

RESEARCH ARTICLE

A disease-associated mutation in the adhesion GPCR BAI2 (*ADGRB2*) increases receptor signaling activity

Ryan H. Purcell¹  | Camilo Toro² | William A. Gahl² | Randy A. Hall¹ 

¹Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia

²NIH Undiagnosed Diseases Program, Office of the Director, NIH, Bethesda, Maryland

Correspondence

Randy A. Hall, Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322

Email: rhall3@emory.edu

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Abstract

Mutations in G protein-coupled receptors (GPCRs) that increase constitutive signaling activity can cause human disease. A de novo C-terminal mutation (R1465W) in the adhesion GPCR BAI2 (also known as *ADGRB2*) was identified in a patient suffering from progressive spastic paraparesis and other neurological symptoms. In vitro studies revealed that this mutation strongly increases the constitutive signaling activity of an N-terminally cleaved form of BAI2, which represents the activated form of the receptor. Further studies dissecting the mechanism(s) underlying this effect revealed that wild-type BAI2 primarily couples to $G\alpha_2$, with the R1465W mutation conferring increased coupling to $G\alpha_i$. The R1465W mutation also increases the total and surface expression of BAI2. The mutation has no effect on receptor binding to β -arrestins, but does perturb binding to the endocytic protein endophilin A1, identified here as a novel interacting partner for BAI2. These studies provide new insights into the signaling capabilities of the adhesion GPCR BAI2/*ADGRB2* and shed light on how an apparent gain-of-function mutation to the receptor's C-terminus may lead to human disease.

KEYWORDS

activation, brain, Gz, $G\beta\gamma$, NFAT, RGS20

1 | INTRODUCTION

Adhesion G protein-coupled receptors (GPCRs) (aGPCRs) are an evolutionarily ancient yet enigmatic family of cell surface receptors. These proteins are widely expressed throughout the body, and loss-of-function mutations to a number of members are associated with human disease (Langenhan, Aust, & Hamann, 2013; O'Hayre et al., 2013). For example, mutations in GPR56 (*ADGRG1*) result in the cortical malformation bilateral frontoparietal polymicrogyria (Piao et al., 2004), mutations in GPR126 (*ADGRG6*) severely disrupt peripheral myelination (Ravenscroft et al., 2015), mutations in VLGR1 (*ADGRV1*) cause deafness and retinitis pigmentosa (Weston, Lujendijk, Humphrey, Moller, & Kimberling, 2004), and a mutation in EMR2 (*ADGRE2*) has been associated with vibratory urticaria (Boyden et al., 2016).

To date, much of the focus on aGPCRs has been concentrated on their strikingly long extracellular amino (N) termini. These regions contain multiple domains, including adhesion folds, and nearly all of the more than 30 human aGPCRs contain a juxtamembrane GPCR Autoproteolysis Inducing (GAIN) domain. This hallmark aGPCR feature has autoproteolytic ability and can sever the receptors into two non-covalently associated protomers—an extracellular N-terminal fragment (NTF) and a C-terminal fragment (CTF) containing the archetypal

seven transmembrane (7-TM) domain (Arac et al., 2012). This autoproteolysis typically occurs in the endoplasmic reticulum, with the cleaved receptor protomers trafficking to the cell surface as a non-covalently associated complex (Krasnoperov et al., 2002).

BAI2 (*ADGRB2*; MIM# 602683) is one of three *ADGRB* subfamily receptors, which are also known as Brain-specific Angiogenesis Inhibitors 1–3 (BAI1–3) (Stephenson, Purcell, & Hall, 2014). These receptors are most abundantly expressed in brain tissue, and BAI1 (*ADGRB1*) and BAI3 (*ADGRB3*) have been shown to have important roles at synapses (Bolliger, Martinelli, & Sudhof, 2011; Duman et al., 2013; Sigoillot et al., 2015). At this point, much less is known about the function of BAI2. Mice lacking BAI2 were found to have no gross deficits, but did exhibit increased hippocampal neurogenesis and resilience to learned-helplessness behavior (Okajima, Kudo, & Yokota, 2011).

While most aGPCRs remain orphan receptors with no known endogenous ligands, substantial progress has been made in understanding the activation mechanisms and signaling activity of many of these receptors. Interestingly, the extraordinarily long N-termini have an inhibitory effect on the constitutive signaling activity of the 7-TM domain in most aGPCRs studied thus far. For many aGPCRs, including BAI1 (Stephenson et al., 2013) and BAI2 (Okajima, Kudo, & Yokota, 2010), and at least six other aGPCRs from five different sub-families

(Kishore & Hall, 2016), removal of the NTF results in a constitutively active receptor. One of the earliest reports of this phenomenon demonstrated that removing the NTF of BAI2 unveils the constitutive activity of the receptor, as measured by the nuclear factor of activated T cells (NFAT) luciferase reporter (Okajima et al., 2010), but this study did not elucidate the G protein alpha subunit ($G\alpha$) to which the receptor primarily couples.

