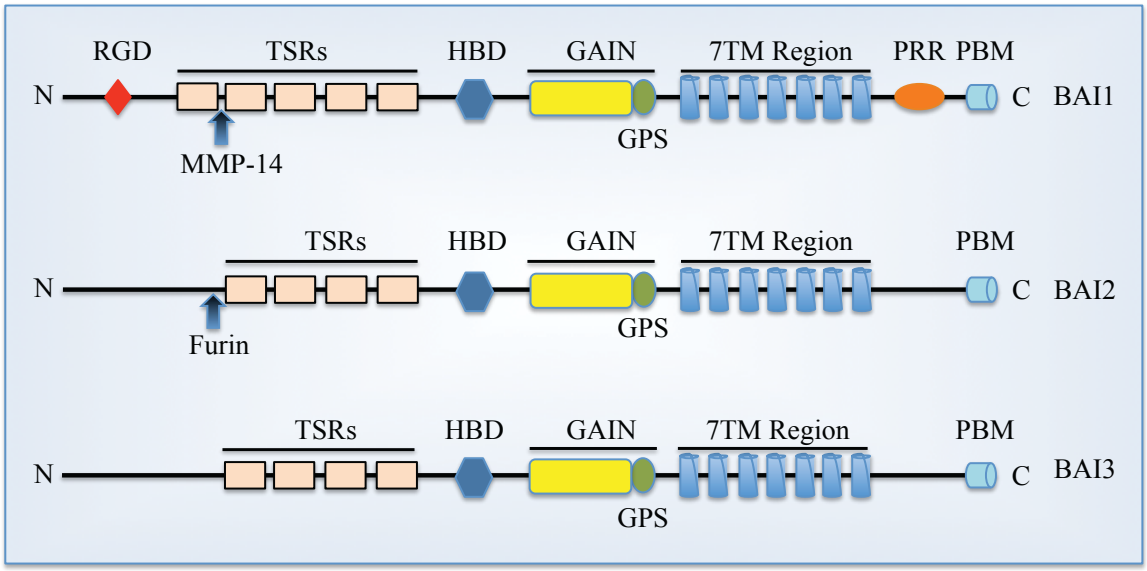
Various adhesion GPCRs have also been implicated in angiogenesis and tumor formation. In most of these cases, interactions of the N-termini of the receptors with integrins have been shown to be important. For example, CD97 interacts with integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ to promote tumor angiogenesis (Wang et al., 2005). GPR124 also interacts with $\alpha_v\beta_3$ to stimulate endothelial angiogenesis, and interestingly this receptor is upregulated during tumor formation (Carson-Walter et al., 2001; Vallon and Essler, 2006). The N-terminus of BAI1 can inhibit endothelial cell proliferation via blocking $\alpha_v\beta_5$ integrins (Koh et al., 2004) and also inhibits angiogenesis through N-terminal interactions with the pro-apoptotic scavenger receptor CD36 (Kaur et al., 2009).

Clearly, adhesion GPCRs are involved in a wide variety of physiological functions and the physiological roles of these receptors are only beginning to be uncovered. Many questions still remain, including identification of ligands for these receptors, elucidation of the signaling pathways utilized, and identification of the mechanisms underlying receptor regulation. As these questions continue to be addressed, novel small molecules may be designed to modulate these receptors, with these synthetic ligands serving both as tools to further dissect the functions of these receptors and as potential novel therapeutics.

1.8 Brain-specific angiogenesis inhibitor subfamily

An intriguing subfamily of adhesion GPCRs is the trio of brain-specific angiogenesis inhibitors: BAI1, BAI2 and BAI3 (Cork and Van Meir, 2011; Park and Ravichandran, 2010). The members of the BAI subfamily contain a variety of conserved domains on both their N-terminal and C-terminal regions (Figure I-4). For example, the N-termini of the BAI subtypes each contain multiple thrombospondin type 1 repeats (TSRs), one hormone-binding domain (HBD) each and one GAIN domain. The BAI1 N-terminus also features an integrin-binding RGD (Arg-Gly-Asp) motif in addition to its five TSRs, whereas the BAI2 and BAI3 N-termini do not possess an RGD motif and have four TSRs. The C-terminus of each BAI subtype ends with a PDZ-binding motif, QTEV (Gln-Thr-Glu-Val). The C-terminus of BAI1 also contains a proline-rich region (PRR), which can bind to Src homology 3 (SH3) domains and WW domains (Kay et al., 2000).

Figure I-4: Schematic representation of the three BAI family members of Adhesion GPCRs. The major domains found in each receptor are shown, and known proteolytic events are also indicated. Abbreviations: PBD, PDZ-binding motif; PRR, proline-rich region; 7TM, 7-transmembrane regions; GPS, GPCR proteolytic side; GAIN, GPCR autoproteolysis-inducing domain; HBD, hormone-binding domain; TSR, thrombospondin type 1 repeats; RGD, Arg-Gly-Asp integrin-binding motif


RGD
TSRs
HBD
GAIN
7TM Region
PRR
PBM
N
C
BAI1
MMP-14
GPS
TSRs
HBD
GAIN
7TM Region
PBM
N
C
BAI2
Furin
GPS
TSRs
HBD
GAIN
7TM Region
PBM
N
C
BAI3
GPS

The BAI subtypes are widely expressed in both fetal and adult brain tissue (Shiratsuchi et al., 1997). BAI1 mRNA is found at high levels in the cerebral cortex, the hippocampus, olfactory bulb, thalamic nuclei and basal ganglia (Koh et al., 2001; Mori et al., 2002; Sokolowski et al., 2011). During development, transcripts for murine BAI1 and BAI2 mRNA peak at postnatal day 10, whereas BAI3 mRNA peaks 1 day after birth (Kee et al., 2004). BAI2 is more ubiquitously expressed than BAI1 during development, exhibiting expression in brain, skin, kidney, skeletal muscle and thymus, but is largely limited to the brain after birth. In contrast, BAI3 is more limited to the central nervous system in all developmental stages, with very low levels also observed in lung, skeletal muscle and testis (Kee et al., 2004; Kee et al., 2002). BAI1 protein is found in neurons, astrocytes, microglia, and macrophages, with the most robust expression observed in neurons and astrocytes (Duman et al., 2013; Mori et al., 2002; Park et al., 2007; Sokolowski et al., 2011). BAI3 is known to be expressed in hippocampal neurons (Lanoue et al., 2013), but the expression profile for BAI2 and BAI3 across different cell types has not yet been characterized as fully as for BAI1.

1.9 Autoproteolysis of brain-specific angiogenesis inhibitor subfamily

One of the defining characteristics of adhesion GPCRs is the presence of a GAIN domain, which is an evolutionarily-conserved region that contains an integral GPS motif (Arac et al., 2012b; Promel et al., 2013). The ability of the BAI subfamily to undergo autoproteolysis appears to be cell-specific, as BAI1 and BAI3 do not readily undergo proteolytic cleavage in HEK293T cells (Arac et al., 2012b; Park and Ravichandran, 2010;

Stephenson et al., 2013). Interestingly, BAI1 has been shown to undergo cleavage at the GPS motif in human malignant glioma cells (Kaur et al., 2005; Kaur et al., 2009) and all three BAI family members are autoproteolytically cleaved in mouse brain lysates (Arac et al., 2012b; Okajima et al., 2010). These findings indicate a possible role for regulatory factors, present in neurons and glia but absent in HEK-293T cells, which modulate the BAI subtypes such that autoproteolytic cleavage can occur. It should be noted that although BAI1 and BAI3 do not readily undergo autoproteolysis in HEK293T cells, these receptors are still efficiently trafficked to the plasma membrane (Arac et al., 2012b; Stephenson et al., 2013), indicating that proteolysis at the GPS motif is not required for proper surface trafficking. These observations are intriguing because, as mentioned earlier, mutations in the GAIN domains of other adhesion GPCRs can cause protein misfolding and improper trafficking, which in some cases results in human disease (Chiang et al., 2011; Jin et al., 2007; Ke et al., 2008; Krasnoperov et al., 2002; Piao et al., 2004).

Upon proteolysis at the GAIN domain, the N-terminus of BAI1 becomes a 120-kDa protein termed Vasculostatin-120, which has been extensively studied with regard to its ability to inhibit angiogenesis and tumor formation (Cork and Van Meir, 2011; Kaur et al., 2005; Kaur et al., 2009). Further cleavage occurs upstream of the GAIN domain by matrix metalloproteinase 14 to produce a 40-kDa fragment, Vasculostatin-40, which also possesses anti-angiogenic properties (Cork et al., 2012). BAI1 was initially identified in a screen for targets of the p53 tumor suppressor (Nishimori et al., 1997), but was subsequently shown to be down-regulated in glioblastoma multiforme via epigenetic regulation independently of p53 expression (Kaur et al., 2003; Zhu et al., 2011). The

anti-angiogenic effects of the BAI1 N-terminus appear to be primarily mediated through interactions of the TSRs with the scavenger receptor CD36, which induces pro-apoptotic signaling upon binding of the BAI1 TSRs (Kaur et al., 2009). This is further regulated by histidine-rich glycoprotein, which can bind to the BAI1 N-terminus and block its interaction with CD36, which allows for pro-angiogenic pathways to predominate (Klenotic et al., 2010).

1.10 Physiological roles of brain-specific angiogenesis inhibitor subfamily

As mentioned earlier, the BAI subtypes possess multiple TSRs on their N-termini. TSRs were first identified as regions of thrombospondin-1 that mediate the anti-angiogenic activity of this secreted protein (de Fraipont et al., 2001). Thus, much of the early research on the BAI subtypes revolved around the ability of these receptors to inhibit experimental angiogenesis and tumor formation (Duda et al., 2002; Kang et al., 2006; Kaur et al., 2005; Nishimori et al., 1997). Restoration of BAI1 can inhibit the growth of tumors derived from gliomas and renal cell carcinomas (Izutsu et al., 2011; Kaur et al., 2009; Kudo et al., 2007; Xiao et al., 2006; Yoon et al., 2005). Moreover, expression of the isolated BAI1 N-terminus (Vasculostatin-120) and the association of its TSRs with CD36 can mimic the anti-angiogenic effects of full-length BAI1 (Cork and Van Meir, 2011; Kaur et al., 2005; Kaur et al., 2009). Thus, the anti-angiogenic action of BAI1 parallels the classical anti-angiogenic action of thrombospondin-1, which interacts with CD36 to induce apoptosis in endothelial cells and thereby inhibit angiogenesis (Dawson et al., 1997; Jimenez et al., 2000).

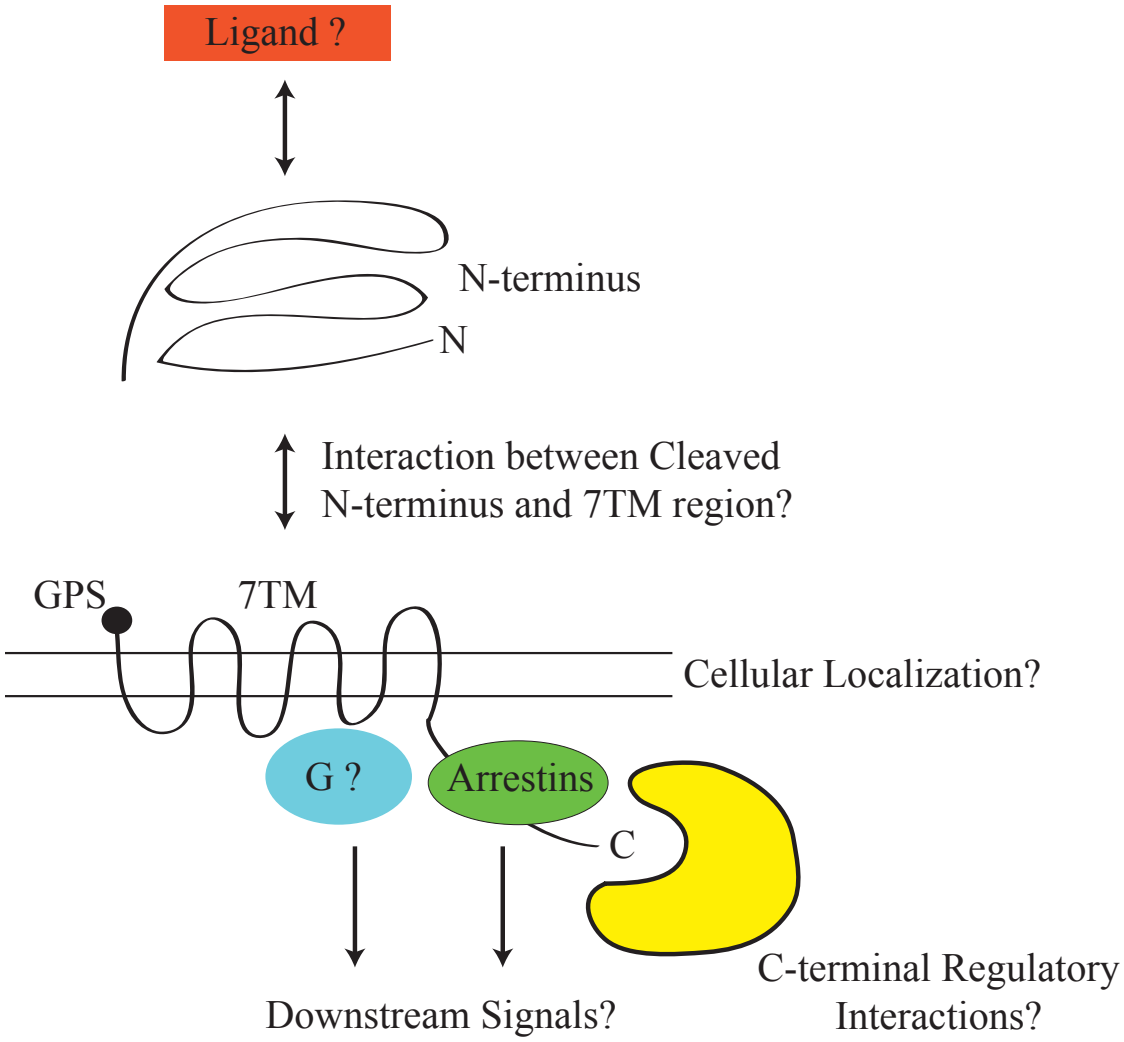
In addition to its role in regulating angiogenesis, a novel role for BAI1 was uncovered following identification of an interaction between the BAI1 C-terminus and the Rac-GEF ELMO-Dock180, a conserved signaling complex known to be important in promoting the internalization of apoptotic cells (Park et al., 2007). BAI1 was identified as a receptor upstream of this signaling module and a key player in the engulfment of cells that have undergone apoptosis by macrophages. One classical feature of apoptotic cells is the exposure of phosphatidylserine on the outer leaflet of the cell membrane (Fadok et al., 1992). The TSRs on the BAI1 N-terminus were found to interact with exposed phosphatidylserine on apoptotic debris, thereby eliciting the activation of the Rac signaling pathway (Park et al., 2007). Activated Rac is known to promote cytoskeletal rearrangement via actin polymerization, allowing for the engulfment and internalization of apoptotic cell debris by macrophages (Gumienny et al., 2001). The ability of BAI1 to bind to externalized phosphatidylserine on apoptotic cells has also been shown to be important for myoblast fusion, as genetic deletion of BAI1 was observed to reduce the size of myofibers and impair muscle regeneration *in vivo* (Hochreiter-Hufford et al., 2013). Additionally, the BAI1 TSRs have been shown to bind to lipopolysaccharides on Gram-negative bacteria to mediate bacterial phagocytosis (Das et al., 2011). Interestingly, a key feature shared by the processes of phagocytosis and angiogenesis is that they are both known to be highly regulated by thrombospondin interactions with CD36 (Silverstein and Febbraio, 2009). Thus, although it has not yet been determined if CD36 plays a role in BAI1-mediated regulation of phagocytosis, it is possible that BAI1 regulates both angiogenesis and phagocytosis via the action of the five

TSRs on the BAI1-NT in a manner that parallels the regulation of angiogenesis and phagocytosis by thrombospondin-1.

1.11 Aim of dissertation research

The primary aims of my dissertation research were to elucidate the signaling pathways downstream of brain-specific angiogenesis inhibitor-1 and also identify mechanisms by which the receptor's signaling is regulated. In this work, my colleagues and I mainly utilized a heterologous overexpression system to study the various downstream signaling pathways activated by BAI1, including both G protein-dependent and -independent signaling and the functional importance of the receptor's cleaved N-terminus. We also uncovered various C-terminal interactions that play regulatory roles with regards to BAI signaling. Furthermore, we explored the cellular localization of BAI1 and the identification of a possible ligand. Findings from my dissertation research indicate that BAI1 is a synaptic receptor that can activate both the Rho and ERK pathways, and the N-terminal and C-terminal regions of BAI1 play major roles in the regulation of signaling activity. A schematic representation of unknown aspects of BAI1 function that were addressed by the work presented in this dissertation is shown in Figure I-5.

Figure I-5: Schematic representation of unknown aspects of BAI1 function addressed in this dissertation. Areas of BAI1 signaling and regulation that were explored in the work described in this dissertation include; functional importance of the receptor's cleaved N-terminus, G protein-mediated signaling, non-G-protein-mediated signaling, regulatory C-terminal interactions, cellular localization of BAI1, and identification of a possible ligand.


Ligand ?
N-terminus
N
Interaction between Cleaved
N-terminus and 7TM region?
GPS
7TM
Cellular Localization?
G ?
Arrestins
C
Downstream Signals?
C-terminal Regulatory
Interactions?

CHAPTER II: BAI1 activation of Rho via G proteins is regulated by the receptor's N-terminus

2.1 Introduction

As previously mentioned, several adhesion GPCRs are known to undergo autoproteolytic cleavage at the GPS motif, with the N-terminal and 7TM regions retaining the ability to remain associated for a certain amount of time. It has been shown for a few of the adhesion receptors that the signaling capabilities of the 7TM region are enhanced following removal of the N-terminus, and a model has been proposed based on these findings suggesting that the NT locks the 7TM region in an inhibited state and upon removal of the NT, the 7TM region undergoes a conformational change allowing for signal transduction (Paavola and Hall, 2012). In the present study, we sought to gain insight into the ability of BAI1 to undergo autoproteolysis in HEK293T cells and native mouse brain tissue. We found that BAI1 readily undergoes autoproteolytic cleavage in native brain tissue homogenate, but this proteolytic event appears to be largely absent when the receptor is transfected into HEK293T cells. We also probed whether the BAI1 N-terminus can associate with the 7TM region, BAI1-ΔNT, when co-transfected into HEK293T cells and found that the two halves of BAI1 do in fact associate.

Regarding BAI subfamily signaling capabilities, BAI2 was found to promote NFAT activation via a promiscuous G protein, $G\alpha_{16}$, revealing an ability of this receptor to couple to G proteins (Okajima et al., 2010). In addition to the G protein-dependent signaling that has been demonstrated for BAI2, several G protein-independent pathways have also been established for the BAI family. The ability of BAI1 to exert effects on intracellular signaling pathways was first shown in studies that identified an interaction between the C-terminus of BAI1 and the intracellular adaptor protein ELMO (Park et al.,

2007). ELMO can interact with Dock180 to form a functional guanine nucleotide exchange factor (GEF), which activates the small GTPase Rac by facilitating the exchange of GDP for GTP (Brugnera et al., 2002; Lu and Ravichandran, 2006). BAI3 can also interact with ELMO-Dock180 to activate the Rac pathway (Lanoue et al., 2013). More recently, BAI1 was shown to stimulate Rac via a mechanism independent of either classical G proteins or the ELMO-Dock180 complex, as the PDZ binding motif of BAI1 was found to interact with the PDZ domain of the Rac-GEF Tiam1 and the polarity protein Par3 (Duman et al., 2013). BAI1 was shown to enhance the synaptic localization of the Tiam1/Par3 complex, thereby leading to increased Rac activation in cultured hippocampal neurons.

Since G protein-dependent signaling for BAI1 remains unknown, we assessed the signaling capabilities of full-length BAI1 and BAI1-ΔNT, a construct lacking the N-terminus at the GPS motif. Specifically, we examined whether these constructs can couple to G proteins and whether the large BAI1 N-terminus might regulate the signaling activity of these receptors. We found that BAI1 couples to $G\alpha_{12/13}$ to activate Rho, and removal of the BAI1 N-terminus results in enhanced receptor signaling to Rho and upregulation of endogenous Cyr61.

2.2 Experimental procedures

Antibodies- Antibodies against HA (Roche), myc (Sigma-Aldrich), BAI1 (Thermo Scientific), and Cyr61 (abcam) were purchased from the manufacturers. A distinct anti-BAI1-CT antibody (#17108) was custom-made by Pocono Rabbit Farm & Laboratory (Canadensis, PA) via injection of rabbits with a peptide (HSLTLKRDKAPKSS) derived from the human BAI1 C-terminus (amino acids 1305-1318), followed by affinity purification.