Despite substantial progress in understanding how aGPCRs are activated, very little is known at this point about how these receptors are regulated. Previous reports have shown aGPCR C-terminal interactions with many different proteins containing PDZ domains (Kreienkamp, Zitzer, Gundelfinger, Richter, & Bockers, 2000; Paavola, Stephenson, Ritter, Alter, & Hall, 2011; Stephenson et al., 2013; Tobaben, Sudhof, & Stahl, 2000) and demonstrated that the constitutively active forms of BAI1 and GPR56 robustly co-immunoprecipitate with β -arrestins (Paavola et al., 2011; Stephenson et al., 2013). However, it remains unclear whether these interactions can fully explain how the receptors are internalized and undergo post-endocytic trafficking.

The present investigation into the signaling activity and regulation of BAI2 was spurred by work from the NIH Undiagnosed Diseases Program (UDP) (Gahl et al., 2016), which identified a de novo mutation (NM_001703.2(*ADGRB2*):c.4393C > T, p.Arg1465Trp) in a human patient suffering from progressive spastic paraparesis and other symptoms. We engineered the Arg to Trp substitution exhibited by this patient into BAI2 expression constructs and studied the effect of this mutation on receptor signaling, trafficking, and protein-protein interactions. Our studies revealed that this mutation increases BAI2 signaling activity and surface expression, and also disrupts interaction with the regulatory protein endophilin A1. Moreover, our results provided evidence that BAI2 predominantly couples to $G\alpha_2$, with the mutation promoting enhanced $G\alpha_1$ coupling.

2 | MATERIALS AND METHODS

2.1 | Variant discovery

Whole blood samples were obtained from the proband and family members in the nuclear pedigree. DNA was extracted utilizing the FLEX STAR automated system (Autogen, Holliston, MA) according to the manufacturer's recommended procedures. After phenol-chloroform DNA purification, samples underwent massively parallel sequencing utilizing the TruSeqV2 Exome Kit on the Illumina HiSeq2000 (Illumina, San Diego, CA) platform for the generation of 101-bp paired-end read. Image analyses and base calling used the Illumina Genome Analyzer Pipeline software with default parameters. Reads were filtered for quality, and aligned to human reference genome NCBI build 37 (hg19) using an in-house developed pipeline based on Novoalign (Novocraft Technologies, Selangor, Malaysia). Variants were called via HaplotypeCaller and GenotypeGVCFs (DePristo et al., 2011; McKenna et al., 2010; Van der Auwera et al., 2013). SnpEff (Cingolani et al., 2012) and a combination of publicly available data sources (EXAC, ESP, 1000 Genomes) were used for variant annotations. Variant filtration captured rare start-

gain/loss, frameshift, nonsynonymous, canonical splice site variants, and intronic variants (± 20 bp) that were consistent with homozygous recessive, compound heterozygous, X-linked, or de novo dominant disease models and segregated to the family's affected status. The variants were then individually inspected using the Integrative Genomics Viewer and compared against publicly available clinical or functional datasets in Online Mendelian Inheritance in Man, Human Gene Mutation Database, and PubMed. BAI2 snake plot (Figure 2A) was constructed using Protter (Omasits, Ahrens, Muller, & Wollscheid, 2014).

2.2 | DNA constructs

Human *ADGRB2* wild-type (WT) and R1465W plasmids were synthesized in pcDNA3.1 vectors (Genscript, Piscataway, NJ). Sequences for BAI2 Δ NT and BAI2 Δ NT-RW (912–1,585) were sub-cloned into pcDNA3.1+ between 5' *KpnI* (AGA CCA TCT ACA TTT GCT GTA CTA GCT CAA CCT CCT) and 3' *EcoRI* (AGA CCA GAA TTC TCA AAC TTC TGT CTG GAA GTC ACC ATC AGG) from each of these templates and sequences were verified (Eurofins Genomics, Louisville, KY). To differentiate endogenous from mutant BAI2, a C-terminal Flag tag was added to BAI2 Δ NT and BAI2 Δ NT-RW via PCR. GFP-EndophilinA1 was a gift from Kozo Kaibuchi (Nagoya University); GST-EndoA1-SH3 was kindly provided by Harvey McMahon (Cambridge University); EE- $G\alpha_2$ and RGS20 (splice variant 2) were purchased from the cDNA Resource Center (cdna.org); HA-RGS2 was gift from John Hepler (Emory University); and HA- β -arrestin2 (Luttrell et al., 1999) was a gift from Robert Lefkowitz (Addgene plasmid # 14692).