Cell Culture and transient plasmid transfections- HEK293T cells (ATCC) used for cell-based assays were cultured and maintained in complete media (DMEM containing 10% FBS and 1% penicillin/streptomycin) at 37 °C at 5% CO_2. Transfections were performed by incubating cells plated on 100mm x 20mm Cell Culture Dishes (Corning) with Lipofectamine 2000 (Invitrogen) and plasmid DNA for 3-5 hours in serum free DMEM. The transfection reaction was stopped by addition of complete media. Experiments were performed 24 hours post-transfection.

Plasmids- Expression vectors for BAI1 wild-type and Myc-BAI1-NT (myc-Vstat120) were previously published (Kaur et al., 2003; Kaur et al., 2005). The BAI1-ΔNT construct was cloned into pcDNA3.1 by creating primers for human BAI1 starting at amino acid 929 and ending at 1584. Primers used for PCR were 5'-GCTAGCATGTTCGCCATCTTAGCCCAGCTC-3' and 5'-AAGCTTTCAGACCTCGGTCTGGAGGTCGAT-3'. The RGSp115 plasmid was a gift from Tohru Kozasa (University of Illinois at Chicago). The GST-RBD (Addgene) and

HA-Rho (Missouri S&T cDNA Resource Center) expression vectors were all obtained from commercial sources.

Western Blot- Samples were run on 4-20% SDS-PAGE gels (Invitrogen) for 2 hours at 130 V and then transferred to a nitrocellulose membrane (Bio-Rad) for 2 hours at 30 V. The membrane was blocked in blot buffer containing 2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM Hepes, at PH 7.4 for 30 minutes at room temperature. The membrane was incubated with primary antibody in blot buffer for 1 h at room temperature, followed by three 5 min washes. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (GE Healthcare) for 30 minutes at room temperature followed by three 5 min washes with blot buffer. Blots were visualized via enzyme-linked chemiluminescence using the Supersignal® West Pico Chemiluminescent Substrate (Pierce).

Preparation of mouse brain homogenate- Brain tissue was extracted from adult mice and homogenized in ice-cold buffer containing 20mM HEPES pH 7.4, 50 mM NaCl, 1 mM EDTA and a protease inhibitor cocktail tablet (Roche Diagnostics). The resulting homogenate was then centrifuged at 2,000 x g for 10 minutes at 4 °C to remove nuclei and cell debris. Membrane proteins were extracted in Tris Buffer (50mM Tris, pH 7.4, 5 mM $MgCl_2$, 1mM EGTA, 150 mM NaCl, 1mM EDTA, one protease inhibitor cocktail tablet and 1% Triton X-100 for 3 hours at 4 °C and debris was cleared by centrifugation.

Co-Immunoprecipitation- Expression vectors for proteins of interest were transfected into HEK293T cells as described above. After 24 hours, cells were resuspended in 500 μL Lysis Buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10mM $MgCl_2$, 1 mM EDTA, 1 protease inhibitor cocktail tablet (Roche Diagnostics) and 2% glycerol]. Cells were rotated in lysis buffer for 30 minutes at 4° C then cell debris was cleared by centrifugation. Soluble lysates were incubated for 60 minutes with 30 μL of protein A/G beads (Thermo Scientific) with corresponding antibody (3 μL) to the protein being immunoprecipitated. Beads were washed 3 times with Lysis Buffer, resuspended in 40 μL 2x sample buffer and heated to 95 °C for 10 minutes.

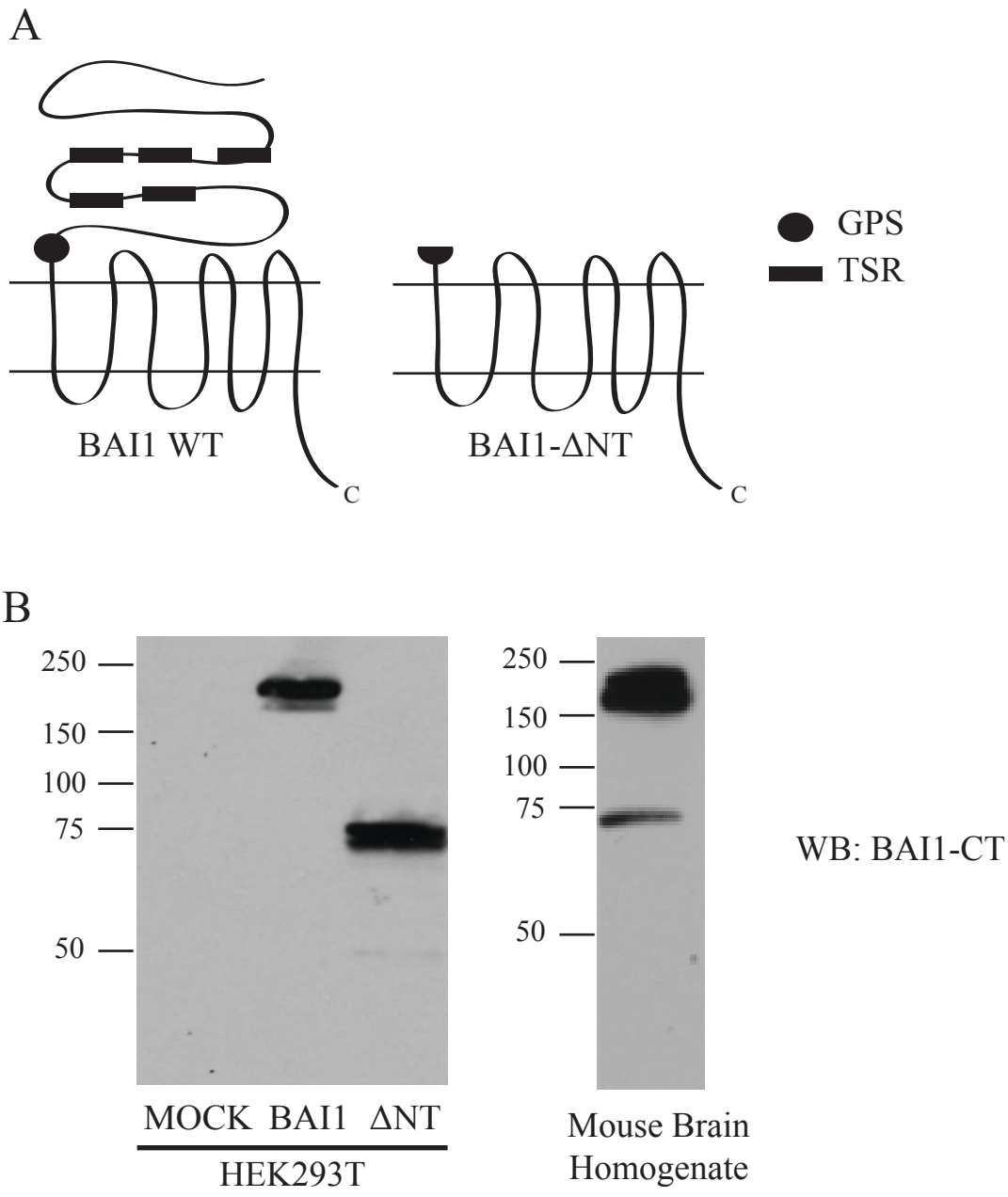
Rhotekin Rho Activation Assay- HEK293T cells were co-transfected with expression plasmids for HA-Rho and either pcDNA3.1, BAI1 wild-type, BAI1-ΔNT, or BAI1-ΔNTΔPDZ with or without RGSp115. As previously described (Paavola et al., 2011), 24 hours post-transfection, cells were resuspended in 500 μL Lysis Buffer. Cells were lysed by slowly rotating on a spinning wheel for 30 minutes at 4° C, then cell debris was cleared by centrifugation. Soluble cell lysates were incubated with 30 μL of GST-Rho Binding Domain (GST-RBD) of Rhotekin coupled to glutathione agarose beads (Sigma). Beads were washed 3 times with Lysis Buffer, resuspended in 40 μL 2x sample buffer and heated to 95 °C for 10 minutes. Active Rho was detected via the Western blot procedure described above. Images were quantified using Image J.

2.3 Results

2.3.1 BAI1 undergoes autoproteolysis in native brain tissue but not in HEK293T cells

Transfection of HEK293T cells with full-length BAI1 resulted in a major band at approximately 200 kDa, which is consistent with past reports that BAI1 does not readily undergo autoproteolysis at the GPS motif in HEK293T cells (Arac et al., 2012b; Park and Ravichandran, 2010). Thus, in order to explore the effect of the NT on signaling by the BAI1 7TM region, we created a truncated version of BAI1 ("ΔNT") lacking the NT region up to the predicted GPS cleavage site (Fig. II-1A). When this construct was transfected into HEK293T cells, which are devoid of endogenous BAI1, the expressed protein migrated at its predicted molecular weight of 71 kDa (Fig. II-1B, left panel). Conversely, when probing mouse brain homogenate with a mouse-specific BAI1 antibody (Thermo Scientific), a prominent, full-length band at approximately 200 kDa was observed, along with a lower band at 71 kDa, the predicted molecular weight of the cleaved 7TM region (Fig. II-1B, right panel). This is consistent with previous reports that BAI1 undergoes autoproteolytic cleavage in human malignant glioma cells (Kaur et al., 2005; Kaur et al., 2009) and all three BAI family members are autoproteolytically cleaved in mouse brain lysates (Arac et al., 2012b; Okajima et al., 2010).

Figure II-1: Expression pattern of BAI1 in HEK293T cells and native brain tissue. (A) Schematic drawing showing full-length BAI1 and the BAI1-ΔNT construct. Rectangles represent the thrombospondin-like repeats on the BAI1-NT and the circle represents the GPS motif. (B) Left panel: Lysates from HEK293T cells transiently transfected with either full-length BAI1 or BAI1-ΔNT were probed with anti-BAI1-CT antibody (Pocono Rabbit Farm & Laboratory). Right panel: Mouse brain homogenate was probed with an anti-BAI1-CT antibody (Thermo Scientific). The sizes of molecular weight markers are indicated in kDa.

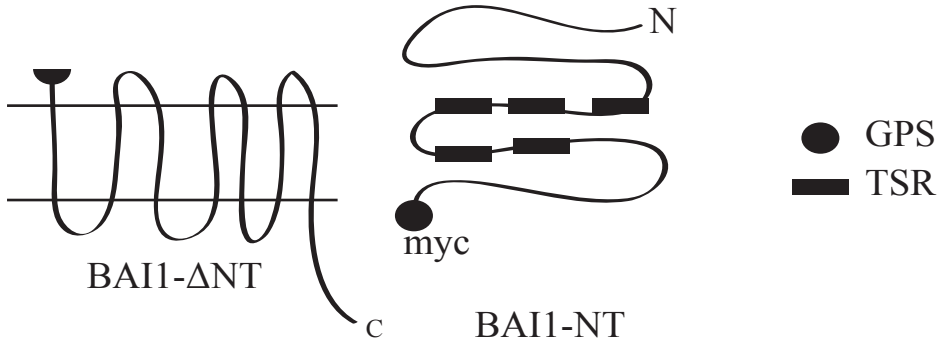

A
GPS
TSR
BAI1 WT
C
BAI1-ΔNT
C
B
250
150
100
75
50
MOCK
BAI1
ΔNT
HEK293T
250
150
100
75
50
Mouse Brain
Homogenate
WB: BAI1-CT

2.3.2 The N-terminus interacts with the 7-transmembrane region of BAI1 when co-expressed in HEK293T cells

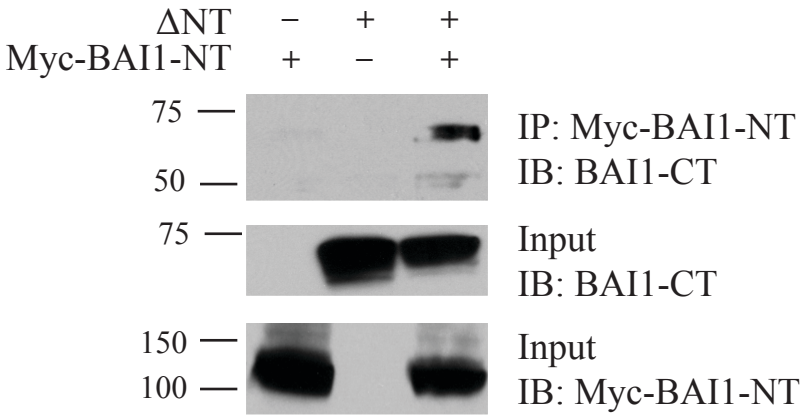
Although BAI1 appears to be cleaved at the GPS motif in brain tissue, it remains undetermined whether the two fragments of BAI1 can remain associated following cleavage of the receptor at the GPS, as has been shown for other adhesion GPCRs (cite references). To determine if the BAI1 7TM region can physically interact with the NT region (also known as Vstat120) following cleavage, we created a truncated version of BAI1 ("ΔNT") lacking the NT region up to the predicted GPS cleavage site (Fig. II-2A). To determine if the BAI1 NT and 7TM regions can interact, we performed co-immunoprecipitation studies utilizing the ΔNT construct and a myc-tagged version of the BAI1-NT (Fig. II-2A), which has been described previously (Kaur et al., 2005; Kaur et al., 2009). As shown in Figure II-2B, immunoprecipitation of Myc-BAI1-NT yielded co-immunoprecipitation of the co-expressed BAI1-ΔNT truncated mutant. These results indicate that the NT and 7TM fragments of BAI1 can physically associate, even when co-transfected into cells as separate constructs.

Figure II-2: the N-terminus of BAI1 associated with the 7TM region when co-transfected into HEK293T cells. (A) Schematic drawing showing the BAI1-ΔNT and the myc-tagged BAI1-NT construct. Rectangles represent the thrombospondin-like repeats on the BAI1-NT and the circle represents the GPS motif. (B) HEK293T cells were transiently transfected with a Myc-tagged expression vector for the N-terminus of BAI1 (Myc-BAI1-NT) in the absence or presence of a second expression vector for BAI1 lacking the N-terminus (BAI1-ΔNT). Immunoprecipitation (IP) was performed with an anti-myc antibody bound to Protein A/G beads. Co-immunoprecipitation of BAI1-ΔNT was detected via immunoblot (IB) with the anti-BAI1-CT antibody. Input lysates were examined as controls for protein amount and integrity.

A


N
GPS
TSR
myc
BAI1-ΔNT
C
BAI1-NT

B


ΔNT − + +
Myc-BAI1-NT + − +
75
50
IP: Myc-BAI1-NT
IB: BAI1-CT
75
Input
IB: BAI1-CT
150
100
Input
IB: Myc-BAI1-NT

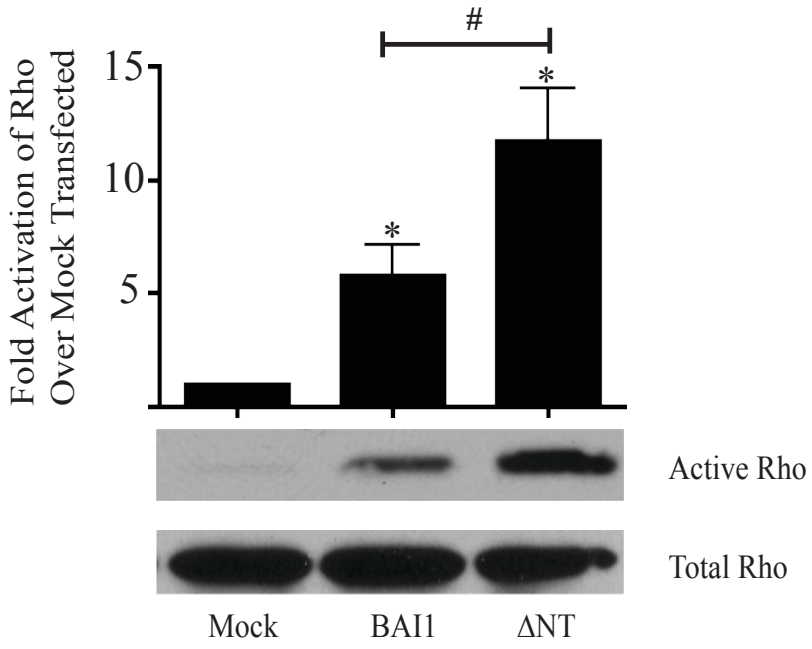
2.3.3 Removal of the BAI1 N-terminus enhances stimulation of Rho and Cyr61 expression via $G\alpha_{12/13}$

To determine whether BAI1 can couple to G proteins, we transfected HEK293T cells with full-length BAI1 and assessed its ability to activate G protein-dependent signaling cascades. Since other adhesion GPCRs, including GPR56 (Iguchi et al., 2008; Paavola et al., 2011) and CD97 (Ward et al., 2011), have been found to robustly couple to $G\alpha_{12/13}$ to activate Rho, we examined potential BAI1-mediated Rho signaling using a rhotekin pull-down assay (Fig. II-3A). This assay uses the Rho binding domain (RBD) of the Rho effector protein, rhotekin; the RBD motif binds specifically to the GTP-bound active form of Rho. When compared to cells transfected with empty vector ("mock"), a significant increase in Rho activation was observed upon transfection with full-length BAI1. Interestingly, transfection of cells with the BAI1-ΔNT mutant resulted in a significantly more robust activation of the Rho pathway than that observed with full-length BAI1 (Fig. II-3A). To determine if the activation of Rho by BAI1 was mediated via receptor coupling to $G\alpha_{12/13}$, co-transfection with the regulator of G protein signaling (RGS) domain of p115RhoGEF (RGSp115) was used as a dominant negative inhibitor for $G\alpha_{12/13}$-dependent signaling (Kozasa et al., 1998). RGSp115 specifically accelerates the GTPase activity of $G\alpha_{12/13}$, but not other G proteins, thereby selectively inhibiting $G\alpha_{12/13}$-mediated signaling (Fig. II-4). As shown in Figure II-3B, co-transfection with RGSp115 dramatically inhibited Rho activation by both full-length BAI1 and the BAI1-ΔNT mutant. To further explore the signaling capacities of both full-length BAI1 and the BAI1-ΔNT mutant, we looked at endogenous upregulation of the extracellular matrix-

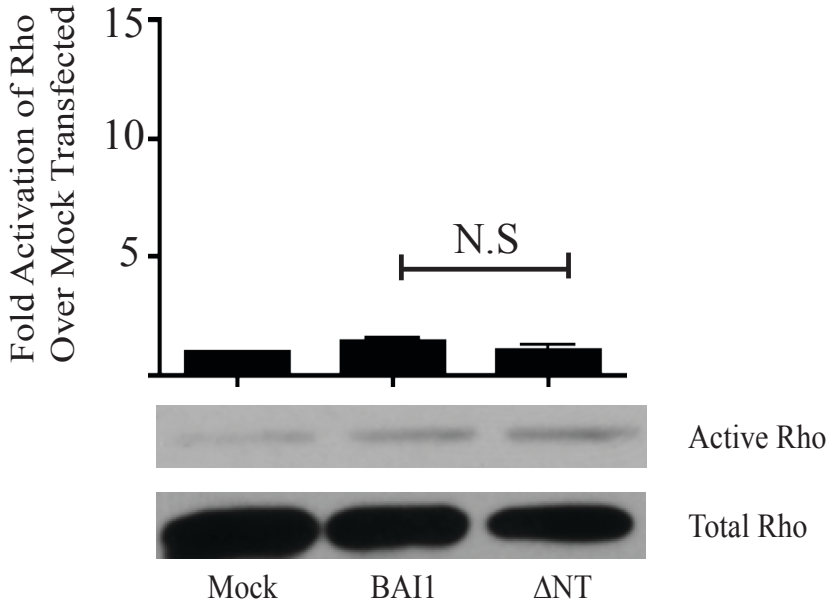
associated protein protein cysteine rich angiogenesis inducer-61 (Cyr61). Cyr61 is involved in a variety of biological activities including angiogenesis, apoptosis, and cellular senescence, mediated mainly through interactions with various integrins (Lau, 2011). Moreover, it has been established that GPCRs that activate Rho also lead to a significant increase of Cyr61, making it an attractive downstream response to examine with regards to both full-length BAI1 and the BAI1-ΔNT mutant signaling capacities (Pendurthi et al., 2002; Sakamoto et al., 2004). As shown figure II-4C, overexpression of both full-length BAI1 and the BAI1-ΔNT mutant resulted in increased expression levels of Cyr61 when compared to mock transfected HEK293T cells. Furthermore, removing the N-terminus of BAI1 resulted in an even greater endogenous increase in Cyr61 compared to full-length receptor, mirroring the data observed for Rho activation.