2.3 | Cell culture

HEK293T/17 cells (ATCC, Manassas, VA) were maintained in a humid, 5% CO₂, 37°C incubator with standard growth medium (DMEM, 10% FBS, 1% penicillin/streptomycin). Transfections utilized Mirus TransIT-L1 (Madison, WI).

2.4 | Western blot

Protein samples were reduced in 1× Laemmli buffer (Bio-Rad, Hercules, CA), electrophoresed in 4%–20% Tris-glycine gels and transferred to nitrocellulose membranes (Bio-Rad). Non-specific binding was blocked with 5% milk (in 50 mM NaCl, 10 mM HEPES pH 7.3, 0.1% Tween-20 (Sigma, St. Louis, MO)) and incubated with primary antibodies for 1 hr at room temperature or overnight at 4°C. The BAI2 C-terminal antibody was purchased from Mab Technologies (Stone Mountain, GA; cat. #BAI2-3), rabbit polyclonal anti-GFP (Rockland, Limerick, PA; cat. # 600-401-215), mouse monoclonal EE antibody from Abcam (Cambridge, MA; cat. #ab73989), and Flag-HRP from Sigma (cat. #A8592). Blots were then washed, incubated with HRP-conjugated secondary antibodies if necessary (GE Healthcare, Pittsburgh, PA) and visualized with Thermo Scientific SuperSignal West solutions on a Li-Cor Odyssey Imager.

2.5 | Cell surface biotinylation

Twenty-four hours following transfection with 2 μ g of receptor DNA, HEK-293T cells were washed in cold PBS+Ca²⁺ and incubated with

5 mM Sulfo-NHS-Biotin (Thermo Scientific, Rockford, IL) or vehicle (PBS+Ca²⁺) for 1 hr on ice. Biotinylation was quenched with 100 mM glycine and cells were harvested in cold PBS. Membranes were ruptured with a rapid freeze-thaw and pelleted at 17,000×g for 15 min at 4°C. The membrane pellet was then re-suspended in 1% Triton X-100 buffer (25 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1× HALT protease, and phosphatase inhibitor (Thermo)) and rotated end-over-end for 45 min to solubilize membrane proteins. The insoluble fraction was then pelleted at 17,000×g for 15 min at 4°C and solubilizes were incubated with streptavidin agarose (Thermo) for 30 min to precipitate biotinylated proteins. Agarose was washed with 1% Triton buffer and proteins were eluted in 2× Laemmli buffer. Biotinylated proteins were detected via Western blot (above).

2.6 | Co-immunoprecipitation

Cells were transfected with 3 μg of receptor and 1 μg of G protein or arrestin and balanced with empty vector (EV) DNA. The following day, cells were harvested and membrane proteins were solubilized in 1% Triton X-100 buffer. Solubilizes were separated (above) and incubated with anti-HA agarose (Sigma), magnetic anti-Flag (Sigma), or protein A/G agarose (Thermo) beads with the indicated primary antibody for 1 hr end-over-end at 4°C. Samples were then washed, eluted, and Western blotted as described above.

2.7 | Endogenous pull-down assay

Animal care and experimental procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the protocol was approved by the Emory University Institutional Animal Care and Use Committee. Mice were euthanized by CO₂ asphyxiation. Brain tissue was harvested and homogenized in ice-cold 150 mM NaCl, 25 mM HEPES, 10 mM MgCl₂, 1 mM EDTA, 1× fresh protease, and phosphatase inhibitor (HALT, Thermo) pH 7.4. Large debris were cleared by centrifugation at 2,000×g at 4°C and supernatant proteins were solubilized with 1% Triton X-100 (Sigma) for 1 hr at 4°C. The insoluble fraction was cleared by centrifugation at 17,000×g for 15 min at 4°C and samples were normalized to 1 mg/ml with harvest buffer by BCA. A portion of the lysates were saved as "input" samples and the remainder was split between GST and GST-endo1-SH3 agarose beads and rotated at 4°C overnight. The following day, beads were washed thoroughly with 1% Triton buffer, eluted with an equal volume of 2× Laemmli buffer, and 20 μl was loaded for SDS-PAGE as described above.