Figure II-3: Removal of the BAI1 N-terminus enhances Rho activation via $G\alpha_{12/13}$ and endogenous Cyr61 in HEK293T cells. (A) Quantification of active RhoA via pull-down with GST-RBD, a recombinant GST fusion protein corresponding to the Rho binding domain of Rhotekin (*, indicates comparison to mock-transfected; #, indicated $p<0.05$, n=6, one-way ANOVA) . *Top Panel:* Western blot analysis of active RhoA after pull-down with GST-RBD beads from HEK293T cells transiently transfected with empty vector ("Mock"), or expression vectors for full-length BAI1 or BAI1-ΔNT. *Bottom Panel:* Western blot analysis of total RhoA levels in the input fraction of the same cells (B) Quantification of active RhoA via pull-down with GST-RBD in the presence of RGSp115 (N.S., not significant, n=3, Student's t test). *Top Panel:* Western blot analysis of active RhoA via pull-down with GST-RBD beads from HEK293T cells co-transfected with RGSp115 and either empty vector (Mock), full-length BAI1 or BAI1-ΔNT. *Bottom Panel:* Western blot analysis of total RhoA levels in same cells. Refer to Figure 1B for representative total expression levels of BAI1 and BAI1-ΔNT in HEK293T cells. (C) Representative Western blot showing changes in endogenous expression of Cyr61 induced by transfection with either mock, full-length BAI1, or BAI1-ΔNT in HEK293T cells (n = 2).

A


Fold Activation of Rho
Over Mock Transfected
15
10
5
#
*
*
Active Rho
Total Rho
Mock
BAI1
ΔNT

B


Fold Activation of Rho
Over Mock Transfected
15
10
5
N.S
Active Rho
Total Rho
Mock
BAI1
ΔNT

C

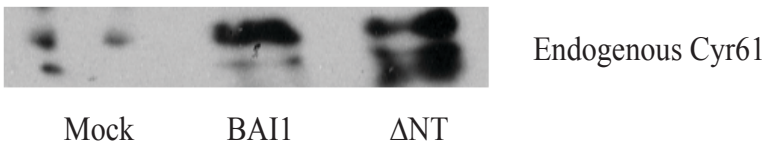
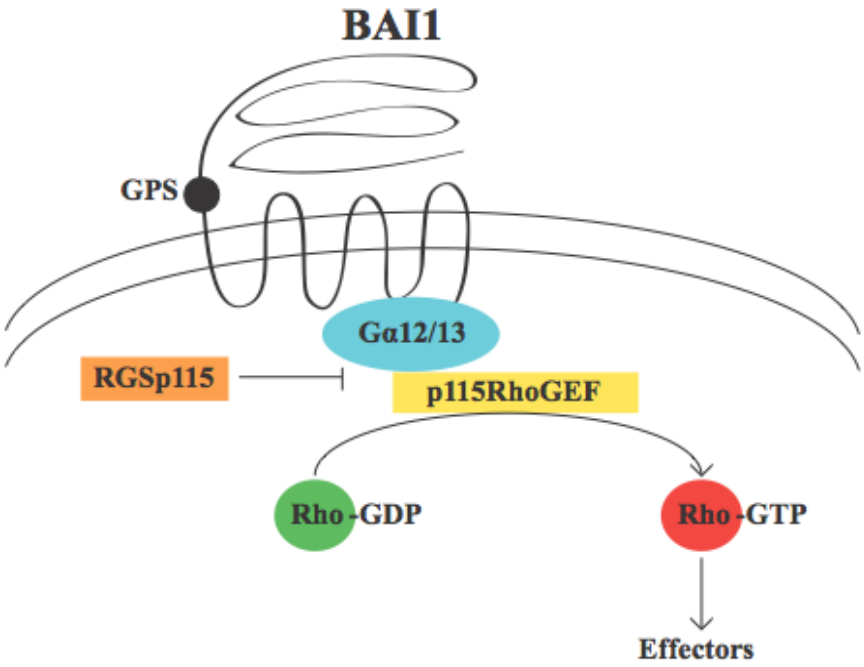

Endogenous Cyr61
Mock
BAI1
ΔNT

Figure II-4: Rho pathway and inhibition of $G\alpha_{12/13}$ by RGSp115. The activation of Rho via $G\alpha_{12/13}$ involves an intermediate GEF protein to enhance guanine-nucleotide exchange, allowing GDP-bound Rho to become the active form, Rho-GTP. Upon activation of a GPCR, subsequent activation of $G\alpha_{12/13}$ occurs allowing for interactions with the regulator of G protein signaling (RGS) homology domain of p115RhoGEF. The catalytic Dbl homology (DH) domain of p115RhoGEF then enhances the exchange of GDP-Rho to GTP-Rho. The (RGS) domain of p115RhoGEF (RGSp115), without the catalytic DH domain, can be used as a dominant-negative inhibitor for $G\alpha_{12/13}$-dependent signaling (Kozasa et al., 1998). RGSp115 specifically accelerates the GTPase activity of $G\alpha_{12/13}$, but not other G proteins, thereby selectively inhibiting $G\alpha_{12/13}$-mediated signaling.


BAI1
GPS
Gα12/13
RGSp115
p115RhoGEF
Rho-GDP
Rho-GTP
Effectors

2.4 Discussion

The studies described in this chapter reveal that the NT and 7TM regions of BAI1 can physically interact with each other when cleaved at the GPS motif. In this regard, BAI1 is analogous to other members of the adhesion GPCR family for which it has been shown that the two fragments can remain associated following GPS cleavage (Paavola and Hall, 2012; Yona et al., 2008b). The studies shown here also provide evidence that BAI1 can activate the Rho pathway through coupling to $G\alpha_{12/13}$, thereby providing clear evidence that BAI1 does in fact couple to G proteins. These findings place BAI1 on the short list of adhesion GPCRs for which coupling to G proteins has been documented: GPR56 (Iguchi et al., 2008; Paavola et al., 2011) and CD97 (Ward et al., 2011) have been shown to activate Rho via $G\alpha_{12/13}$, latrophilin-1 has been shown to couple to $G\alpha_q$ and $G\alpha_o$ (Lelianova et al., 1997; Rahman et al., 1999), GPR133 (Bohnekamp and Schoneberg, 2011) and GPR114 (Gupte et al., 2012) have been shown to increase cAMP production through coupling to $G\alpha_s$, and GPR97 has been shown to couple to $G\alpha_o$ (Gupte et al., 2012).

Our data showing BAI1-mediated Rho activation is interesting when considered in conjunction with the previously identified G protein-independent activation of Rac via BAI1 (Duman et al., 2013; Park et al., 2007). Both Rho and Rac are Rho-family GTPases known to be associated with cytoskeletal rearrangements through their regulation of the actin dynamics (Nobes and Hall, 1995). One major downstream effector of Rho is Rho-associated coiled-coil kinase (ROCK), which directly phosphorylates myosin light chain phosphatase and LIM kinase, both regulators of actin

cytoskeleton (Spiering and Hodgson, 2011). The phosphorylation state of myosin light chain has a direct effect on cellular contractility, whereas LIM kinase phosphorylates Cofilin to regulate actin nucleation and severing (Riento and Ridley, 2003; Spiering and Hodgson, 2011). Furthermore, Rho can also activate mammalian Diaphanous formin (mDia), which regulates actin polymerization and microtubule stabilization (Bartolini et al., 2008). Given the data linking BAI1 to both Rho and Rac activation, it seems apparent that BAI1 may play a key role in the regulation of actin dynamics to exert control over a variety of actin-dependent cellular processes including migration, phagocytosis, and stabilization of subcellular processes such as dendritic spines and cilia.

Further evidence for BAI1-mediated activation of Rho was found in the studies presented in this chapter with observations of BAI1-mediated increases in endogenous expression of Cyr61, a downstream effector of Rho. One interesting aspect of enhanced Cyr61 expression in connection to BAI1 is the ability of Cyr61 to induce apoptosis and thus act as a tumor suppressor. Cyr61 is a secreted protein that has been shown to promote apoptotic pathways in human cancer cell lines (Chien et al., 2004; Todorovic et al., 2005). Cyr61 appears to induce apoptosis by increasing expression of Bax and Bad, both pro-apoptotic proteins, as well as enhancing caspase activity (Walsh et al., 2008). These data provide a possible unidentified signaling mechanism utilized by BAI1 to promote inhibition of angiogenesis and tumor formation.

The data shown here demonstrate that removal of the BAI-NT results in enhanced receptor signaling activity to both Rho and Cyr61, suggesting that the association of the BAI1-NT with the receptor's 7TM regions results in suppression of BAI1 signaling activity. It is interesting to note that while removal of the BAI1-NT resulted in enhanced

activation of Rho, full-length BAI1 also resulted in significant activation of Rho over the Rho activity observed in mock-transfected cells. There are a couple of potential explanations for this observed effect. First, BAI1 could simply have a certain level of constitutive activity, or ligand-independent signaling capability (Rosenbaum et al., 2009), and removal of the NT might enhance this constitutive activity. Alternatively, HEK293T cells may express an endogenous ligand that activates full-length BAI1 by engaging the NT and inducing a conformation change. A third potential explanation for the increase in Rho seen upon transfection of full-length BAI1 is that the receptor may be a ligand for itself. The N-termini of other adhesion GPCRs have been show to undergo homophilic trans-interactions resulting in activation of receptor signaling, for example in the cases of Celsr2 & Celsr 3 (Shima et al., 2007) and GPR56 (Paavola et al., 2011), and it is possible that this mechanism of activation is conserved among adhesion GPCRs.

In addition to the findings shown here for BAI1, apparently inhibitory actions resulting from the N-termini of adhesion receptors interacting with the receptors' 7TM regions have previously been reported for CD97 (Ward et al., 2011), GPR56 (Paavola et al., 2011) and BAI2 (Okajima et al., 2010). These congruent findings from work on four different receptors suggest a potentially general mechanism of activation for adhesion GPCRs, in which a receptor undergoes autoproteolysis, the NT and 7TM regions remain associated for a period of time, and then ultimately extracellular ligands bind to the NT and promote its disengagement from the 7TM region, which relieves the inhibitory constraint imposed by the NT and promotes receptor signaling activity. Other models of adhesion GPCR signaling have been proposed (Arac et al., 2012a; Langenhan et al., 2013), as for example it has been suggested that the NT and 7TM regions of EMR2 are

inactive when isolated, but signal upon interaction within lipid rafts (Huang et al., 2012). Further work will be needed to clarify the activation mechanisms of adhesion GPCRs, but it is interesting to note that all current models focus on the importance of a dynamic interplay between the NT and 7TM regions in regulating receptor signaling activity.

CHAPTER III: BAI1 C-terminal interactions and regulation of receptor signaling

3.1 Introduction

In the previous chapter, we showed the first evidence of G protein-mediated signaling by BAI1, leading to activation of Rho and increased levels of Cyr61. Removal of the BAI1-NT resulted in potentiated receptor signaling, indicating a novel mechanism of activation wherein removal of the N-terminus relieves inhibitory constraint on the 7TM region allowing G protein-coupling and subsequent signaling to downstream effectors. Aside from N-terminal regulation of signaling capabilities, we were also interested in elucidating the role of the BAI1 C-terminus (CT) with regards to signaling. Aside from G proteins, there are a number of GPCR-interacting proteins that can regulate these receptors in a variety of ways. As previously mentioned, β-arrestins can associate with GPCRs to not only attenuate G protein-mediated signaling but also act as signaling modules themselves by recruiting signaling complexes to GPCRs (Luttrell and Lefkowitz, 2002). Furthermore, β-arrestins can act as adaptor proteins for E3 ubiquitin ligases, increasing the ubiquitination of GPCRs (Shenoy and Lefkowitz, 2011). E3 ubiquitin ligases covalently attach ubiquitin, a low molecular weight protein, to the amino group of C-terminal lysine residues on GPCRs. Ubiquitination of GPCRs is a highly regulated post-translational modification that dictates the trafficking of activated receptors into various endocytic pathways.

Proteins with PDZ domains can also interact with PDZ-binding motifs at the extreme C-termini of GPCRs. These interactions are known to regulate GPCRs in numerous ways, including controlling receptor trafficking, subcellular localization, direct mediation of signaling, and allosteric modulation of receptor pharmacology (Hall and

Lefkowitz, 2002). For example, the PDZ domain-containing protein Na+/H+ exchanger regulatory factor-2 (NHERF-2) interacts with mGluR5 to enhance its PLCβ-mediated signaling pathway (Paquet et al., 2006), and NHERF-1 directs trafficking of the β2-adrenergic receptor, as mutations in the receptor that disrupt the interaction results in greater targeting to lysosomes following agonist stimulation (Cao et al., 1999). Moreover, the homer1 scaffold protein regulates mGluR5 targeting to dendritic spines (Ango et al., 2000), and the receptor activity-modifying proteins (RAMPs) can dictate the pharmacological properties of the calcitonin receptor-like receptor (CRLR) (McLatchie et al., 1998). The ability of GPCRs to interact with a variety of interacting proteins permits cell-specific fine-tuning that allows the receptors to adapt to changes in the cellular environment depending on the context.

Given this wealth of previous work on GPCR regulation by CT-interacting partners, we studied the potential regulation of BAI1 signaling by the receptor's CT. The BAI1-CT contains a putative PDZ-binding motif (QTEV) and has been reported to bind to two PDZ domain-containing scaffold proteins (Lim et al., 2002; Shiratsuchi et al., 1998a). However, nothing is known at present about the functional significance of these interactions. We sought to more comprehensively explore BAI1 associations with PDZ scaffolds and also assess the potential importance of these interactions in the regulation of receptor signaling. Since the studies shown in the previous chapter provided evidence for BAI1 signaling via G proteins, we were also interested in the ability of BAI1 to interact with β-arrestins. We found that the PDZ-binding domain on the BAI1 C-terminus interacts with a variety of PDZ domains and can regulate signaling. We also found the removal of the BAI1 N-terminus enhances association with β-arrestin2 and also

increases receptor ubiquitination. Moreover, we also found that BAI1 interactions with β-arrestin2 are further enhanced by removal of the receptor's PDZ-binding motif.

3.2 Experimental procedures

Antibodies and Reagents- Antibodies against HA (Roche), FLAG (Sigma-Aldrich), β-arrestin2 (Cell Signaling), phospho-ERK (Santa Cruz), total ERK (Cell Signaling Technology) were purchased from the manufacturers. A distinct anti-BAI1-CT antibody (#17108) was custom-made by Pocono Rabbit Farm & Laboratory (Canadensis, PA) via injection of rabbits with a peptide (HSLTLKRDKAPKSS) derived from the human BAI1 C-terminus (amino acids 1305-1318), followed by affinity purification. Gallein was purchased from Santa Cruz and AG1478 from EMD Millipore.

Cell Culture and transient plasmid transfections- HEK293T cells (ATCC) used for cell-based assays were cultured and maintained in complete media (DMEM containing 10% FBS and 1% penicillin/streptomycin) at 37 °C at 5% CO_2. Transfections were performed by incubating cells plated on 100mm x 20mm Cell Culture Dishes (Corning) with Lipofectamine 2000 (Invitrogen) and plasmid DNA for 3-5 hours in serum free DMEM. The transfection reaction was stopped by addition of complete media. Experiments were performed 24 hours post-transfection.

Plasmids- Expression vectors for BAI1 wild-type (Kaur et al., 2003; Kaur et al., 2005). The BAI1-ΔNTΔPDZ was cloned into pcDNA3.1 by creating primers from BAI1-ΔNT starting at amino acid 929 and ending at amino acid 1581. Primers used for PCR were 5'-GCTAGCATGTTCGCCATCTTAGCCCAGCTC-3' and 5'-AAGCTTTCACTGGAGGTCGATGATGTCCTG-3'. The GST-BAI1-CT30 construct

was made by cloning the sequence corresponding to the last 30 amino acids in the C-terminus of BAI1 into the pGEX-4T1 vector using the following primers: 5'-GAATTCCCGCTGGAGCTTCGCAGCGTGGAGTGG-3' and 5'-CTCGAGTCAGACCTCGGTCTGGAGGTCGATGAT-3'. The HA-ubiquitin construct was a gift from Keqiang Ye (Emory University). The GST-RBD (Addgene), Flag-β-arrestin2 (Addgene), and HA-Rho (Missouri S&T cDNA Resource Center) expression vectors were all obtained from commercial sources.

Western Blot- Samples were run on 4-20% SDS-PAGE gels (Invitrogen) for 2 hours at 130 V and then transferred to a nitrocellulose membrane (Bio-Rad) for 2 hours at 30 V. The membrane was blocked in blot buffer containing 2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM Hepes, at PH 7.4 for 30 minutes at room temperature. The membrane was incubated with primary antibody in blot buffer for 1 h at room temperature, followed by three 5 min washes. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (GE Healthcare) for 30 minutes at room temperature followed by three 5 min washes with blot buffer. Blots were visualized via enzyme-linked chemiluminescence using the Supersignal® West Pico Chemiluminescent Substrate (Pierce). For phospho-ERK assessment, membranes were blocked in Odyssey blocking buffer and then incubated overnight with shaking at 4°C with primary antibodies prepared in equal parts blocking buffer and PBS + 0.1% Tween-20. Nitrocellulose membranes were washed three times in PBS with 0.1% Tween-20 and incubated with Alexa-fluor anti-mouse 700nm conjugated secondary antibody (1:20,000; Invitrogen) and anti-rabbit 800nm conjugated secondary antibody (1:20,000; Li-Cor) for

30 minutes in equal parts blocking buffer and wash buffer. Blots were again washed three times and rinsed in PBS until being visualized on an Odyssey Imaging System (Li-Cor). Images were quantified using Image Station and ImageJ.

Co-Immunoprecipitation- Expression vectors for proteins of interest were transfected into HEK293T cells as described above. After 24 hours, cells were resuspended in 500 μL Lysis Buffer [1% Triton X-100, 150 mM NaCl, 25 mM Hepes, 10mM $MgCl_2$, 1 mM EDTA, 1 protease inhibitor cocktail tablet (Roche Diagnostics) and 2% glycerol]. Cells were rotated in lysis buffer for 30 minutes at 4° C then cell debris was cleared by centrifugation. Soluble lysates were incubated for 60 minutes with 30 μL of protein A/G beads (Thermo Scientific) with corresponding antibody (3 μL) to the protein being immunoprecipitated. Beads were washed 3 times with Lysis Buffer, resuspended in 40 μL 2x sample buffer and heated to 95 °C for 10 minutes.

Rhotekin Rho Activation Assay- HEK293T cells were co-transfected with expression plasmids for HA-Rho and either pcDNA3.1, BAI1 wild-type, BAI1-ΔNT, or BAI1-ΔNTΔPDZ with or without RGSp115. As previously described (Paavola et al., 2011), 24 hours post-transfection, cells were resuspended in 500 μL Lysis Buffer. Cells were lysed by slowly rotating on a spinning wheel for 30 minutes at 4° C, then cell debris was cleared by centrifugation. Soluble cell lysates were incubated with 30 μL of GST-Rho Binding Domain (GST-RBD) of Rhotekin coupled to glutathione agarose beads (Sigma). Beads were washed 3 times with Lysis Buffer, resuspended in 40 μL 2x sample buffer

and heated to 95 °C for 10 minutes. Active Rho was detected via the Western blot procedure described above. Images were quantified using Image J.

PDZ Domain Proteomic Array- The PDZ domain proteomic array has been previously described (Fam et al., 2005; He et al., 2006). Briefly, nylon membranes were spotted with bacterially produced recombinant His/S-tagged PDZ domain fusion proteins at a concentration of 1 μg per bin. Either control GST or a GST fusion protein corresponding to the last 30 amino acids of BAI1 were overlaid at a concentration of 100 nM in blot buffer overnight at 4 °C. The arrays were then washed three times with blot buffer and incubated with horseradish peroxidase-conjugated anti-GST antibody (Amersham Biosciences). Interactions of the GST fusion proteins and the PDZ domains were visualized by chemiluminescence using the ECL kit (Pierce).

GST Fusion Protein Pull-down Assay- GST fusion proteins were purified from bacteria using glutathione-Sepharose 4B beads (Sigma) and resuspended in resuspension buffer [50 mM NaCl, 10 mM Hepes and 1 mM EDTA and 1 protease inhibitor cocktail tablet (Roche Diagnostics)]. Equal amounts of fusion proteins conjugated on beads were incubated with 500 μL of soluble lysates from HEK293T cells transfected with BAI1-ΔNT or BAI1-ΔNTΔPDZ for 60 minutes at 4 °C with rotation. The beads were extensively washed in Lysis Buffer and proteins were eluted from beads in 40 μL 2x sample buffer. Proteins were then resolved on SDS-PAGE and detected via Western blot as mentioned above.

ERK Phosphorylation Assay- HEK293T cells were transfected with pcDNA3.1, ΔNT, or ΔNTΔPDZ expression vectors with or without MAGI-3 expression vectors. 24 hours post-transfection cells were serum starved in Dulbecco's Modified Eagle Medium (Gibco) for 2 hours. For experiments with Gallein and AG 1478, cells were treated during the serum starvation with 100 uM of Gallein or AG 1478 in dimethyl sulfoxide, which was used as a vehicle in untreated cells. Cells were then placed on ice and washed with ice cold PBS. Following serum starvation, cells were harvested in 1.5x sample buffer and briefly sonicated. Protein lysates were resolved on SDS-PAGE and proteins were visualized via Western blot and the Odyssey Imaging System as described above.

Cell Surface Biotinylation- HEK293T cells were transfected with BAI1 wild-type, ΔNT, or ΔNTΔPDZ expression vectors. 24 hours post-transfection cells were placed on ice and washed with ice cold PBS three times. Cells were then incubated with 10 mM Sulfo-NHS-SS-Biotin (Thermo Scientific) in PBS on ice for 1 hour and then washed three times with PBS + 100 mM glycine. Cells were resuspended in 500 μL Lysis Buffer and lysed by slowly rotating on a spinning wheel for 30 minutes at 4° C. Cell debris was cleared by centrifugation and soluble cell lysates were incubated with 30 μL of streptavidin agarose beads (Thermo Scientific) for 1 hour. Beads were washed 3 times with Lysis Buffer, resuspended in 40 μL 2x sample buffer and heated to 95 °C for 10 minutes. Biotinylated constructs were detected via the Western blot procedure described above.

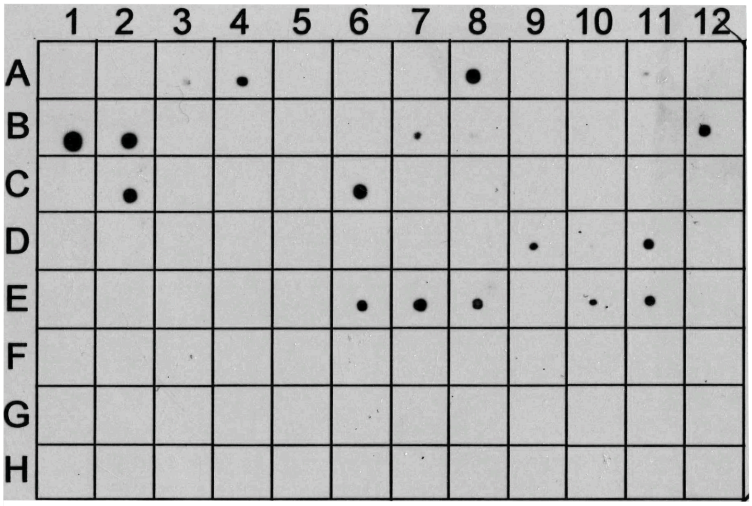
3.3 Results

3.3.1 BAI1 interacts with a variety of PDZ domains from synaptic proteins

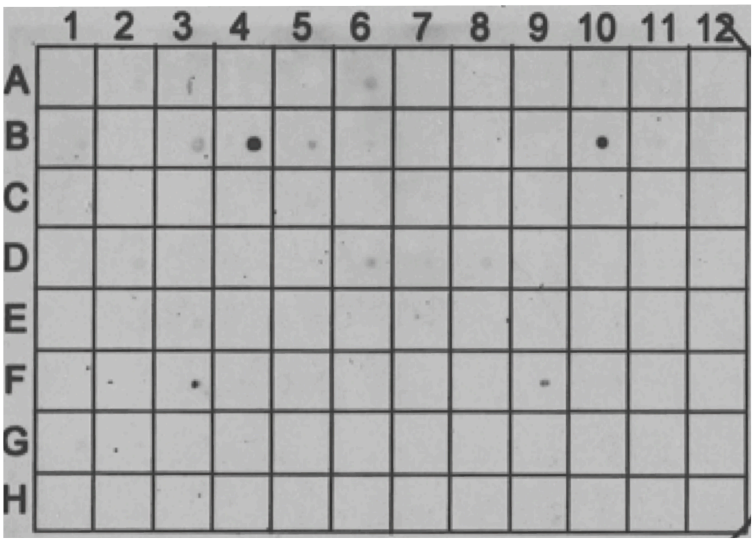
Since the C-terminus of BAI1 contains a consensus sequence for possible interactions with PDZ domains (amino acids T-E-V), we screened a recombinant GST fusion protein corresponding to the last 30 amino acids of the BAI1-CT ("BAI1-CT30") against a proteomic array of 96 distinct PDZ domains in order to assess potential BAI1 interacting partners. As shown in Figure III-1A, interactions of the BAI1-CT30 were detected for PDZ1+2 of PSD-95 (bin B7) and PDZ4+5 of MAGI-1 (bin A4), consistent with previous reports (Lim et al., 2002; Shiratsuchi et al., 1998a). Additionally, novel associations of the BAI1-CT were seen with PDZ domains from MAGI-2 (bin A8), MAGI-3 (bins B1 and B2), INADL (bin B12), SAP97 (bin C2), Chapsyn-110 (bin C6), MALS-1 (bin D9), Densin-180 (bin D11) PAPIN 1 (bin E11), and several syntrophins (bins E6-8, E10; Fig. III-1C). In contrast to these positive hits detected with the BAI1-CT30, only light non-specific labeling of the array was observed upon overlay of control GST at extended exposures, as seen in Figure III-1B.

Figure III-1: The C-terminus of BAI1 interacts with a variety of PDZ domains from synaptic proteins. A recombinant GST fusion protein corresponding to the last 30 amino acids of BAI1 (GST-BAI1-CT30) (A) or control GST (B) was overlaid at 100 nM onto a proteomic array containing 96 distinct PDZ domains. The data shown are representative of 3 independent experiments. (C) List of PDZ domains that interact with the GST-BAI1-CT30 fusion protein. The complete list of the PDZ proteins on this array has been described previously (He et al., 2006).

A



B



C

A3: MAGI-1 PDZ3
A4: MAGI-1 PDZ4+5
A8: MAGI-2 PDZ4
B1: MAGI-3 PDZ4
B2: MAGI-3 PDZ5
B7: PSD-95 PDZ1+2
B12: INADL PDZ5
C2: SAP97 PDZ1+2
C6: CHAP110 PDZ1+2
D9: MALS-1 PDZ
D11: DENSIN-180
E6: α1-SYNTROPHIN
E7: β1-SYNTROPHIN
E8: β2-SYNTROPHIN
E10: γ2-SYNTROPHIN
E11: PAPIN 1

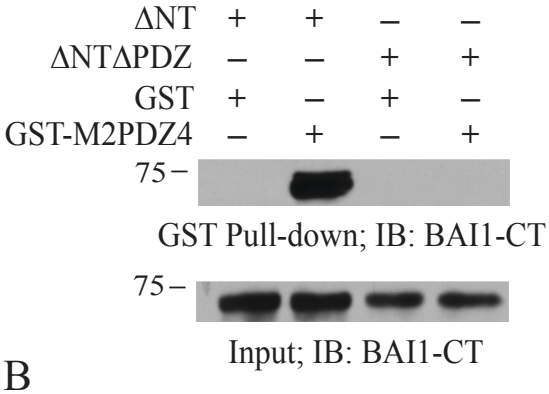
3.3.2 Removal of BAI1 PDZ-binding motif attenuates receptor signaling to Rho

To gain insights into the functional effects that PDZ interactions might have on the signaling and regulation of BAI1, the last three amino acids of the BAI1-CT (T-E-V) were truncated from the constitutively-active BAI1-ΔNT construct ("BAI1-ΔNTΔPDZ"). To verify that removal of the BAI1 PDZ-binding motif indeed disrupts receptor interaction with PDZ domains, we utilized a pull-down assay with a recombinant GST fusion protein corresponding to PDZ4 from MAGI-2, which was one of the strongest interactions detected in the screen of the PDZ domain proteomic array (Fig. III-IA, bin A8). As shown in Figure III-2A, a strong association of MAGI-2 PDZ4 with the BAI1-ΔNT mutant was evident, whereas no detectable interaction was observed between MAGI-2 PDZ4 and the BAI1-ΔNTΔPDZ mutant, indicating that removal of the T-E-V sequence from the CT of BAI1 ablates the receptor's capacity to interact with PDZ domains. A representative SDS-PAGE gel stained with Coomassie Brilliant Blue is shown in Figure III-2B to illustrate purification of GST-MAGI-2 PDZ4 and GST alone.

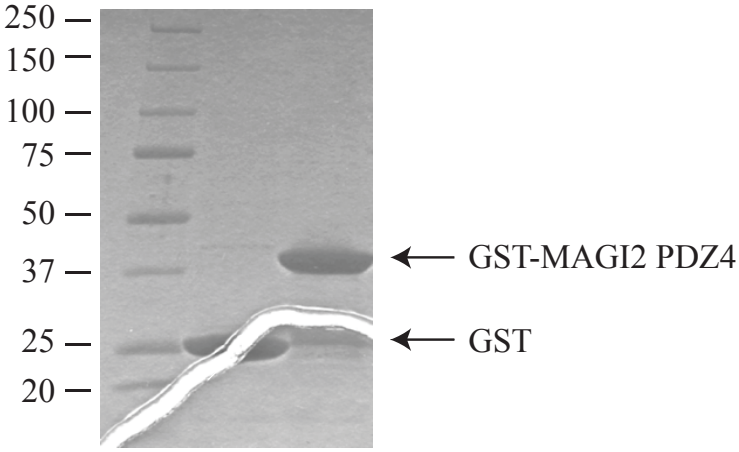
Next, the BAI1-ΔNTΔPDZ mutant receptor was assessed in terms of its ability to activate Rho. As shown in Figure III-2C, truncation of the BAI1 PDZ-binding motif greatly attenuated the ability of BAI1-ΔNT to activate Rho. No significant increase (1.3 +/- 0.4 fold) in Rho activation compared to mock-transfected cells was observed following transfection with the BAI1-ΔNTΔPDZ expression vector. This represents the first data indicating that the C-terminus of BAI1, specifically the PDZ-binding motif, regulates receptor signaling through G proteins.

Figure III-2: Removal of the PDZ-binding motif from constitutively active BAI1 inhibits interaction with PDZ 4 domain of MAGI-2 and attenuates signaling to Rho. (A) A recombinant GST-MAGI-2 PDZ4 (GST-M2-PDZ4) fusion protein pulls down transfected BAI1-ΔNT but not the BAI1-ΔNTΔPDZ mutant from solubilized HEK293T cell lysates. (B) Representative SDS-PAGE gel showing expression and purification of GST-MAGI-2 PDZ4 and GST. (C) Quantification of active RhoA via pull-down with GST-RBD (n=4). *Top Panel:* Immunoblot (IB) analysis of active RhoA following pull-down with GST-RBD beads from HEK293T cells transfected with empty vector (Mock) or expression vector for BAI1-ΔNTΔPDZ. *Bottom Panel:* Western blot analysis of total RhoA levels in the same cells.

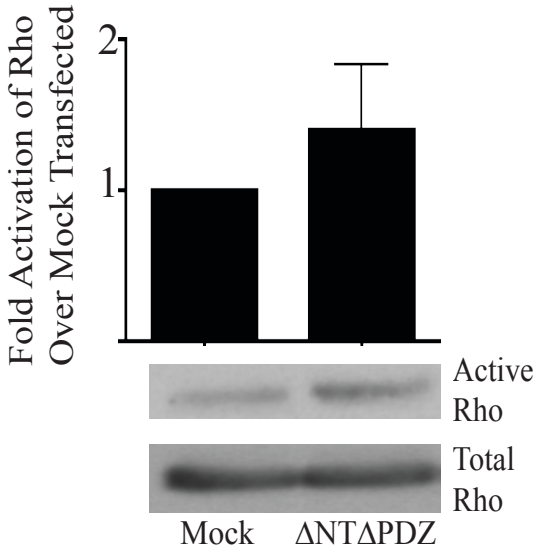
A


ΔNT + + – –
ΔNTΔPDZ – – + +
GST + – + –
GST-M2PDZ4 – + – +
75 –
GST Pull-down; IB: BAI1-CT
75 –
Input; IB: BAI1-CT

B


250 –
150 –
100 –
75 –
50 –
37 –
25 –
20 –
GST-MAGI2 PDZ4
GST

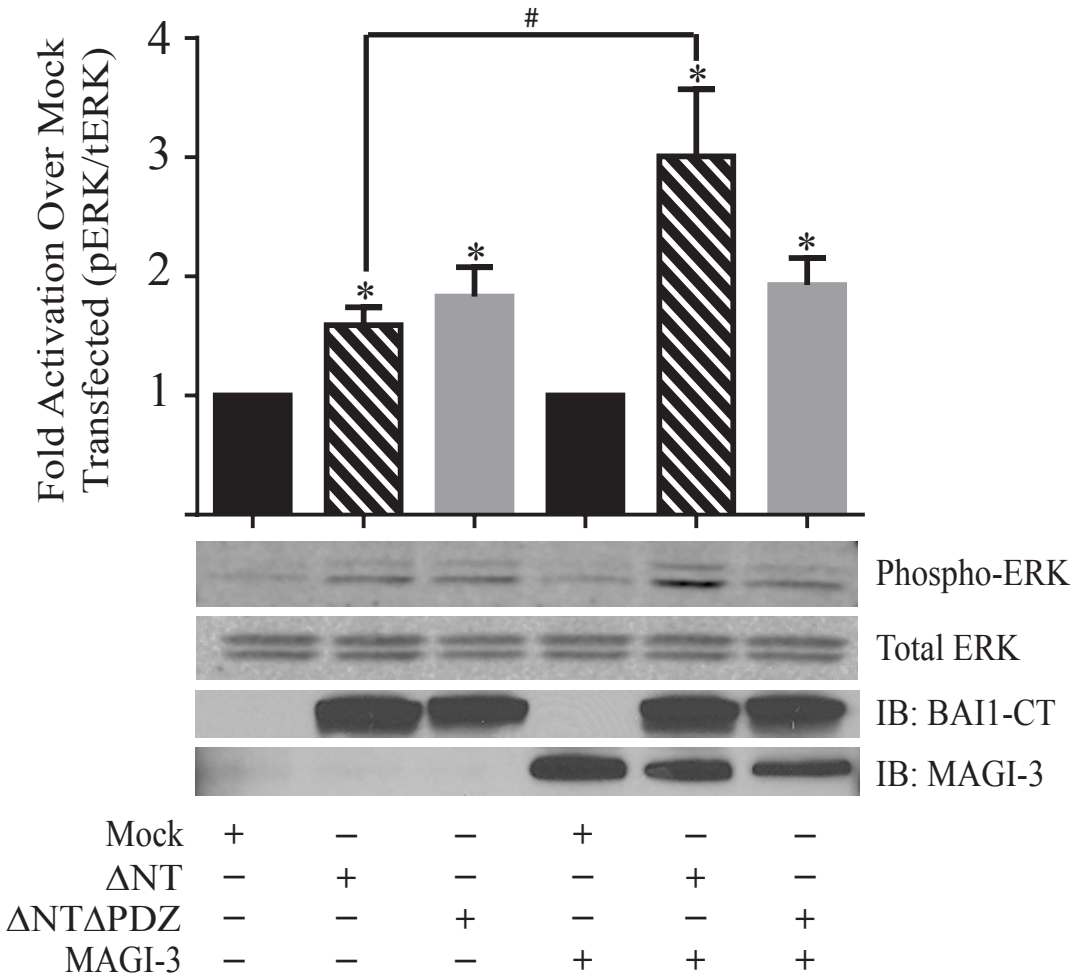
C


Fold Activation of Rho
Over Mock Transfected
2
1
Active
Rho
Total
Rho
Mock
ΔNTΔPDZ

3.3.3 BAI1 signaling to ERK is potentiated by MAGI-3

In addition to studying BAI1-mediated activation Rho, we assessed other possible downstream effectors of BAI1 that might be regulated by C-terminal interactions. We found that transfection of BAI1-ΔNT into HEK293T cells resulted in a significant increase in ERK phosphorylation (Fig. III-3). Interestingly, BAI1-ΔNTΔPDZ caused a comparable increase in pERK, indicating that removal of the PDZ-binding motif exerts distinct effects on different BAI1-activated signaling pathways. Since MAGI-3 was found to robustly interact with BAI1 (Figure III-2A, bins B1 and B2) and has been shown to regulate signaling to ERK by a variety of GPCRs including the β_1AR (He et al., 2006), β_2AR (Yang et al., 2010), LPA(2) (Zhang et al., 2007), and frizzled (Yao et al., 2004), we tested the effects of MAGI-3 co-expression on BAI1-mediated pERK signaling. Strikingly, MAGI-3 potentiated pERK levels when co-expressed with BAI1-ΔNT, but had no effect at all on pERK stimulation mediated by BAI1-ΔNTΔPDZ. This provides evidence that BAI1 interactions with different PDZ domains can differentially regulate receptor signaling to distinct pathways.

Figure III-3: BAI1-mediated ERK signaling is enhanced by MAGI-3 co-expression. Quantification of phosphorylated ERK (pERK) to total ERK (tERK) from HEK293T cells transiently transfected with expression vectors for pcDNA3.1, BAI1-ΔNT or BAI1-ΔNTΔPDZ with or without an expression vector for HIS-V5-MAGI-3 (*, indicates comparison to mock-transfected; #, indicates $p<0.05$, n=7, one-way ANOVA). *Top panel:* Immunoblot analysis of pERK. *Second panel:* Immunoblot analysis of tERK. *Third panel:* Immunoblot analysis of BAI1 from whole cell lysates. *Bottom panel:* Immunoblot analysis of MAGI-3 from whole cell lysates.


Fold Activation Over Mock Transfected (pERK/tERK)
4
3
2
1
#
*
*
*
*
Phospho-ERK
Total ERK
IB: BAI1-CT
IB: MAGI-3
Mock + – – + – –
ΔNT – + – – + –
ΔNTΔPDZ – – + – – +
MAGI-3 – – – + + +

3.3.4 BAI1 activation of ERK is not mediated by $G\alpha_{12/13}$, Gβγ, or transactivation of EGFR

As shown in the previous section, transfection of BAI1-ΔNT and BAI1-ΔNTΔPDZ into HEK293T cells results in a comparable increase in ERK phosphorylation (Fig. III-3). We next wanted to determine if this observed ERK activation was downstream of the BAI1-$G\alpha_{12/13}$-Rho pathway elucidated in the experiments described in Chapter 2. Therefore, we co-transfected the RGSp115 construct with BAI1-ΔNT and BAI1-ΔNTΔPDZ into HEK293T. As shown in Figure III-4A, addition of RGSp115 had no effect on the ability either BAI1-ΔNT or BAI1-ΔNTΔPDZ to increase phosphorylation of ERK, indicating that BAI1-mediated ERK activation is not dependent on BAI1 coupling to $G\alpha_{12/13}$.