2.8 | Luciferase assays

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

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

3 | RESULTS

3.1 | A mutation in ADGRB2 is associated with human disease

The patient was examined at 46 years of age. She indicated a history of progressive gait difficulties and urinary urgency since age 15. A diagnosis of multiple sclerosis was entertained at age 18, and she began using a wheelchair at age 20. At age 23, neurological evaluation recorded mild bilateral optic nerve atrophy, bilateral nystagmus, and normal upper extremities strength but severe weakness and spasticity in the lower extremities. By age 35, weakness in upper extremities was evident. A suprapubic catheter was placed at age 41, and a baclofen pump was placed later the same year. She has been regarded as totally disabled since age 41.

The patient has undergone repeated diagnostic studies, chiefly with attention to the question of multiple sclerosis. Cranial MR is normal, but spinal cord MR shows atrophy, especially the thoracic cord (Figure 1). Electromyogram/nerve conduction velocity (EMG/NCV) was abnormal with evidence of a distal axonal neuropathy. Brain stem auditory and somatosensory evoked potential indicate central conduction abnormalities. Visual evoked potentials are prolonged, suggesting optic nerve dysfunction. Her EMG suggests a length-dependent motor neuropathy or neuronopathy with chronic denervation findings.

Retina and eye exam are otherwise unremarkable. She has evidence of severe spastic quadriparesis and is totally wheelchair bound. The patient has undergone therapeutic trials of prednisone, cytoxan, and Rocephin therapy, although none appeared to provide any substantial effects. The disease has been slowly progressive.

The NIH UDP performed whole exome sequencing on the trio of the patient (proband) and her parents. Sequencing revealed a de novo mutation in *ADGRB2/BAI2* (BAI2) in the patient: NM_001703.2(*ADGRB2*):c.4393C > T, resulting in p.Arg1465Trp (ClinVar accession number SCV000583612; [https://www.ncbi.nlm.nih.gov/clinvar/?term=ADGRB2\[gene\]](https://www.ncbi.nlm.nih.gov/clinvar/?term=ADGRB2[gene])). This R1465W mutation is located in the middle of a highly conserved region of the BAI2 C-terminus (Figure 2A–C) and has a CADD score of 22.7, indicating a high likelihood that the mutation is deleterious (Kircher et al., 2014). Thus, given that the whole exome sequencing did not reveal any other mutations that might plausibly account for the observed pathology, we engineered this mutation into human BAI2 expression plasmids and explored whether the R1465W mutation might alter BAI2 function.

3.2 | p.R1465W increases signaling activity and surface expression

To test potential effects of the BAI2 p.R1465W (RW) mutation on receptor insertion in the plasma membrane, we assessed total and cell surface expression of the full-length and truncated (Δ NT) forms of the both the WT and mutant receptors using a surface biotinylation

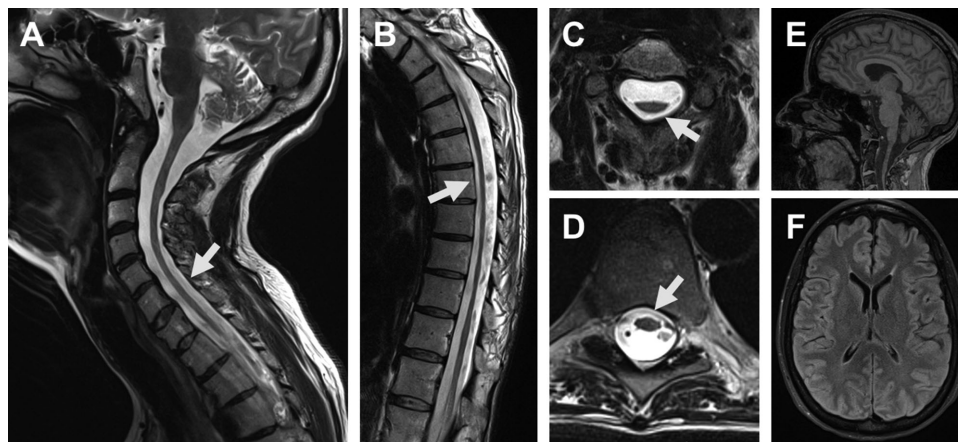


FIGURE 1 Clinical presentation. (A) Sagittal T2-weighted MRI of the cervical and (B) thoracolumbar spinal cord. (C) Axial T2 MRI images of the spinal cord at the C6 and (D) T5 levels. (E) FLAIR sagittal and (F) axial images of the brain. Arrows demonstrate the significant reduction in spinal cord cross sectional dimensions