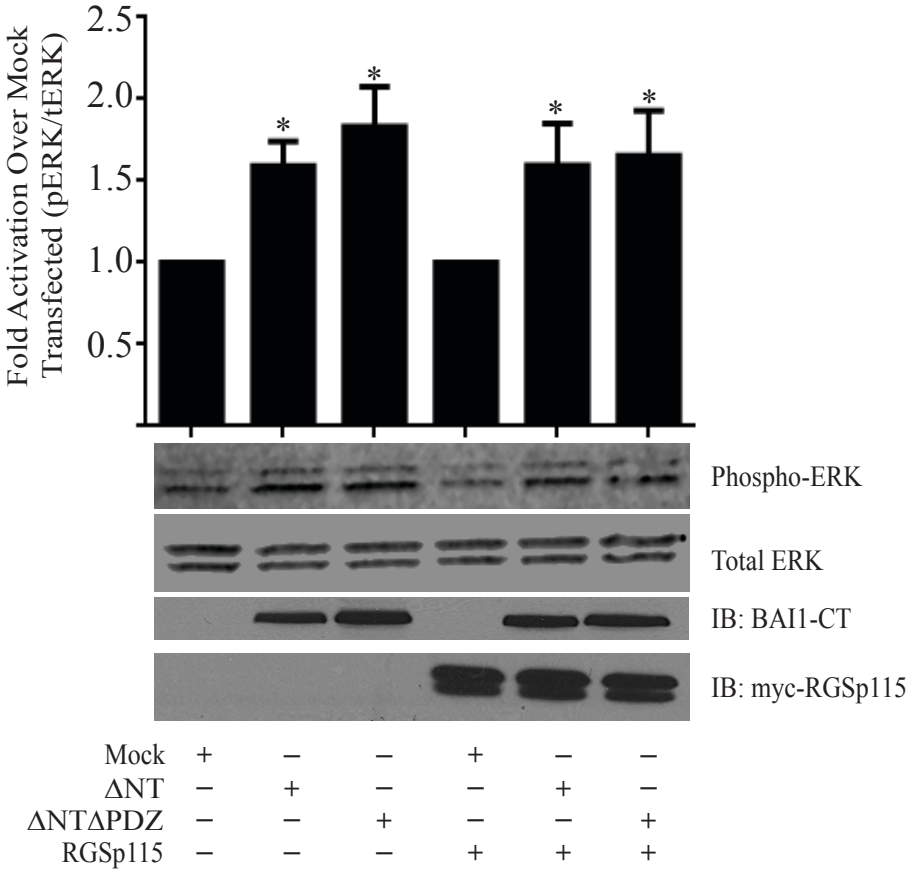
GPCRs can activate ERK using a multitude of pathways, involving both the Gα and Gβγ subunits, interactions with β-arrestins and/or transactivation of receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR) (DeWire et al., 2007; Goldsmith and Dhanasekaran, 2007; Gutkind and Offermanns, 2009). To explore the possibility of BAI1-mediated ERK activation via Gβγ subunits, we utilized the Gβγ inhibitor gallein. As figure III-4B shows, no significant differences were observed between phosphorylation of ERK by BAI1-ΔNT in gallein-treated versus untreated conditions, indicating downstream activation of ERK is not dependent on liberation of Gβγ subunits.

Another pathway by which GPCRs can activate ERK is through transactivation of receptor tyrosine kinases, with EGFR as the RTK that most commonly mediates

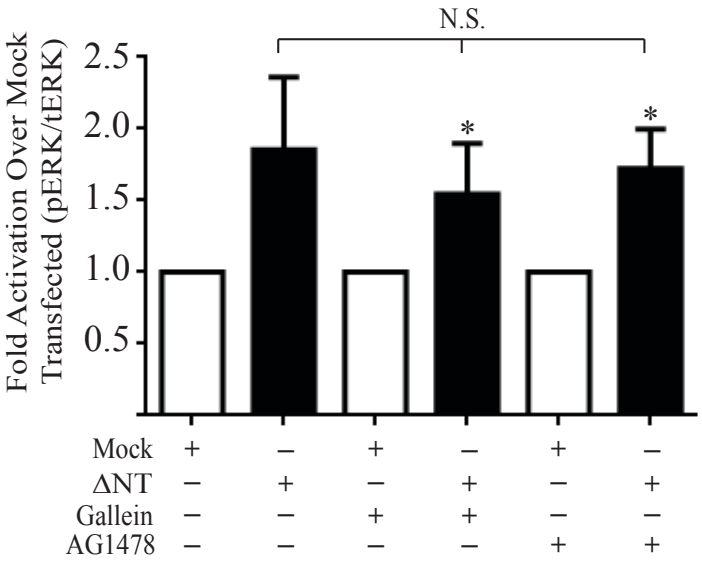
mitogenic signaling by GPCRs (Liebmann, 2001). Therefore, we used the EGFR inhibitor AG 1478 to determine if BAI1-mediated transactivation of EGFR was necessary for the observed BAI1-induced phosphorylation of ERK. HEK-293T cells transfected with BAI1-ΔNT exhibited comparably enhanced pERK levels in the absence and presence of AG 1478 pre-treatment, suggesting that transactivation of EGFR is not required for BAI1-mediated signaling to ERK in HEK293T cells.

Figure III-4: BAI1-mediated ERK signaling is not dependent on $G\alpha_{12/13}$, $G\beta\gamma$, or transactivation of EGFR. (A) Quantification of phosphorylated ERK (pERK) to total ERK (tERK) from HEK293T cells transiently transfected with expression vectors for pcDNA3.1, BAI1-ΔNT or BAI1-ΔNTΔPDZ with or without an expression vector for myc-RGSp115 (* indicates $p<0.05$ compared to mock, n=5, one-way ANOVA). *Top panel:* Immunoblot analysis of pERK. *Second panel:* Immunoblot analysis of tERK. *Third panel:* Immunoblot analysis of BAI1 from whole cell lysates. *Bottom panel:* Immunoblot analysis of myc-RGSp115 from whole cell lysates. (B) Quantification of phosphorylated ERK (pERK) to total ERK (tERK) from HEK293T cells transiently transfected with expression vectors for pcDNA3.1, BAI1-ΔNT with or without treatment with Gallein or AG 1478 (* indicates $p<0.05$ compared to mock, n=4, one-way ANOVA).

A


Fold Activation Over Mock Transfected (pERK/tERK)
2.5
2.0
1.5
1.0
0.5
*
*
*
*
Phospho-ERK
Total ERK
IB: BAI1-CT
IB: myc-RGSp115
Mock + – – + – –
ΔNT – + – – + –
ΔNTΔPDZ – – + – – +
RGSp115 – – – + + +

B

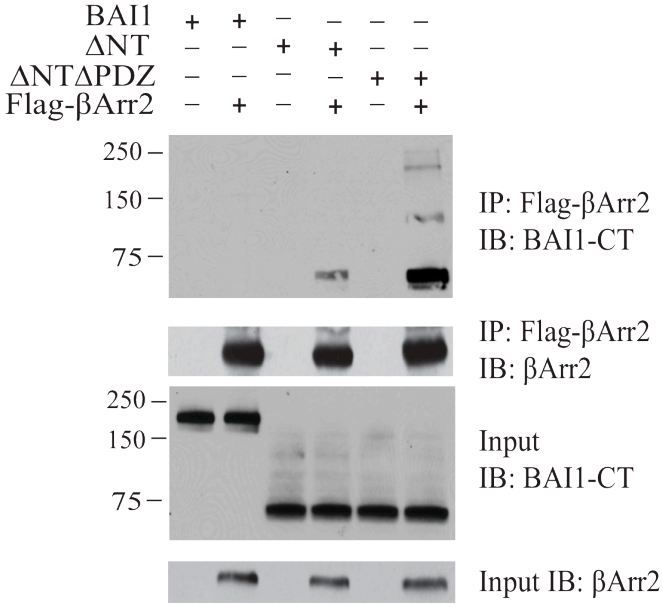

N.S.
Fold Activation Over Mock Transfected (pERK/tERK)
2.5
2.0
1.5
1.0
0.5
*
*
Mock + – + – + –
ΔNT – + – + – +
Gallein – – + + – –
AG1478 – – – – + +

3.3.5 Removal of the BAI1 N-terminus enhances association with β-arrestin2 and receptor ubiquitination

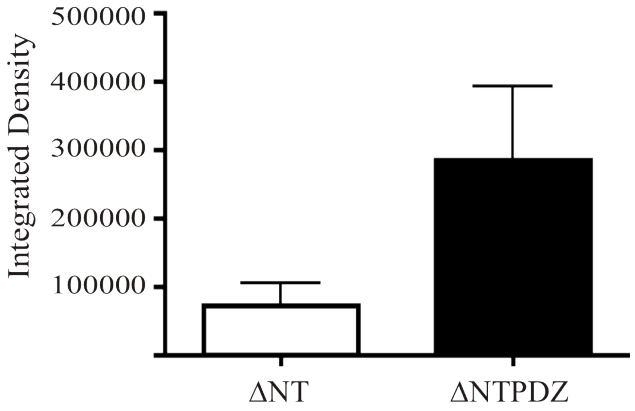
Previous studies have shown that constitutively-active receptors often exhibit robust interactions with β-arrestins, which dampen signaling by preventing receptor coupling to G proteins and promoting receptor internalization (Ferrari and Bisello, 2001; Mhaouty-Kodja et al., 1999). Thus, we assessed potential interactions of BAI1, BAI-ΔNT and BAI1-ΔNTΔPDZ with arrestins. As shown in Figure III-5A, a significant interaction was seen between BAI1-ΔNT and β-arrestin2. In contrast, full-length BAI1 did not detectably bind to β-arrestin2. Interestingly, the BAI1-ΔNTΔPDZ mutant exhibited a level of β-arrestin2 interaction that was even more robust (3.5-fold higher as shown in Figure III-5B) than that observed with the BAI1-ΔNT mutant, suggesting that PDZ protein binding to the C-terminus may negatively regulate β-arrestin2 interactions. Since constitutively-active receptors can also be heavily ubiquitinated (Shenoy, 2007; Shenoy et al., 2001), we co-transfected full-length BAI1, BAI1-ΔNT, or BAI1-ΔNTΔPDZ with HA-ubiquitin to determine levels of receptor ubiquitination. No observable ubiquitination of full-length BAI1 was seen. In contrast, significant and comparable levels of ubiquitination were observed for both BAI1-ΔNT and BAI1-ΔNTΔPDZ (Fig. III-5C), indicating that removal of the BAI1-NT results in enhanced receptor ubiquitination whereas removal of the BAI1 PDZ-binding motif does not affect receptor ubiquitination.

Figure III-5: Removal of both the BAI1-NT and PDZ-binding motif enhances association with β-arrestin2, but only NT removal of enhances receptor ubiquitination. (A) HEK293T cells were transiently transfected with expression vectors for full-length BAI1, BAI1-ΔNT or BAI1-ΔNTΔPDZ in the absence or presence of Flag-β-arrestin2. Immunoprecipitation (IP) was performed with anti-Flag antibody coupled to agarose beads. Co-immunoprecipitated BAI1 was detected by Western blot with anti-BAI1-CT antibody. (B) Quantification of integrated density for association of BAI1-ΔNT or BAI1-ΔNTΔPDZ with Flag-β-arrestin2 (C) Expression vectors for full-length BAI1, BAI1-ΔNT or BAI1-ΔNTΔPDZ were transiently co-transfected into HEK293T cells with an HA-ubiquitin (HA-Ub) expression vector. Immunoprecipitation was performed with anti-HA antibodies coupled to agarose beads. Co-immunoprecipitated BAI1 was visualized with anti-BAI1-CT antibody.

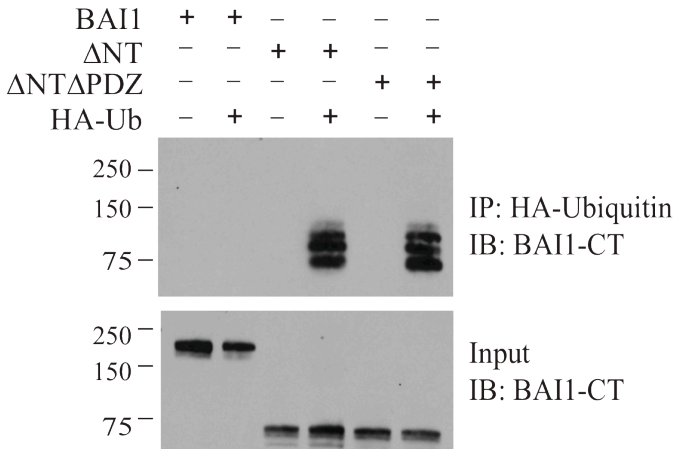
A


BAI1 + + – – – –
ΔNT – – + + – –
ΔNTΔPDZ – – – – + +
Flag-βArr2 – + – + – +
250
150
75
IP: Flag-βArr2
IB: BAI1-CT
IP: Flag-βArr2
IB: βArr2
250
150
75
Input
IB: BAI1-CT
Input IB: βArr2

B


Integrated Density
500000
400000
300000
200000
100000
ΔNT
ΔNTPDZ

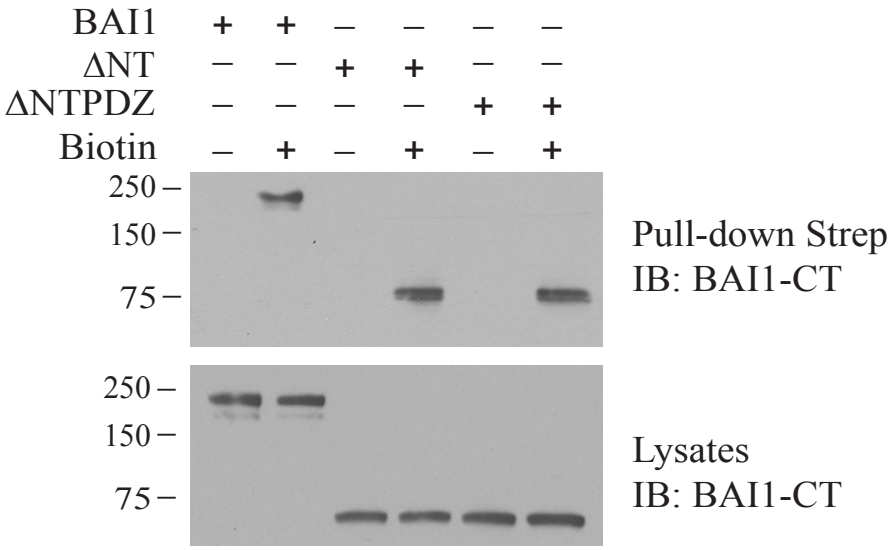
C


BAI1 + + – – – –
ΔNT – – + + – –
ΔNTΔPDZ – – – – + +
HA-Ub – + – + – +
250
150
75
IP: HA-Ubiquitin
IB: BAI1-CT
250
150
75
Input
IB: BAI1-CT

3.3.6 All BAI1 constructs are expressed equally at the plasma membrane

The differences described above in the signaling activity, arrestin binding and ubiquitination of full-length BAI1, BAI1-ΔNT and BAI1-ΔNTΔPDZ could be due to differences in the targeting of the WT vs. mutant receptors to the plasma membrane. Thus, the plasma membrane insertion of the WT vs. mutant receptors was assessed in cell surface biotinylation studies. These experiments revealed very comparable levels of plasma membrane localization between full-length BAI1, BAI1-ΔNT and the BAI1-ΔNTΔPDZ mutant (Fig. III-6).

Figure III-6 All BAI1 constructs are expressed equally at the plasma membrane. HEK293T cells were transiently transfected with full-length BAI1, BAI1-ΔNT or BAI1-ΔNTΔPDZ expression vectors and incubated with 10 mM Sulfo-NHS-ss-Biotin ("Biotin"). Biotinylated proteins were pulled down with streptavidin beads ("Strep") to determine cell surface expression, and both pull-down samples and whole cell lysates ("Lysates") were visualized via Western blot using the anti-BAI1-CT antibody.


BAI1 + + – – – –
ΔNT – – + + – –
ΔNTPDZ – – – – + +
Biotin – + – + – +
250
150
75
Pull-down Strep
IB: BAI1-CT
250
150
75
Lysates
IB: BAI1-CT

3.4 Discussion

Screens of a PDZ domain proteomic array revealed that the CT of BAI1 can bind to a variety of PDZ domains from synaptic proteins. Along with the previously-reported BAI1 interactions with PSD-95 (Lim et al., 2002) and MAGI-1 (Shiratsuchi et al., 1998a), novel interactions were seen in our PDZ domain screen with MAGI-2, MAGI-3, Densin-180, SAP97 (DLG1), MALS-1 (Veli-1) and several syntrophins. All of these proteins are localized to synapses (Albrecht and Froehner, 2002; Apperson et al., 1996; Cho et al., 1992; Danielson et al., 2012; Ito et al., 2012; Jo et al., 1999; Valtschanoff et al., 2000). The ability of the BAI1-CT to selectively interact with PDZ domains from synaptic proteins suggests the possibility that BAI1 itself may be localized to synapses.

In terms of the functional significance of BAI1/PDZ interactions, truncation of the BAI1 PDZ-binding motif resulted in an attenuation of receptor signaling to Rho but had no effect on receptor stimulation of pERK, revealing that PDZ scaffold interactions with BAI1 can differentially regulate receptor coupling to the various signaling pathways downstream of the receptor. The functional importance of the PDZ-binding motif in regulating BAI1 signaling was further demonstrated by experiments revealing that MAGI-3 co-expression potentiated pERK activation by BAI1 only when the PDZ-binding motif was present. BAI1 signaling to ERK in these experiments was not mediated by $G\alpha_{12/13,}$ as co-expression with RGSp115 had no effect on BAI1-mediated increases in pERK. It is possible that BAI1 can increase pERK via coupling to G proteins other than $G\alpha_{12/13.}$ As mentioned in Chapter 1, GPCRs that couple to $G\alpha_{12/13}$ often possess the ability to signal through other G proteins, including $G\alpha_q$ and $G\alpha_{i/o}$, both

of which can mediate signaling to ERK (Goldsmith and Dhanasekaran, 2007; Riobo and Manning, 2005). Liberation of Gβγ subunits also did not appear to be involved in BAI1 signaling to ERK, as gallein, a small molecule inhibitor of Gβγ, did not attenuate BAI1-mediated pERK increases. Finally, as GPCRs can sometimes stimulate ERK via transactivation of RTKs (Liebmann, 2001), we tested whether BAI1 signaling to ERK might be mediated via transactivation of EGFR. However, no differences in BAI1-mediated ERK activation in the presence of the EGFR inhibitor AG1478 were observed. Thus, BAI1 stimulation of the ERK pathway did not seem to involve coupling to $G\alpha_{12/13}$, liberation of Gβγ subunits or transactivation of EGFR.

In terms of the mechanism(s) by which PDZ interactions might influence BAI1 signaling to ERK, we observed that removal of the BAI1 PDZ-binding motif resulted in a substantial increase in receptor association with β-arrestin2. Since arrestins are known to impair G protein coupling to many different GPCRs (Reiter and Lefkowitz, 2006), and also elicit their own signaling pathways, notably activation of ERK (DeWire et al., 2007), it is possible that PDZ interactions with BAI1 can modulate receptor signaling by controlling the access of arrestins to the receptor's cytoplasmic regions. Conversely, or perhaps concurrently, PDZ scaffolds might also recruit positive regulators of various signaling pathways into a complex with BAI1, thereby enhancing receptor signaling in a cell type-specific manner.

It is likely that the various PDZ domain-containing proteins shown to interact with the CT of BAI1 have a variety of cell-specific effects on receptor signaling, trafficking and localization. For example, one of the interacting partners from the array, densin-180, has been shown to control the trafficking of mGluR5 in the postsynaptic

density (Carlisle et al., 2011). Two of the other BAI1-interacting partners have been shown to differentially regulate agonist-induced internalization of the β1-adrenergic receptor: MAGI-2 promotes internalization, whereas PSD-95 reduces internalization of β1-AR (Xu et al., 2001). Furthermore, MAGI-3 can regulate ERK signaling by β_1AR (He et al., 2006), β_2AR (Yang et al., 2010), LPA(2) (Zhang et al., 2007), and frizzled (Yao et al., 2004). Another BAI1-interacting partner, SAP97, has been shown to regulate both the endocytosis and signaling to ERK of the corticotropin-releasing factor receptor 1 (Dunn et al., 2013). Interestingly, β2-syntrophin was shown to regulate Tiam1-Rac activity during cell-cell junction formation (Mack et al., 2012), and BAI1 was found to interact with Tiam1 to activate Rac (Duman et al., 2013). It is highly probable that BAI1 is found in different populations of neurons expressing distinct sets of these interacting PDZ partners, with these interactions differentially regulating BAI1 signaling, trafficking and subcellular localization depending on the cellular context.

The data shown in this chapter provide the first evidence that BAI1 is ubiquitinated. This observation is especially interesting since the ubiquitination appears to be activity-dependent, as ubiquitination was only seen upon removal of the NT, which correlates with enhanced receptor signaling. The first evidence of agonist-stimulated ubiquitination of a mammalian GPCR was reported for the β2-adrenergic receptor (Shenoy et al., 2001) and has subsequently been shown for a variety of other GPCRs including dopamine D4 receptor (Rondou et al., 2008), group 1 mGluRs (Moriyoshi et al., 2004), and melanocortin receptors (Cooray et al., 2011). Upon ubiquitination, receptors are often internalized into endosomes where the ubiquitin moieties may be removed, resulting in recycling back to the plasma membrane, or alternatively the

ubiquitin moieties can remain intact, resulting in degradation of the receptor (Marchese and Trejo, 2013). The ubiquitination of BAI1 may play a role in controlling receptor stability, as degradation could be a major mechanism by which BAI1 signaling is turned off, analogous to the importance of ubiquitination of PAR receptors following their irreversible proteolytic activation (Jacob et al., 2005). Furthermore, ubiquitination of GPCRs and the cellular machinery involved may also play a role in dictating receptor signaling. For example, ubiquitination was shown to play a role in CXCR4-induced ERK signaling, as ubiquitiantion was found to localize CXCR4 and its signaling components to plasma membrane microdomains that allow for enhanced pERK signaling (Malik et al., 2012). Therefore, ubiquitination may regulate both the stability and signaling of BAI1. The differential regulation of BAI1 by PDZ interactions, β-arrestin association, and ubiquitination in distinct cell types and/or at different points in development will be an intriguing area for future exploration.