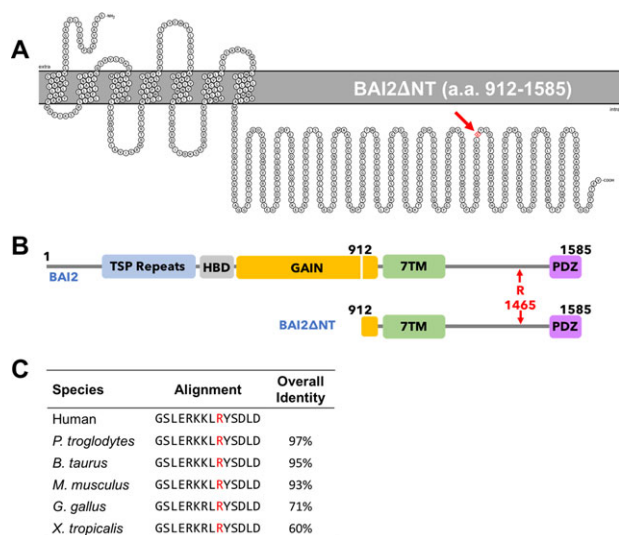


FIGURE 2 BAI2 p.R1465W. (A) Topological amino acid diagram of BAI2ΔNT with an arrow indicating the location of R1465. (B) Domain structure schematics of full-length BAI2 and BAI2ΔNT. (C) R1465 is in an ultra-conserved region of the BAI2 cytoplasmic domain. (TSP, thrombospondin; HBD, hormone-binding domain; GAIN, GPCR autoproteolysis-inducing domain; PDZ, PSD95/Dlg1/ZO-1 domain binding motif)

approach. These studies revealed that the BAI2ΔNT p.R1465W mutant exhibited significantly increased surface expression over WT BAI2ΔNT (Figure 3A; one-sample t-test of surface expression normalized to WT BAI2ΔNT: BAI2ΔNT-RW = 2.06 ± 0.15 , $P = 0.0019$, $n = 5$). Total expression of BAI2ΔNT-RW also appeared to be increased, but was not statistically significant compared with WT BAI2ΔNT ($P = 0.0637$).

To investigate the role of the p.R1465W mutation on BAI2 signaling activity, we over-expressed full-length and cleavage-mimicking (ΔNT) forms of the WT and mutant receptors and assessed activation of a panel of luciferase reporters. Serum response factor (SRF) and NFAT were the most likely outputs based on homology to BAI1 (Stephenson et al., 2013) and a previous report about BAI2 (Okajima et al., 2010).

We found that transfection of full-length BAI2 into HEK-293T cells did not result in activation of either reporter, either for the WT or mutant forms of the receptor (Figure 3B, SRF data not shown). However, BAI2ΔNT robustly activated NFAT luciferase (one-way ANOVA, $F(4, 15) = 66.61$, Sidak *post-hoc* test vs. EV, $P < 0.0001$, $n = 4$), consistent with previous findings (Okajima et al., 2010). Furthermore, we observed that the p.R1465W mutation significantly potentiated this signaling activity to NFAT luciferase ($P = 0.0001$ vs. BAI2ΔNT, $n = 4$).

3.3 | BAI2ΔNT signals to NFAT luciferase via $G\beta\gamma$ and calcium channel activation

To shed light on the signaling pathway by which BAI2ΔNT activates NFAT luciferase, a number of different inhibitors were deployed. The BAI2ΔNT signal to NFAT luciferase was strongly inhibited by the $G\beta\gamma$ subunit inhibitor gallein (Lehmann, Seneviratne, & Smrcka, 2008) (Figure 3C; two-way ANOVA, main effect of inhibitor treatment, $F(3, 56) = 10.06$, Holm-Sidak *post-hoc* test BAI2ΔNT $P < 0.001$ vs. vehicle for gallein, BAI2ΔNT-RW $P < 0.0001$ vs. vehicle for gallein, $n = 4$). Neither WT- nor mutant BAI2ΔNT-induced signaling was inhibited by the PLC β inhibitor U73122, which blocks signaling downstream of $G\alpha_q$ -coupled receptors, and only signaling by the mutant receptor BAI2ΔNT-RW showed statistically significant sensitivity to pertussis toxin (PTX) (Figure 3C; BAI2ΔNT-RW $P < 0.01$ vs. vehicle, $n = 4$), which inhibits $G\alpha_{i/o}$ -mediated signaling.

To further assess the importance of $G\beta\gamma$ liberation on BAI2ΔNT signaling to NFAT luciferase, we co-expressed GRK2-CT (β ARKct), which can bind to and inhibit the activity of $G\beta\gamma$ subunits (Koch, Inglese, Stone, & Lefkowitz, 1993). Like the gallein treatment, GRK2-CT inhibited the activity of BAI2ΔNT-RW (Figure 3D; two-way ANOVA, main effect of receptor \times GRK2-CT interaction $F(3, 9) = 7.378$, Sidak *post-hoc* test BAI2ΔNT-RW+GRK2-CT $**P = 0.0016$ vs. vehicle). Furthermore, we observed that the activation of NFAT luciferase by both WT and p.R1465W mutant BAI2ΔNT was almost completely blocked by the calcium channel inhibitor SKF96365 (Figure 3D; two-way ANOVA, main effect of SKF96365 $F(1, 32) = 29.95$, Holm-Sidak *post-hoc* test

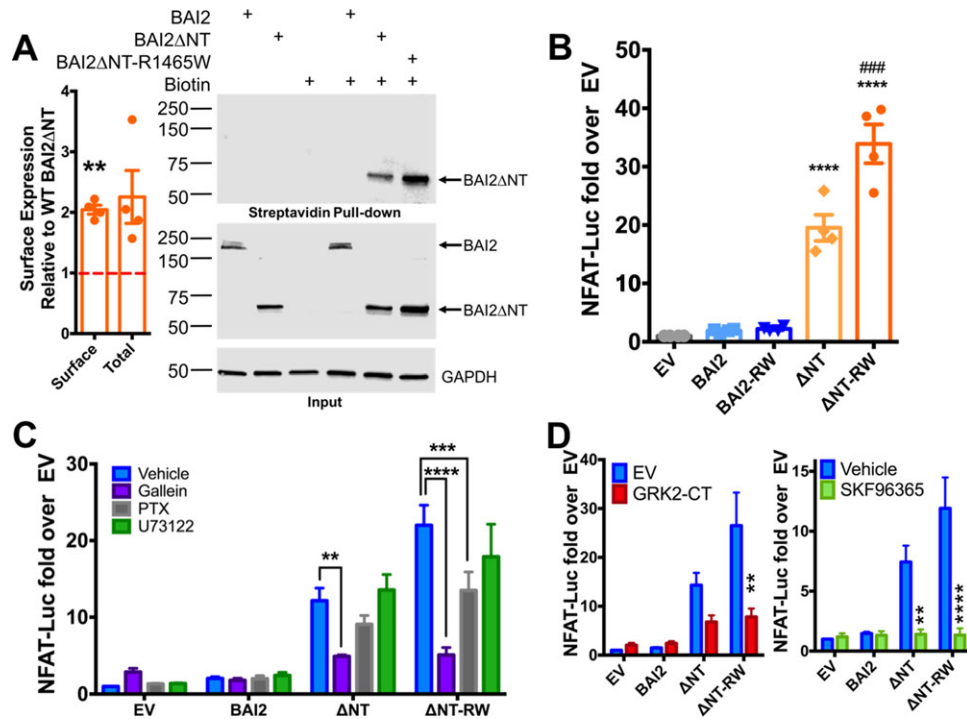


FIGURE 3 BAI2 p.R1465W increases signaling activity and surface expression. (A) The R1465W mutation increased surface and total expression of BAI2ΔNT, as assessed by surface biotinylation (top, total protein expression bottom). Inset: one sample t-test compared with WT = 1.00 (Surface $**P < 0.01$ vs. B2ΔNT, $n = 5$). (B) BAI2ΔNT activated NFAT luciferase (one-way ANOVA, Sidak multiple comparisons BAI2ΔNT $****P < 0.0001$ vs. empty vector (EV) condition, $n = 4$). The R1465W mutation had no effect on the full-length receptor (BAI2-RW) but significantly potentiated the activity of BAI2ΔNT (### $P = 0.0001$ vs. BAI2ΔNT, $n = 4$). (C) The NFAT luciferase signal from BAI2ΔNT and BAI2ΔNT-RW was abolished by the $G\beta\gamma$ inhibitor gallein (second bar of each set; $**P < 0.01$ vs. B2ΔNT-vehicle condition, $****P < 0.0001$ vs. BAI2ΔNT-RW vehicle $n = 4$). The mutant receptor was sensitive to pertussis toxin (PTX, 100 ng/ml) but the wild-type receptor was not (third bar of each set; $***P = 0.001$ vs. BAI2ΔNT-RW vehicle condition, $n = 4$). Neither receptor was inhibited by 10 μ M U73122 (fourth bar of each set). (D) A transfectable $G\beta\gamma$ inhibitor, GRK2-CT (left), significantly inhibited the NFAT signal from BAI2ΔNT-RW (GRK2-CT-treated is second bar of each set; two-way ANOVA, BAI2ΔNT-RW $**P = 0.001$ vs. EV, $n = 4$). The calcium channel inhibitor SKF96365 (right, 50 μ M) completely blocked the NFAT signal from both receptors (SKF96365-treated is second bar of each set; two-way ANOVA, BAI2ΔNT $**P > 0.01$, BAI2ΔNT-RW $****P < 0.0001$, $n = 5$)

BAI2ΔNT $**P = 0.0011$ vs. vehicle, BAI2ΔNT-RW $****P < 0.0001$ vs. vehicle). These results indicate that the NFAT reporter activation by BAI2ΔNT is almost entirely due to $G\beta\gamma$ liberation and activation of a calcium channel.