CHAPTER IV: BAI1 is a component of the postsynaptic density

4.1 Introduction

Excitatory synapses are characterized by an electron-dense postsynaptic membrane thickness known as the postsynaptic density (PSD), which is comprised of a variety of receptors, ion channels, scaffold proteins, signaling molecules and cytoskeletal components (Feng and Zhang, 2009; Sheng and Hoogenraad, 2007). A majority of these excitatory synapses occur on small projections emanating from dendrites, known as dendritic spines, which are highly dynamic structures important for synaptogenesis, synaptic plasticity, learning and memory (Bourne and Harris, 2008). Synaptic plasticity is the ability of synapses to modulate their strength depending on activity. At many synaptic connections in the CNS, strong stimuli can result in an enhancement of synaptic strength, known as long-term potentiation (LTP), which correlates with spine enlargement and increased insertion of AMPA-type glutamate receptors (Matsuzaki et al., 2004). Conversely, weak stimuli can result in a decrease of synaptic strength known as long-term depression (LTD), which correlates with dendritic spine shrinkage and decreased insertion of AMPA-type glutamate receptors (Zhou et al., 2004).

Rapid morphological changes observed in dendritic spines during synaptic plasticity are controlled by changes in the actin cytoskeleton, which is mediated by many regulatory proteins, including the Rho GTPases (Schubert et al., 2006). Synaptogenesis and plasticity are highly regulated by Rho GTPases (Tolias et al., 2011), with the relevant upstream effectors of these proteins still being discovered. The studies shown in the preceding chapters have revealed that Rho activity can be activated downstream of BAI1. Along with Rho activation, we also observed BAI1-mediated increases in pERK, which

is also known to play a key role in controlling the formation and stabilization of dendritic spines (Goldin and Segal, 2003).

Although it is known that BAI1 is highly expressed in neurons, the function and subcellular localization of this receptor within neurons has remained elusive. Evidence towards a role of BAI1 as a synaptic protein has been implicated by the BAI1 C-terminus interaction with the IRSp53 (Oda et al., 1999), which is a key regulator of dendritic spines (Choi et al., 2005) that is thought to play a role in autism spectrum disorder (ASD) (Toma et al., 2011). Utilizing a PDZ domain proteomic array, we also uncovered a variety of C-terminal interacting partners for BAI1, all of which are known to be synaptic proteins, including PSD-95, a scaffold protein that regulates spine formation and shape (El-Husseini et al., 2000).

Further implicating the members of the BAI subfamily in synaptic regulation is the observation that the N-terminus of BAI3 can interact with the secreted protein C1ql3, with C1ql3 treatment of primary hippocampal neurons resulting in a decreased density of excitatory synapses in a BAI-specific manner (Bolliger et al., 2011). Interestingly, BAI3 was also shown to play a role in the regulation of dendrite arborization in cultured neurons, and *in vivo* knockdown of BAI3 resulted in an immature dendritic phenotype (Lanoue et al., 2013). These data all point to a role for the BAI family as regulators of synaptic morphology and function.

In the studies described in this chapter, we sought to assess the subcellular localization of BAI1 within neurons. We found that BAI1 is localized in synaptosomes and is highly enriched in the postsynaptic density, supporting a role for BAI1 as synaptic receptor. We also utilized mice lacking endogenous BAI1 to further explore the role of

BAI1 within the PSD. We observed that BAI1-knockout (KO) mice exhibit altered levels of multiple PSD proteins that play important roles in synaptic function. Moreover, since the N-terminal TSRs are conserved among the BAI family, we also explored the ability of C1ql3 to interact with BAI1. We uncovered a novel interaction between C1ql3 and BAI1, suggesting a possible role of C1ql3 as a ligand for BAI1.

4.2 Experimental Procedures

Antibodies- Antibodies against PSD-95 (Thermo Scientific), synaptophysin (Abcam), BAI1 (Thermo Scientific), CaMKII (Upstate Cell Signaling), MAGI-3 (Abcam), densin-180 (Santa Cruz), protein phosphatase 1 (Abcam), actin (Sigma), calcineurin (Abcam), GluN2b (Millipore), GluA1 (Millipore) were purchased from manufacturer. A distinct anti-BAI1-CT antibody (#17108) was custom-made by Pocono Rabbit Farm & Laboratory (Canadensis, PA) via injection of rabbits with a peptide (HSLTLKRDKAPKSS) derived from the human BAI1 C-terminus (amino acids 1305-1318), followed by affinity purification.

Cell Culture and transient plasmid transfections- HEK293T cells (ATCC) used for cell-based assays were cultured and maintained in complete media (DMEM containing 10% FBS and 1% penicillin/streptomycin) at 37 °C at 5% CO_2. Transfections were performed by incubating cells plated on 100mm x 20mm Cell Culture Dishes (Corning) with Lipofectamine 2000 (Invitrogen) and plasmid DNA for 3-5 hours in serum free DMEM. The transfection reaction was stopped by addition of complete media. Experiments were performed 24 hours post-transfection.

Plasmids- Expression vectors for BAI1 wild-type (Kaur et al., 2003; Kaur et al., 2005) and the GST-C1ql3 plasmid was a gift from Thomas Sudhoff (Stanford University).

Western Blot- Samples were run on 4-20% SDS-PAGE gels (Invitrogen) for 2 hours at 130 V and then transferred to a nitrocellulose membrane (Bio-Rad) for 2 hours at 30 V. The membrane was blocked in blot buffer containing 2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM Hepes, at PH 7.4 for 30 minutes at room temperature. The membrane was incubated with primary antibody in blot buffer for 1 h at room temperature, followed by three 5 min washes. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (GE Healthcare) for 30 minutes at room temperature followed by three 5 min washes with blot buffer. Blots were visualized via enzyme-linked chemiluminescence using the Supersignal® West Pico Chemiluminescent Substrate (Pierce).

Co-immunoprecipitation of PSD-95/BAI1 complex from mouse brain homogenate- Brain tissue was extracted from adult mice and homogenized in ice-cold buffer containing 20mM HEPES pH 7.4, 50 mM NaCl, 1 mM EDTA and a protease inhibitor cocktail tablet (Roche Diagnostics). The resulting homogenate was then centrifuged at 2,000 x g for 10 minutes at 4 °C to remove nuclei and cell debris. Membrane proteins were extracted in Tris Buffer (50mM Tris, pH 7.4, 5 mM $MgCl_2$, 1mM EGTA, 150 mM NaCl, 1mM EDTA, one protease inhibitor cocktail tablet and 2% *n*-Dodecyl-β-D-maltoside (Sigma) for 3 hours at 4 °C and debris was cleared by centrifugation. The resulting supernatant was rotated at 4° C with Protein A/G (Thermo Scientific) with or without anti-PSD-95 antibody (Thermo Scientific) overnight. Protein A/G beads were washed 3 times with ice-cold buffer containing 20mM HEPES pH 7.4, 50 mM NaCl, 1 mM EDTA and a protease inhibitor cocktail tablet, resuspended in 40 μL 2x sample

buffer and heated to 95 °C for 10 minutes. BAI1 and PSD-95 were detected via the Western blot procedure described above.

GST Fusion Protein Pull-down Assay- GST fusion proteins were purified from bacteria using glutathione-Sepharose 4B beads (Sigma) and resuspended in resuspension buffer [50 mM NaCl, 10 mM Hepes and 1 mM EDTA and 1 protease inhibitor cocktail tablet (Roche Diagnostics)]. Equal amounts of fusion proteins conjugated on beads were incubated with 500 μL of soluble lysates from HEK293T cells transfected with full-length BAI1 for 60 minutes at 4 °C with rotation. The beads were extensively washed in Lysis Buffer and proteins were eluted from beads in 40 μL 2x sample buffer. Proteins were then resolved on SDS-PAGE and detected via Western blot as mentioned above.

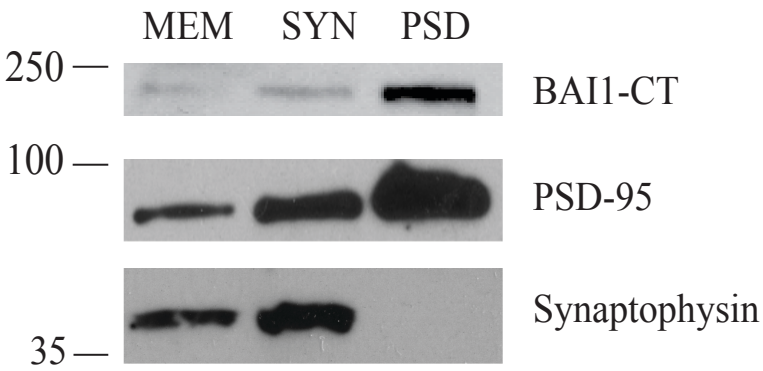
Preparation of Synaptosomes and Postsynaptic Density Fractions- As previously described (Dunkley et al., 2008), brain tissue was extracted from adult mice and homogenized in an ice-cold isotonic sucrose solution. The resulting homogenate was then centrifuged at 1,000 x g for 10 minutes at 4 °C to remove nuclei and cell debris. The supernatant was then placed on a Percoll (Sigma-Aldrich)/sucrose gradient and centrifuged at 31,000 x g for 5 minutes at 4 °C. The fraction located at the interface between the 15% and 23% Percoll gradient was extracted, diluted in ice-cold sucrose/EDTA [0.32 M sucrose, 1 mM EDTA, 5 mM Tris, Ph7.4], and centrifuged at 20,000 x g for 30 minutes at 4 °C. The pellet was resuspended in an isotonic buffer yielding the synaptosome fraction. The postsynaptic density fraction was then isolated via a 1% Triton X-100 extraction followed by high-speed centrifugation at 4 °C.

4.3 Results

4.3.1 BAI1 is enriched in the postsynaptic density

Since BAI1 is known to be present in neurons, and all of the PDZ domains identified as BAI1-CT interactors in the screens of the PDZ proteomic array were from synaptic proteins, we assessed whether BAI1 might be enriched in synaptosomal (SYN) and/or postsynaptic density (PSD) fractions of mice brains. As shown in Fig. IV-1, these studies revealed BAI1 to be somewhat enriched in synaptosomes, but most strongly concentrated in PSD fractions, very similar to the pattern of enrichment observed for PSD-95, a known postsynaptic marker and one of the BAI1-interacting proteins detected in our proteomic screen. In contrast, immunoreactivity for synaptophysin, a presynaptic marker, was found to be enriched in synaptosomes, but completely absent from the PSD fractions, thereby verifying the purity of the PSD fraction prepared for these experiments.

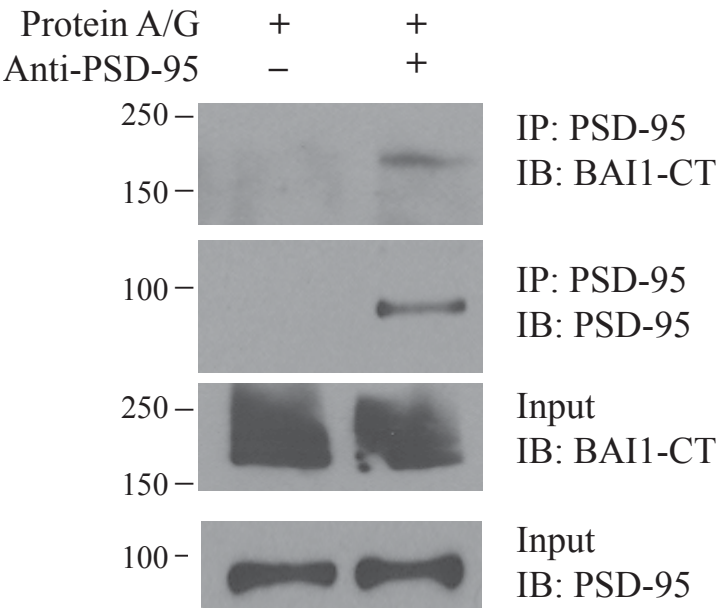
Figure IV-1: BAI1 is enriched in the postsynaptic density indicating new role of BAI1 as a synaptic GPCR. Synaptosome and postsynaptic density fractions were prepared from adult mice brains via a Percoll/sucrose gradient and 1% Triton X-100 extraction. Lysed crude membrane ("MEM"), synaptosome ("SYN"), and postsynaptic density ("PSD") fractions were probed via Western blot with antibodies for BAI1-CT (Thermo Scientific), PSD-95, and synaptophysin. Molecular weights of markers are indicated in kDa.


MEM
SYN
PSD
250
100
35
BAI1-CT
PSD-95
Synaptophysin

4.3.2 BAI1 co-immunoprecipitates with the postsynaptic density marker PSD-95

The most intensively-studied of all the PDZ proteins that were found to interact with the BAI1-CT in the PDZ proteomic array screen is PSD-95, which is one of the most abundant proteins in the PSD and known to be crucial in the regulation of spine formation and synaptic function (Opazo et al., 2012). To assess whether an interaction between BAI1 and PSD-95 might occur in native brain tissue, we performed co-immunoprecipitation experiments examining whether complexes of BAI1 and PSD-95 could be detected in mouse brain homogenates. As shown in Figure IV-2, BAI1 was found to robustly co-immunoprecipitate with PSD-95, providing evidence for complex formation between these proteins in native brain tissue.

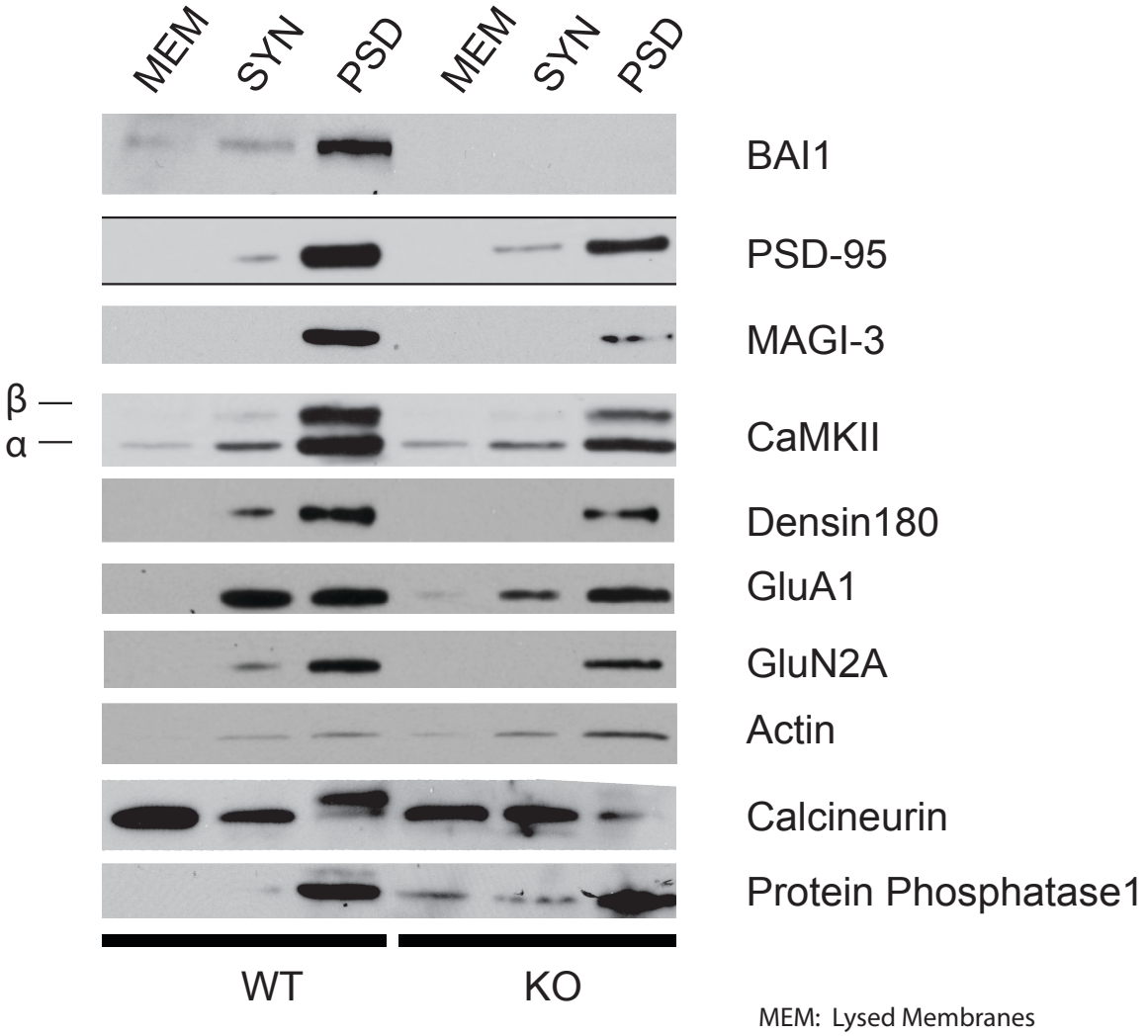
Figure IV-2: BAI1 co-immunoprecipitates with postsynaptic density marker PSD-95. Co-immunoprecipitation of BAI1 and PSD-95 from mouse brain homogenates. Crude membrane fractions were collected by homogenization and centrifugation. Membrane proteins were solubilized in 2% Dodecyl-β-D-maltoside, immunoprecipitated with Protein A/G beads +/- anti-PSD-95 antibodies and probed via Western blot analyses with anti-BAI1-CT (Thermo Scientific) and anti-PSD-95 antibodies.


Protein A/G + +
Anti-PSD-95 – +
250
150
IP: PSD-95
IB: BAI1-CT
100
IP: PSD-95
IB: PSD-95
250
150
Input
IB: BAI1-CT
100
Input
IB: PSD-95

4.3.3 Genetic knockdown of BAI1 alters expression levels of postsynaptic density associated proteins

Since we observed that BAI1 is enriched in the postsynaptic density and interacts with a variety of synaptic proteins known to be involved in PSD maintenance and structure, we sought to determine if genetic deletion of BAI1 in mice might have any effect on other proteins enriched in the PSD. Thus, PSD fractions were prepared from WT mice as well as BAI1 knockout (BAI1-KO) mice that were developed in the laboratory of Erwin Van Meir. An approximately 50 percent reduction PSD-95 levels was observed in the PSD fractions from the BAI1-KO mice relative to WT PSD fractions (Figure IV-3). Furthermore, dramatic reductions in the levels of MAGI-3 and densin-180, which like PSD-95 were found in our proteomic screens to interact with BAI1, were also observed in the PSD fractions from BAI1-KO mice. Additionally, modest reductions in the BAI1-KO PSD fraction were also observed for Ca2+/calmodulin-dependent protein kinase α and β isoforms, calcineurin (protein phosphatase 2A), the α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluA1, and the *N*-Methyl-D-aspartate (NMDA) receptor subunit GluN2A. In contrast, no differences were seen in the levels of protein phosphatase 1, and there was a modest increase in actin levels in the PSD fractions from the BAI1-KO mice.

Figure IV-3: Genetic knockout of BAI1 alters the expression levels of various proteins involved in the regulation of the postsynaptic density. Synaptosome and postsynaptic density fractions were prepared from adult mice brains via a Percoll/sucrose gradient and 1% Triton X-100 extraction. Lysed crude membrane ("MEM"), synaptosome ("SYN"), and postsynaptic density ("PSD") fractions from both wild-type mice and BAI1-KO mice were probed via Western blot with antibodies for BAI1-CT (Thermo Scientific), PSD-95, MAGI-3, CaMKII, Densin180, AMPA receptor subunit GluA1, NMDA receptor subunit GluN2A, actin, protein phosphatase 2 (calcineurin), and protein phosphatase 1. The positions and sizes of molecular mass markers are indicated in kDa.