3.4 | BAI2 couples to $G\alpha_z$

NFAT luciferase is a common readout downstream of $G\alpha_q$ signaling (Hill, Baker, & Rees, 2001) but can also report activity from $G\alpha_{12/13}$ (Nishida et al., 2007). In addition, $G\beta\gamma$ -mediated activity is most typically due to $G\alpha_i$ activation (Smrcka, 2008). We have previously reported that ADGRB1/BAI1, a $G\alpha_{12/13}$ -coupled receptor, activates NFAT luciferase and therefore, given a high degree of similarity in the 7TM region, considered $G\alpha_q$, $G\alpha_{12/13}$ and $G\alpha_i$ as the most likely candidates to be the cognate G protein for BAI2 (Kishore, Purcell, Nassiri-Toosi, & Hall, 2016). However, we observed no interactions between BAI2ΔNT and $G\alpha_q$ or $G\alpha_{13}$ in co-immunoprecipitation experiments (Figure 4A). In contrast, we observed that the $G\alpha_{i/o}$ -family member $G\alpha_z$ robustly co-immunoprecipitated with BAI2ΔNT, with WT and p.R1465W mutant BAI2ΔNT immunoprecipitating $G\alpha_z$ to a similar extent (Figure 4B).

To further test the possibility that BAI2 might signal through $G\alpha_z$ coupling, we assessed BAI2ΔNT signaling to NFAT luciferase in the presence of co-transfection with the $G\alpha_z$ -specific regulator of G protein signaling RGS20 (RGSZ1) (Glick, Meigs, Miron, & Casey, 1998). As a control, we co-expressed a related RGS protein, RGS2, which acts specifically on $G\alpha_q$ (Heximer, Watson, Linder, Blumer, & Hepler, 1997). RGS2 had no effect on BAI2ΔNT or BAI2ΔNT-RW signaling to NFAT. In contrast, RGS20 strongly increased the activity of BAI2ΔNT and BAI2ΔNT-RW (Figure 4C; two-way ANOVA $F(3, 36) = 54.56$, $P < 0.0001$, $n = 4$, Tukey *post-hoc* test BAI2ΔNT vs. mock $P < 0.0001$, BAI2ΔNT-RW vs. mock $P < 0.001$).

Based on the findings described above showing that signaling by the R1465W mutant but not WT BAI2ΔNT was significantly inhibited by the $G\alpha_{i/o}$ -inhibitor PTX, we hypothesized that R1465W mutant BAI2ΔNT might possess an enhanced ability to couple to $G\alpha_{i/o}$ in addition to $G\alpha_z$. To test this hypothesis, we performed co-immunoprecipitation experiments assessing WT BAI2ΔNT and BAI2ΔNT-RW interactions with $G\alpha_{i1}$. No interaction was observable between WT BAI2ΔNT and $G\alpha_{i1}$, but substantial co-immunoprecipitation of $G\alpha_{i1}$ was observed with the BAI2ΔNT p.R1465W mutant (Figure 4D).