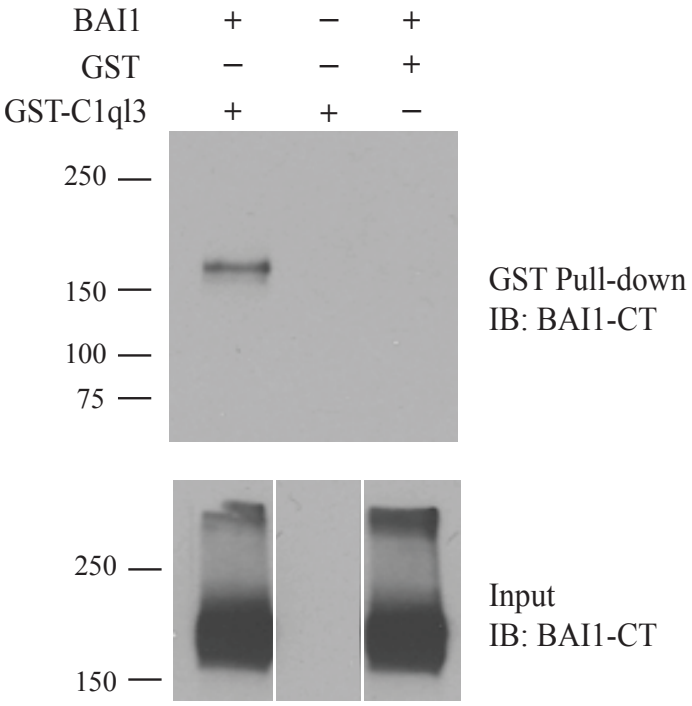


MEM: Lysed Membranes
SYN: Synaptosomes
PSD: Postsynaptic Density

4.3.4 BAI1 interacts with the synaptic protein C1ql3

As mentioned in Chapter 1, the TSRs on the N-terminus of BAI3 were shown to be capable of high-affinity binding to complement-like C1ql proteins (Bolliger et al., 2011). Since the TSRs are fairly well-conserved between the BAI family members, we sought to determine if C1ql proteins might be able to interact with BAI1. Therefore, we utilized a pull-down assay with a recombinant GST fusion protein corresponding to the globular C1q domain of C1ql3. As shown in Figure IV-4, a robust association was observed between GST-C1ql3 and BAI1, whereas no interaction was seen between GST alone and BAI1.

Figure IV-4: BAI1 interacts with the synaptic protein C1ql3. GST-C1ql3 fusion protein, but not GST alone, pulls down transfected full-length BAI1 from solubilized HEK293T cell lysates. Representative image shown for input was cropped from the same Western blot. Molecular mass markers (in kDa) are shown on the left.


BAI1 + − +
GST − − +
GST-C1ql3 + + −
250
150
100
75
GST Pull-down
IB: BAI1-CT
250
150
Input
IB: BAI1-CT

4.4 Discussion

The ability of the BAI1-CT to selectively interact with PDZ domains from synaptic proteins, as shown in the studies described in Chapter 3, suggested that BAI1 itself might be localized to synapses. Indeed, we found that BAI1 is highly enriched in postsynaptic density fractions prepared from adult mouse brain and that complexes between BAI1 and PSD-95 could be co-immunoprecipitated from brain tissue. Furthermore, it is interesting to note that a reduction in PSD-95 was found in the postsynaptic density fractions of BAI1-KO mice. It has been established that PSD-95 knockout mice exhibit aberrant synaptic plasticity and deficiencies in learning and memory (Migaud et al., 1998), which are also observed in BAI1-KO mice (Zhu et al, in submission). Along with PSD-95, the levels of two other BAI1-CT-interacting proteins, MAGI-3 and Densin-180, were also reduced in the postsynaptic density fractions of BAI1-KO mice. Furthermore, slight reductions were seen for CaMKII, calcineurin, the AMPA receptor subunit GluA1, and NMDA subunit GluN2A. All of these proteins are key mediators of dendritic spine formation, maintenance, and regulation of synaptic plasticity (Fortin et al., 2012).

AMPA and NMDA receptors are particularly important to discuss in context of synaptic plasticity. As previously mentioned, synaptic plasticity is the strengthening or weakening of synapses depending on stimuli. Briefly, high frequency stimulation of a synapse results in strong elevations of postsynaptic Ca^{2+} currents through NMDA receptors, which leads to phosphorylation-dependent mechanisms resulting in AMPA receptor insertion into the PSD and actin cytoskeleton rearrangements leading to spine

growth. Conversely, low frequency stimulation results in lower levels of postsynaptic Ca^{2+} through NMDA receptors, which leads to dephosphorylation-dependant mechanisms resulting in decrease AMPAR insertion into the plasma membrane and spine shrinkage (Malenka and Bear, 2004; Sanderson and Dell'Acqua, 2011). The level of AMPA receptors anchored at the postsynaptic density is important because this dictates the relative strength of synaptic transmission at the synapse (Opazo et al., 2012).

It is interesting to note that many of the PSD proteins were decreased in the fractions from BAI1-KO mice. Since the samples were normalized for total protein between WT and BAI1-KO mice it would presumably be impossible for all proteins to be decreased, as decreases in the relative proportion of one protein must necessarily be counterbalanced by increases in the relative proportion of another protein. In this regard, it should be noted that the levels of actin, which is one of the most abundant proteins in the PSD (Walikonis et al., 2000), did markedly increase in the BAI1-KO mice compared to the WT. It is possible that the BAI1-KO mice have an overall dysregulation of the actin cytoskeleton, presumably due to aberrations of Rho, Rac, and/or ERK signaling, with this dysregulation resulting in increased PSD-associated actin and reduced PSD localization of other key PSD proteins. Aside from signaling, BAI1 may also be serving as a key scaffold for various PDZ proteins that are important in maintaining the architecture of the PSD. For example, we found a novel interaction between BAI1 and densin-180, one of the proteins that is reduced in the PSD fractions from BAI1-KO mice. Densin-180 has been shown to interact with CaMKII (Strack et al., 2000), which is also reduced in BAI1-KO mice, and CaMKII is another major synaptic protein that regulates plasticity and has a direct impact on the actin cytoskeleton (Hoffman et al., 2013).

Our observation that BAI1 is highly enriched in the PSD is particularly interesting when considered in combination with our signaling data, which reveal that BAI1 can activate Rho via coupling to $G\alpha_{12/13}$. Rho GTPases are well-established regulators of neuronal migration, synapse formation, PSD structure and spine morphogenesis (Tolias et al., 2011), as well as synaptic plasticity and memory formation (Rex et al., 2009). Along with Rho activation, we also observed BAI1-mediated increases in pERK, with this signaling being markedly enhanced in the presence of MAGI-3. Interestingly, ERK is known to play a key role in controlling the formation and stabilization of dendritic spines (Goldin and Segal, 2003), thereby regulating synaptic plasticity, learning and memory (Giovannini, 2006; Sweatt, 2004). These data together are providing evidence towards a role for BAI1 in regulation and maintenance of the postsynaptic density. To further support this role, the BAI1-KO mice also have reduced lengths of their PSDs, along with altered hippocampal plasticity and defects in spatial learning and memory (Zhu et al, in submission). Taken together, these data suggest a role for BAI1 in the regulation and maintenance of the postsynaptic density.

We also uncovered an interaction between BAI1 and the complement-like protein C1ql3. The C1ql family members (1-4) are approximately 27-35 kDa secreted proteins related to the C1q proteins of the complement system and the Cbln subfamily, which have been collectively termed “transneuronal cytokines” (Iijima et al., 2010; Yuzaki, 2008). Each C1ql protein contains a C-terminal C1q globular domain as well as a collagen-like domain and two cysteine residues near the signal sequence, which allow for homo- and heterodimerization (Iijima et al., 2010). The C1ql proteins are highly expressed in the central nervous system, with C1ql3 mRNA found most prominently in

the brain and kidney (Iijima et al., 2010; Yuzaki, 2008). In the brain, the C1ql subfamily is mainly expressed in neurons and C1ql2 and C1ql3 are highly expressed in the dentate gyrus of the hippocampus (Iijima et al., 2010). Bolliger *et al.* reported that incubating primary hippocampal neurons with low concentrations of C1ql3 decreased the density of excitatory synapses (Bolliger et al., 2011). This detrimental effect of C1ql3 on excitatory synapse density was inhibited by addition of a BAI3 fragment containing TSRs, which is the interaction site between BAI3 and C1ql3. Interestingly, no significant difference in the length and branching of dendrites was observed upon incubation with C1ql3. These data indicate a possible role for C1ql proteins affecting synapse density via a BAI-dependent mechanism. Future studies to uncover how C1ql3 might affect BAI1 signaling and the physiological importance of this interaction will be of great interest. It is possible that C1ql3 regulates BAI1 in multiple ways. For instance, C1ql3 might activate BAI1-mediated signaling, thus acting as a traditional agonist. It is also possible that C1ql3 might preferentially activate just one of the pathways that BAI1 has been shown to signal through, thereby acting as a biased agonist (Zheng et al., 2010). Alternatively, C1ql3 could potentially be an antagonist, disrupting the interaction between BAI1 and a different molecule that acts as an agonist. Furthermore, C1ql3 could be an inverse agonist, which might attenuate constitutive activity that we observed for BAI1. The BAI1-C1ql3 interaction could also conceivably mediate stabilization of BAI1 at the plasma membrane by regulating association with intracellular interacting partners, such as β-arrestins, PDZ domain-containing proteins, or levels of ubiquitination. Aside from the functional aspects of this interaction, further defining the regions of interaction

between C1ql3 and the N-terminus of BAI1 may help facilitate the development of novel BAI family modulators with possible clinical relevance.

CHAPTER V: Further discussion and future directions

5.1 Signaling and mechanism of activation for BAI1

The research presented in this dissertation has shed light on the signaling and regulation of brain-specific angiogenesis inhibitor-1. For example, this work has provided the first evidence that BAI1 can couple to G proteins. Specifically, we demonstrated that BAI1 can couple to $G\alpha_{12/13}$ to increase activation of the Rho pathway. Previous studies had demonstrated BAI2 signaling via an over-expressed promiscuous G protein, $G\alpha_{16}$ (Okajima et al., 2010). However, the work shown here represents the first evidence for coupling of a member of the BAI sub-family to endogenous G proteins and furthermore sheds light on the G protein coupling preferences of BAI1. G protein-independent mechanisms of signaling have also been demonstrated for BAI1, notably an interaction between the C-terminus of BAI1 and the intracellular adaptor protein ELMO, which interacts with Dock180 to form a functional GEF that activates Rac (Park et al., 2007). BAI3 can also interact with ELMO-Dock180 to activate the Rac pathway (Lanoue et al., 2013). Furthermore, BAI1 has recently been shown to signal to Rac through distinct C-terminal interactions with the Rac-GEF Tiam1 and the polarity protein Par3 (Duman et al., 2013). The work shown here also revealed a $G\alpha_{12/13}$-independent signaling pathway mediated by BAI1 to increase phosphorylation of ERK, although further research will be necessary to determine the molecular mechanism(s) underlying BAI1-mediated ERK activation.

The studies presented here demonstrated that the N-terminus of BAI1 can remain associated with the 7TM region non-covalently, consistent with reports from work on other adhesion GPCRs indicating that the N-terminal and 7TM regions can remain

associated for some period of time following autoproteolysis at the GPS motif (Gray et al., 1996; Krasnoperov et al., 2002; Krasnoperov et al., 1997; Kwakkenbos et al., 2002; Lin et al., 2004; Paavola et al., 2011). Removing the N-terminus of BAI1 greatly enhanced BAI1-mediated activation of Rho and also enhanced receptor interaction with β-arrestin2 and receptor ubiquitination. Taken together, this set of observations suggests that removal of the N-terminus results in activation and downstream signaling of BAI1. An increase in activity following removal of the N-terminus has been displayed for other adhesion GPCRs including GPR56 (Paavola et al., 2011), CD97 (Ward et al., 2011), and another BAI family member, BAI2 (Okajima et al., 2010). This confluence of findings suggests a possible conserved mechanism of activation for adhesion GPCRs, wherein removal or conformational change of the N-terminus by a ligand may relieve an inhibitory restriction being imposed on the 7TM region of the receptor, thus allowing for the initiation of downstream receptor signaling.

The work shown here revealed that BAI1 signaling can be regulated by PDZ protein interactions with the C-terminal PDZ-binding motif of BAI1. Removal of the PDZ-binding motif of BAI1 resulted in attenuation of Rho activation, but had no apparent effect on the receptor's ability to activate the ERK pathway. This indicates that the PDZ-binding motif of BAI1 may play a role in differentially regulating receptor coupling to the various signaling pathways. Moreover, addition of MAGI-3 potentiated BAI-mediated downstream phosphorylation of ERK only when the PDZ-binding motif was present. The elucidation of multiple new PDZ-binding motif interacting proteins for BAI1 has opened the door for extensive new research with regards to C-terminal regulation of trafficking, localization, signaling, and allosteric modulation of this

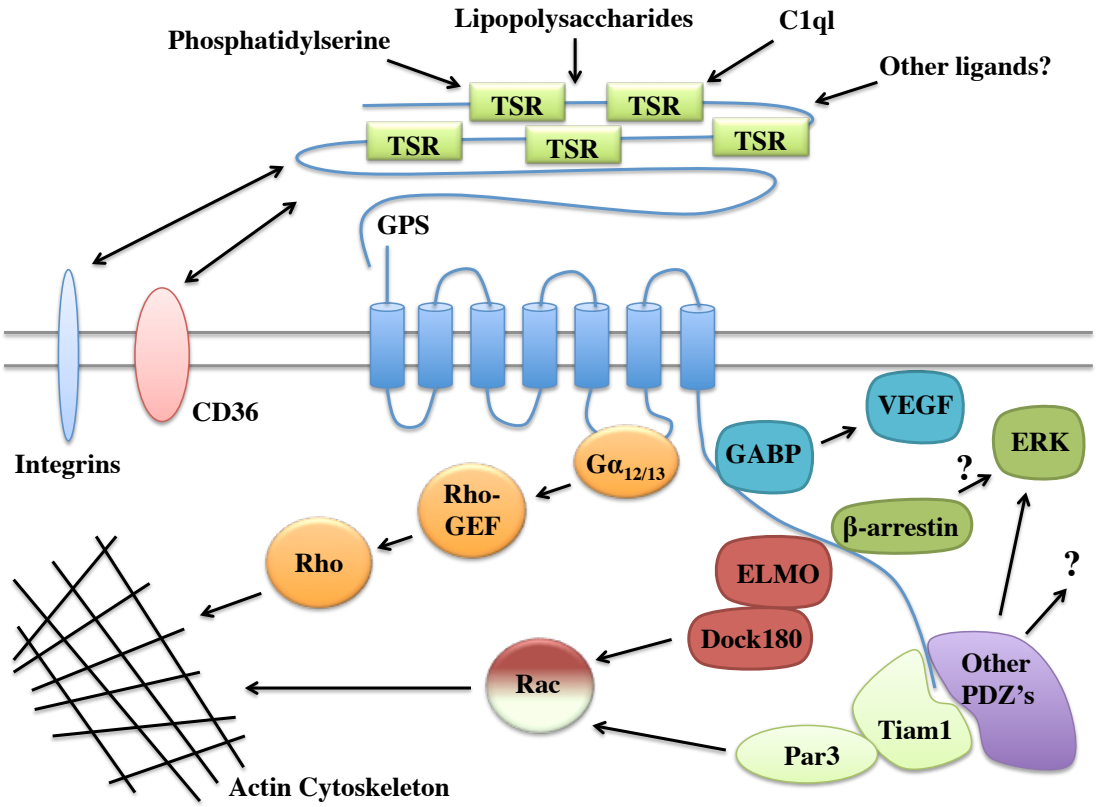
receptor. A comprehensive list of known binding partners of the BAI subtypes is presented in Table V-1, and a schematic representing various signaling pathways and known-binding partners of the BAI family of adhesion GPCRs is shown in Figure V-1.

Table V-1: Comprehensive list of interacting partners that have been identified for BAI1-3. The region of interaction and functional significance are indicated for each partner.

	NT Interaction	Region	Function
BAI1	CD36	TSRs	Inhibits angiogenesis (Kaur et al., 2009)
	$\alpha_v\beta_5$ Integrin	TSRs	Anti-proliferation of endothelial cells (Koh et al., 2004)
	Phosphatidylserine	TSRs	Engulfment of apoptotic cells (Park et al., 2007)
	MMP-14	Between TSR 1&2 AAs S326-L327	Cleaves BAI1 NT (Cork et al., 2012)
	Lipopolysaccharides	TSRs	Bacterial Internalization (Das et al., 2011)
BAI2	Furin	AAs R296-S297	Cleaves BAI1 NT (Okajima et al., 2010)
BAI3	C1q-like Proteins	TSRs	Regulation of synaptic density (Bolliger et al., 2011)
	CT Interaction	**Region**	**Function**
BAI1	ELMO	α-helix region	Rac GEF (Park et al., 2007)
	TIAM1	PDZ-Binding Motif	Rac GEF (Duman et al., 2013)
	Par3	PDZ-Binding Motif	Cellular Polarity (Duman et al., 2013)
	MAGI-3	PDZ-Binding Motif	Potentiate ERK (Stephenson et al., 2013)
	MAGI-1, MAGI-2, PSD-95, INADL, SAP97, Chapsyn-110,	PDZ-Binding Motif	Unknown (Lim et al., 2002), (Stephenson et al.,

	MALS-1, Densin-180, PAPIN, Syntrophins		2013), (Shiratsuchi et al., 1998a)
	β-arrestin2	C-terminus	Signal regulation (Stephenson et al., 2013)
	BAP-2/IRSp53	Proline rich region	Unknown (Oda et al., 1999)
	BAP-3	C-terminus	Unknown (Shiratsuchi et al., 1998b)
	BAP-4	C-terminus	Unknown (Koh et al., 2001)
BAI2	Glutaminase interacting protein	C-terminus	Unknown (Zencir et al., 2011)
	GA-binding protein gamma	C-terminus	Transcriptional regulation of VEGF (Jeong et al., 2006)
BAI3	ELMO	α-helix region	Rac GEF (Lanoue et al., 2013)

Figure V-1: Various signaling pathways and known-binding partners of the BAI family of adhesion GPCRs. BAI1 activates Rac via an ELMO/Dock180 C-terminal interaction upon binding to exposed phosphatidylserine on apoptotic cells (Park et al., 2007) and lipopolysaccharides (Das et al., 2011). BAI1 can also activate Rac via Tiam1/Par3 complex association with the C-terminal PDZ-binding motif (Duman et al., 2013). BAI1 was shown to activate Rho via $G\alpha_{12/13}$ activation of p115RhoGEF and downstream phosphorylation of ERK possibly through association with β-arrestins (Stephenson et al., 2013). BAI1 has also been shown to bind to a variety of PDZ domains that may also regulate signaling and localization (Stephenson et al., 2013). BAI2 suppresses vascular endothelial growth factor (VEGF) expression via interaction with GA-binding protein gamma (GABP) (Jeong et al., 2006). BAI2 was found to promote NFAT activation via a promiscuous G protein, $G\alpha_{16}$ (not shown) (Okajima et al., 2010). BAI3 was also found to activate Rac through ELMO (Lanoue et al., 2013) and bind to C1ql proteins via its N-terminal TSRs (Bolliger et al., 2011). Furthermore, the N-terminus of BAI1 has also been shown to interact with CD36 (Kaur et al., 2009) and integrins (Koh et al., 2004). Although the BAI subfamily of adhesions GPCRs are highly conserved, the generality of these interactions is not known.


Phosphatidylserine
Lipopolysaccharides
C1ql
Other ligands?
TSR
TSR
TSR
TSR
TSR
GPS
CD36
Integrins
Gα12/13
Rho-GEF
Rho
GABP
VEGF
ERK
?
β-arrestin
ELMO
Dock180
Rac
Other PDZ's
?
Tiam1
Par3
Actin Cytoskeleton

5.2 Possible roles for BAI1 in the postsynaptic density

The work presented in this dissertation also provides evidence towards a role of BAI1 as a synaptic receptor. Most of the research on BAI1 until recently has focused on the ability of this receptor to inhibit experimental angiogenesis and tumor formation (Duda et al., 2002; Kang et al., 2006; Kaur et al., 2005; Kaur et al., 2009; Nishimori et al., 1997) and inhibit the growth of tumors derived from gliomas and renal cell carcinomas (Izutsu et al., 2011; Kaur et al., 2009; Kudo et al., 2007; Xiao et al., 2006; Yoon et al., 2005). Moreover, BAI1 was also identified in macrophages as a receptor upstream of the Rac-GEF ELMO-Dock180 pathway that plays a critical role in the engulfment of cells that have undergone apoptosis (Park et al., 2007). However, given that BAI1 is expressed most abundantly in neurons in the CNS (Shiratsuchi et al., 1997) the lack of information about the subcellular localization and signaling capability of BAI1 in neurons represented a major gap in our understanding of this receptor. We demonstrated that BAI1 is enriched in the postsynaptic density and can interact with a variety of synaptic proteins, including PSD-95 and MAGI-3, and that BAI-KO mice exhibit reduced levels of these proteins and other important synaptic proteins in PSD fractions. Furthermore, we uncovered a novel interaction between BAI1 and the complement-like protein C1ql3, providing the first evidence of a possible BAI1 ligand with known physiological effects on neurons.

In parallel with the research described in this dissertation, Duman and colleagues found BAI1 to be enriched in dendritic spines and demonstrated that BAI1 directs Par3 localization to spines to activate Rac1 via Tiam1 and the Par3 polarity complex (Duman

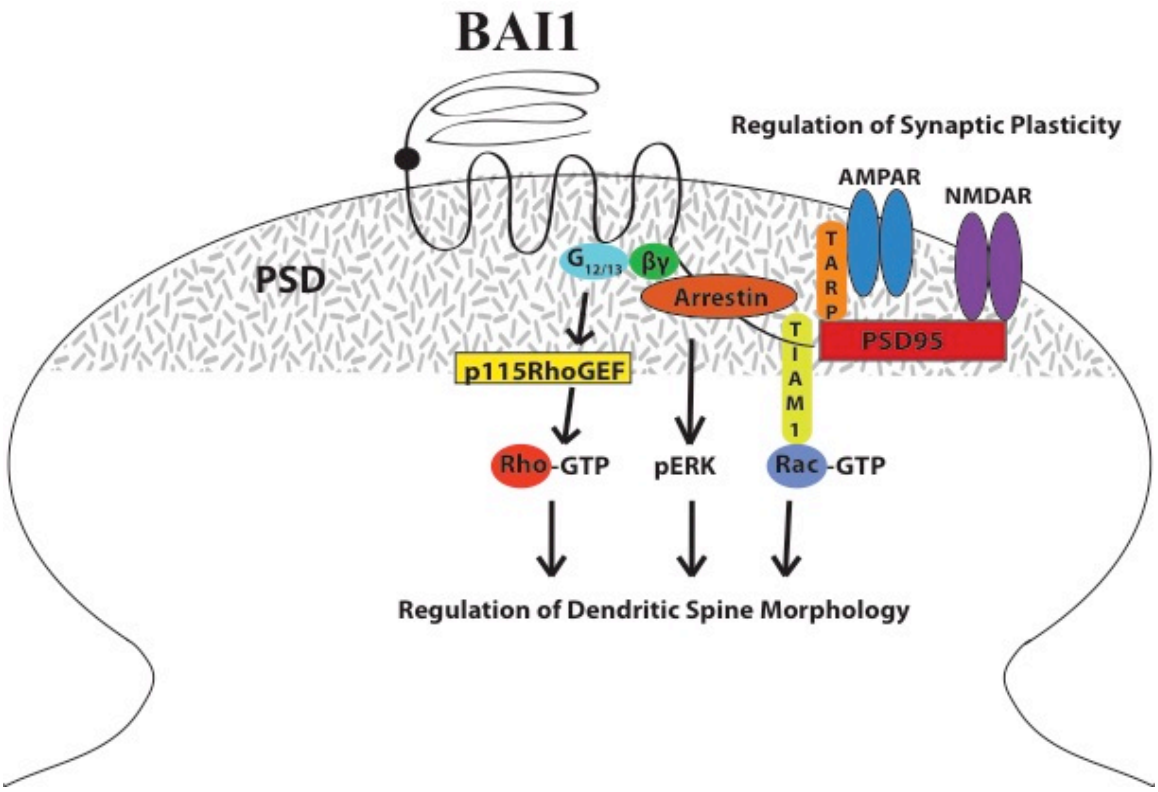
et al., 2013). The work from Duman *et al.* also showed that knockdown of BAI1 in cultured neurons caused a reduction in spine density and less mature phenotype of the remaining spines. Since Rho GTPases are known regulators of dendritic spine maintenance and synaptic plasticity (Rex et al., 2009; Tolias et al., 2011), and BAI1 has been shown to signal to both Rho and Rac, the downstream signaling pathways to which BAI1 can couple are consistent with a role for this receptor in the regulation of spine maturation and PSD formation.

Aside from the signaling capabilities of BAI1, we have uncovered a variety of C-terminal PDZ domain interactions for BAI1, and it is plausible that BAI1 might influence dendritic spine morphology and synaptogenesis in part by controlling the spatial orientation of these various synaptic PDZ partners. For example, PSD-95 is a well-known regulator of spine morphology and synaptic plasticity (Migaud et al., 1998; Steiner et al., 2008) and interacts with a variety of proteins that modulate actin dynamics (Fortin et al., 2012). Furthermore, PSD-95 plays a major role in receptor trafficking within the postsynaptic density for both AMPA receptors and their associated TARP proteins (Opazo et al., 2012) and NMDA receptors (van Zundert et al., 2004), underlying the regulation of synaptic plasticity. Newly emerging evidence indicates that BAI1 is regulating the stability of PSD-95 by interacting with and inhibiting the activity of Mdm2, an E3 ubiquitin ligase, which correlates with decreases PSD-95 in the PSD of BAI1-KO mice (Zhu et al., in submission).

Another BAI1-interacting protein, SAP97, plays a crucial role in sorting and delivery of NMDA receptors to the synapse (Jeyifous et al., 2009) and overexpression of SAP97 increases the synaptic localization of both NMDA and AMPA receptors (Howard

et al., 2010). Moreover, Chapsyn-110 (also known as PSD-93) interacts with the BAI1-CT and has been shown to enhance cell surface expression of GluN2A and GluN2B subunits of the NMDA receptor (Cousins et al., 2008) and enhance AMPA receptor synaptic currents (Elias et al., 2006). Another possible novel interaction observed was found between BAI1-CT and densin-180, a synaptic scaffold protein that plays a role in dendrite and arborization (Quitsch et al., 2005). In addition to the aforementioned PDZ domain-mediated interactions, the BAI1 C-terminus has also been demonstrated to interact with the insulin receptor substrate 53 (IRSp53; also known as 'BAI1-associated protein 2" or BAIAP2) (Oda et al., 1999). When this interaction was identified, little was known about the cellular functions of IRSp53 and no physiological significance was established for its interaction with BAI1. Over the past decade, however, IRSp53 has been demonstrated to be a key regulator of dendritic spines (Choi et al., 2005) and suggested to play a role in autism spectrum disorder (ASD) (Toma et al., 2011). Thus, the ability of BAI1 to signal to the Rho, Rac, and ERK pathways and the interactions of BAI1 with numerous important synaptic proteins suggest a possible role for BAI1 as a master regulator of dendritic spine morphology and PSD formation & maintenance (Figure V-2).

Figure V-2: Novel role of BAI1 as a regulator of the postsynaptic density. BAI1 is enriched in the postsynaptic density and can signal to Rho, ERK and Rac. BAI1 can also interact with a variety of synaptic PDZ proteins that are known to play crucial roles in spine morphology, PSD maintenance and regulation of synaptic plasticity.


BAI1
Regulation of Synaptic Plasticity
AMPAR
NMDAR
PSD
$G_{12/13}$
βγ
Arrestin
TARP
PSD95
p115RhoGEF
TIAM1
Rho-GTP
pERK
Rac-GTP
Regulation of Dendritic Spine Morphology

5.3 C1ql3 as a possible endogenous ligand for BAI1

Various N-terminal binding partners have been uncovered for several members of the adhesion GPCR family, but only a few of these interacting partners have been found to induce receptor signaling (Paavola and Hall, 2012). For example, collagen III was shown to interact with the N-terminus of GPR56 and elicit activation of Rho in NIH 3T3 cells (Luo et al., 2011). Furthermore, the N-termini of Celsr2 and Celsr3 were shown to undergo homophilic trans-trans interactions to stimulate calcium transients (Shima et al., 2007) and N-termini of GPR56 were shown to undergo homophilic trans-trans interactions to activate Rho via $G\alpha_{12/13}$ (Paavola et al., 2011). Within the BAI family, the BAI1 N-terminus was shown to interact with phosphatidylserine via its TSRs to elicit activation of Rac through a C-terminal interaction with the ELMO/Dock-180 complex (Park et al., 2007), though it remains to be determined if this interaction can also promote G protein-dependent signaling. The BAI3 N-terminal TSRs were also found to interact with the complement-like secreted proteins C1ql1-4, (Bolliger et al., 2011). The effects of C1ql interaction on BAI3-mediated signaling are not yet known, but it was found that incubation of cultured hippocampal neurons with C1ql3 resulted in a robust decrease in synaptic density that could be blocked by addition of the TSR fragment of BAI3 (Bolliger et al., 2011). Due to the fairly high sequence conservation between the TSRs of the BAI family N-termini, we tested whether C1ql3 could interact with BAI1 and found that these two proteins can indeed associate. This finding suggests that C1ql3 and the other C1ql proteins may be ligands for the BAI family of adhesion GPCRs. Future studies will be

necessary to determine if association with C1ql3 can provoke or alter BAI1-mediated signaling responses.

5.4 Potential drug development strategies for BAI1

As described in the first chapter, G protein-coupled receptors in general are outstanding drug targets. Since adhesion GPCRs are the largest family of orphan GPCRs and play important roles in a diverse range of physiological functions, these receptors are especially attractive targets for novel therapeutics. Achieving an understanding of these receptors with regard to potential ligands and signaling pathways is an important step toward the targeting of these receptors for therapeutic purposes. The identification of signaling outputs that can be utilized in high-throughput screening approaches is an important step in promoting the therapeutic targeting of a receptor, and one goal of the studies described in this dissertation was to elucidate a G protein-dependent pathway that can be used as an output for BAI1 signaling. High-throughput screening to find small-molecule agonists for adhesion GPCRs is indeed tractable, as recent screens of the adhesion GPCR GPR97 revealed beclomethasone dipropionate as a small molecule ligand for GPR97 (Gupte et al., 2012). The advances described in this dissertation may facilitate similar screens in the future to identify novel small molecule ligands for BAI1.

In addition to developing drug-like compounds that directly activate BAI1, it might also be possible to target BAI1 regulators and/or the signaling pathways downstream of the receptor for therapeutic purposes. It is possible that small molecules could be developed that target the region of interaction between BAI1 and endogenous

ligands, including the TSRs on the BAI1 N-terminus. Furthermore, small molecules could be developed to regulate the interaction between the N-terminus of BAI1 and the 7TM region, since we have shown that the NT/7TM association modulates receptor signaling activity. Additionally, drug-like compounds that disrupt PDZ interactions with BAI1 could be developed to modify signaling and/or receptor localization. Small molecules that bind to PDZ domains and block PDZ-mediated interactions are already in development for certain PDZ domains (Han et al., 2008), so this approach could be applied to some of the PDZ proteins that associate with BAI1. Moreover, small molecules that bind to the GPS motif to accelerate or inhibit GPS cleavage might also be predicted to alter receptor signaling and/or trafficking, and thus the GPS motif of BAI1 and other adhesion GPCRs could provide a unique therapeutic target that is not found in other GPCRs.

5.5 Future directions: further elucidation of the role of BAI1 at the postsynaptic density

Regulation of dendritic spine maintenance and synaptic plasticity is a complex process that involves a variety of signaling pathways and regulatory proteins. Understanding all of the processes involved is of critical importance not only to understanding normal physiology, but also with regards to understanding neurological disorders that have a basis in aberrant synapse formation and morphology. The studies presented in this dissertation, along with recent work with collaborators (Zhu et al., in submission) and from other labs (Duman et al., 2013) are beginning to uncover a role for

BAI1 at the synapse. Further elucidation of BAI1 regulation, signaling capabilities and synaptic function are likely to be topics of significant future research interest. For example, since there are three members of the BAI subfamily that are present throughout development of the brain to various degrees, there may be some level of redundancy with regards to the function of these receptors. To assess this, it may be beneficial to create double or triple BAI-KO mice to determine the level of overlap in function between the various BAI members. Furthermore, in order to further elucidate the importance of BAI1 signaling in dendritic spine development, it would be interesting to utilize the BAI1-KO mice and restore various BAI1 mutants to observe the effects on spine morphology. For example, restoring a constitutively active BAI1 lacking the N-terminus could provide insight into the effect of enhanced BAI1 signaling on dendritic spines, mimicking the use of an agonist. Moreover, if the constitutively active BAI1 only results in enhanced activation of Rho, but has no effect on Rac signaling, it would provide insights regarding the effects of Rho-specific signaling through BAI1 in dendritic spines. Similarly, restoration of a BAI1 mutant lacking the PDZ-binding motif would provide mechanistic insights into the importance of PDZ-mediated BAI1 effects on dendrite spine maintenence and formation.

Uncovering a ligand that regulates BAI1 activity would greatly enhance the understanding of BAI1 and allow for advances in drug development. As the studies presented here have shown BAI1 has the ability to signal to Rho and ERK, and previous research has indicated an ability to activate Rac (Duman et al., 2013; Park et al., 2007), further elucidation of the factor(s) controlling these various signaling pathways would shed light on the role that BAI1 may be playing as a synaptic receptor. We have also

identified a variety of novel C-terminal interacting proteins for BAI1, all of which are localized at the synapse, and discovering how these various interactions might play cell-specific roles in regulating BAI1 function would also be of great interest.

5.6 Future directions: BAI1 as a drug target for diseases associated with aberrant synapse formation and cancer

The aforementioned effects of the members of the BAI subfamily on dendritic spines may be of clinical interest because abnormalities in spine density and morphology are associated with a range of human diseases (Blanpied and Ehlers, 2004; Fiala et al., 2002). For example, Fragile X syndrome is the most common inherited form of mental retardation and is characterized by the development of abnormally long and thin dendritic spines in the cerebral cortex and hippocampus (Irwin et al., 2000). Furthermore, one of the key pathological features of Parkinson's disease is the loss of dendritic spines in the striatum (Irwin et al., 2000; Villalba and Smith, 2010), and alterations in spine morphology have been linked to schizophrenia and other psychiatric disorders (Costa et al., 2001). Chronic drug abuse has also been shown to result in dramatic changes to spine density and morphology in brain regions associated with reward and addiction (Russo et al., 2010). Treatment of Fragile X syndrome and ASD is mainly behavioral, although pharmacological intervention can be used to manage some of the associated symptoms, such as seizures (Hagerman et al., 2009; Myers and Johnson, 2007). Treatment of Parkinson's disease and schizophrenia rely mainly on the use of dopamine modulators, such as levodopa and haloperidol, respectively. While these medications do provide

some relief from the symptoms caused by these neurological disorders, they do not fully address the underlying pathology. New approaches to rescue deficits in dendritic spine morphology and maturation may be beneficial as adjuncts to the current therapeutic approaches. Given the large body of evidence linking spine dysregulation with disease, it is of critical importance to elucidate the underlying elements that regulate dendritic spine development and morphology in order to identify novel targets for therapeutic interventions for these disorders. Thus, the recent studies demonstrating regulation of dendritic spine development and morphology by the members of the BAI subfamily mark these receptors as potentially exciting new therapeutic targets for the treatment of psychiatric and neurological disorders associated with dendritic spine pathology.

Clinical interest in the BAI subtypes has also arisen from genetic studies linking these receptors to various psychiatric and neurological disorders. For example, BAI1 was recently possibly implicated in ASD, as the BAI1 gene was localized to a hot spot for germline mutations in patients with autism (Michaelson et al., 2012). Moreover, BAI1 interacts with PSD-95 (Lim et al., 2002; Stephenson et al., 2013)(Zhu et al., in submission), and there is evidence that regulation of PSD-95 may be a common downstream feature of multiple genes associated with autism (Tsai et al., 2012). Additionally, single-nucleotide polymorphisms in BAI3 (DeRosse et al., 2008) as well as changes in BAI3 copy number (Liao et al., 2012) have been linked to the development of schizophrenia, and BAI3 expression has been found to be regulated by treatment with lithium, a drug that is used to treat psychiatric conditions such as bipolar disorder and certain types of schizophrenia (McCarthy et al., 2012; McQuillin et al., 2007). Thus, converging lines of evidence from *in vitro*, *in vivo* and genetic studies suggest that BAI1-

3 may play roles in a variety of psychiatric and neurological disorders and thus may serve as important new therapeutic targets in the future treatment of these diseases.

Since *in vitro* (Duman et al., 2013) reduction of BAI1 results in morphological disturbances to dendritic spines and *in vivo* (Zhu et al. in submission) knockout of BAI1 results in aberrant synaptic plasticity and deficits in spatial learning and memory, positively modulating BAI1 signaling may reverse these disturbances which are mirrored in a variety of neurological disorders mentioned above. It can be envisioned that small molecule BAI1 agonists could potentially promote dendritic spine stabilization and rescue spines from immature phenotypes. Furthermore, positive allosteric modulators that enhance the interaction between BAI1 and various PDZ-interacting proteins, such as PSD-95, may promote stabilization of the postsynaptic density and subsequent spine morphology. Since BAI1 can signal to both Rho and Rac, both of which regulate the actin cytoskeleton, it may be possible to develop biased agonists that specifically activate one pathway or the other in order to fine-tune cellular responses in a manner that would maximize clinical benefit.

In addition to targeting the BAI subtypes for the treatment of psychiatric and neurological disorders, it might also be tractable to target these receptors for the treatment of certain types of cancer. Multiple cancer-associated somatic mutations have been found for each member of the BAI subfamily (Cork and Van Meir, 2011; Kan et al., 2010). These somatic mutations have been suggested to play roles in lung, ovarian and breast cancers, and down-regulation of BAI1 is associated with pulmonary adenocarcinomas, gastric and colorectal cancers, and primary gliomas (Fukushima et al., 1998; Hatanaka et al., 2000; Kaur et al., 2003; Miyamoto et al., 2007; Yoshida et al.,

1999). Given that somatic mutations and down-regulation of BAI1 appear to play a role in various forms of cancer, it is important to understand the factors that control BAI1 expression in order to potentially allow for restoration of BAI1 expression for therapeutic benefit. Recently, BAI1 expression in glioblastoma multiforme was found to be regulated via epigenetic silencing induced by overexpression of methyl-CpG–binding domain protein 2 (MBD2), which is a key mediator of epigenetic gene regulation (Zhu et al., 2011). Interestingly, knockdown of MBD2 resulted in reactivation of BAI1 gene expression and increased anti-angiogenic activity, indicating that inhibition of MBD2 is a promising drug target for restoration of BAI1 in gliomas and subsequent inhibition of tumor-associated angiogenesis.

5.7 Concluding remarks

The work described in this dissertation has provided new knowledge concerning the signaling pathways and mechanisms of regulation for the adhesion GPCR BAI1. We have also uncovered a previously-unappreciated role for BAI1 as a synaptic protein and regulator of postsynaptic density formation. Prior to this work, very little was understood about the signaling capabilities of BAI1 and the role that this receptor might be playing in neurons, as most previous work on BAI1 had focused on the capacity of the receptor's N-terminus to inhibit angiogenesis and tumor growth. Here we present novel findings with regards to the ability of BAI1 to activate a G protein-dependent pathway, specifically $G\alpha_{12/13}$-dependent activation of the Rho pathway. Additionally, we show that removal of the BAI1 N-terminus greatly enhances receptor signaling, which may be a common

conserved mechanism of activation among the adhesion GPCRs, given that similar observations have been made for various other family members, including GPR56 (Paavola et al., 2011), CD97 (Ward et al., 2011), and another BAI subfamily member, BAI2 (Okajima et al., 2010). Furthermore, we provide evidence that the N-terminus of BAI1 can remain associated with the 7TM region for a certain amount of time, which is also a shared feature with other adhesion GPCRs, including CD97 (Gray et al., 1996), CIRL (Krasnoperov et al., 2002; Volynski et al., 2004), GPR56 (Paavola et al., 2011), and BAI2 (Okajima et al., 2010).

The research presented here also resulted in the identification of a variety of potential novel BAI1 C-terminal interacting partners, all of which are synaptic proteins. The diversity of PDZ interactions found for BAI1 suggest the possibility of extensive cell-specific regulation of BAI1 signaling and localization. Furthermore, we have made the novel observation of BAI1 enrichment in the postsynaptic density, indicating a role for BAI1 as a synaptic receptor. Interestingly, PSD fractions from BAI1-deficient mice (Zhu et al. in submission) were found to exhibit a perturbed composition of proteins, including changes to a variety of proteins known to regulate synaptogenesis and synaptic plasticity. Additionally, we identified a possible ligand for BAI1 be demonstrating an interaction with C1ql3, a complement-like protein known as a regulator of synaptogenesis (Bolliger et al., 2011).

In conclusion, the work presented here sheds light on the mechanism of activation for BAI, the signaling pathways downstream of the receptor and the mechanisms by which BAI1 signaling activity may be regulated. Furthermore, we have advanced the understanding of the physiological roles played by BAI1 via demonstration that BAI1 is a

synaptic receptor. The findings described in this dissertation therefore provide new insights into the fundamental biology of BAI1. These insights might be useful in the future therapeutic targeting of BAI1 for the treatment of psychiatric and neurological diseases arising from aberrant spine morphology.

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