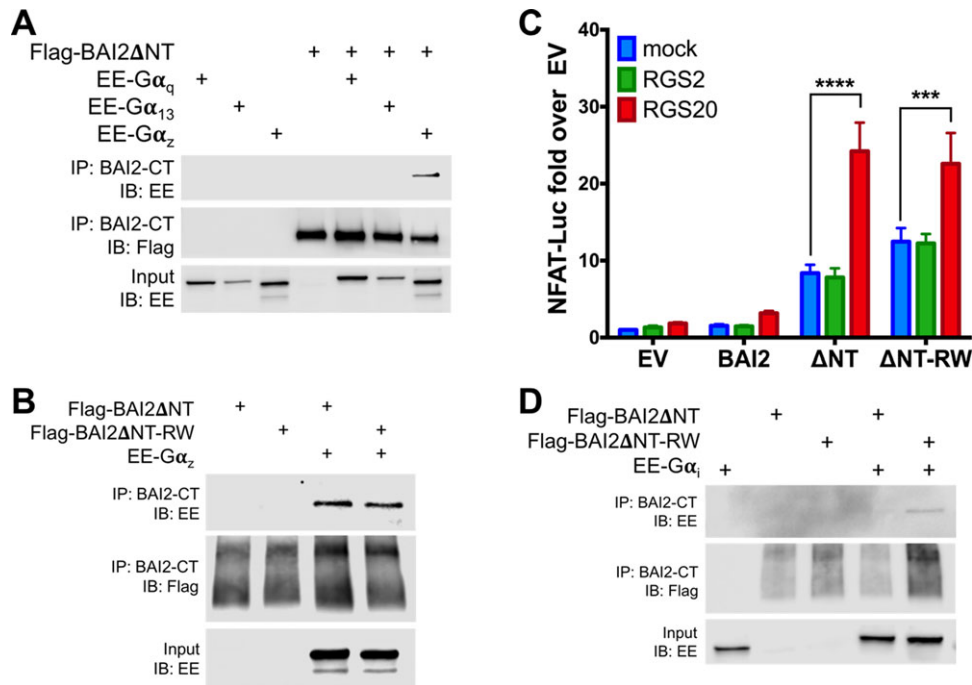


FIGURE 4 Coupling of BAI2 to G proteins. (A) Among a panel of G protein α subunits, only $G_{\alpha z}$ detectably co-immunoprecipitated with BAI2 Δ NT ($n = 4$). (B) Both WT and R1465W forms of BAI2 Δ NT co-immunoprecipitated with $G_{\alpha z}$ and there was no significant difference in their ability to interact ($n = 5$). (C) The $G_{\alpha q}$ -specific RGS protein RGS2 (second bar of each set) had no effect on WT or RW signaling, but the $G_{\alpha z}$ -specific RGS20 (third bar of each set) increased both BAI2 Δ NT and BAI2 Δ NT-RW activity (two-way ANOVA, Tukey test BAI2 Δ NT-RGS20 **** $P < 0.0001$ vs. BAI2 Δ NT-mock, BAI2 Δ NT-RW *** $P = 0.001$ vs. BAI2 Δ NT-RW-mock, $n = 4$). (D) Mutant BAI2 Δ NT-RW interacted with $G_{\alpha 1}$ but no interaction with the WT receptor was detected ($n = 3$)

3.5 | p.R1465W mutation disrupts BAI2 interaction with endophilin A1

The activity and surface expression of GPCRs can be regulated by β -arrestins, which bind to active receptors and often mediate their internalization and desensitization (Reiter & Lefkowitz, 2006). Moreover, we have previously found that cleavage-mimicking forms of BAI1/ADGRB1 and GPR56/ADGRG1 strongly interact with β -arrestins (Paavola et al., 2011; Stephenson et al., 2013). Indeed, we observed that BAI2 Δ NT robustly co-immunoprecipitates with β -arrestin2, with the R1465W mutation having no effect on this interaction (Figure 5A, $n = 3$).

The β -arrestin-mediated pathway is not the sole mediator of GPCR internalization (Ferguson, 2001). The membrane-binding BAR- and SH3-domain-containing protein endophilin A1 (SH3-GL2) has been shown to interact with certain GPCRs (Tang et al., 1999) and can mediate GPCR internalization in a rapid, clathrin-independent manner (Boucrot et al., 2015). Therefore, we tested whether endophilins could interact with BAI2 by performing pull-down assays using the SH3 domain of endophilin A1 fused to glutathione-S-transferase (GST), or GST alone as a control, to pull down BAI2 Δ NT. We found that WT BAI2 Δ NT robustly interacted with the endophilin A1 SH3 domain, whereas interaction with BAI2 Δ NT-RW was significantly reduced relative to WT (Figure 5B; unpaired t -test, WT binding = 6.46 ± 0.59 vs. RW = 0.48 ± 0.22 , $P < 0.0001$, $n = 4$).

We hypothesized that if endophilin A1 is in fact an important regulator of BAI2 signaling, then co-transfection should limit the

signaling activity of BAI2 Δ NT. Indeed, we found that in 96-well format NFAT luciferase assays, as little as 2 ng of endophilin A1 DNA significantly reduced the signaling of BAI2 Δ NT and BAI2 Δ NT-RW (Figure 5C; two-way ANOVA, main effect of endo1 transfection $F(1, 12) = 22.45$, $P = 0.0005$, Sidak *post-hoc* test BAI2 Δ NT and BAI2 Δ NT-RW not significantly different from EV with endo1 co-transfection, $n = 3$). Both BAI2 Δ NT WT and RW receptors were significantly inhibited by endophilin A1 over-expression, suggesting that even low levels of endophilin over-expression are sufficient to overcome the binding deficit of the mutant receptor.

The members of the brain-specific angiogenesis inhibitor (BAI1-3/ADGRB1-3) sub-family of receptors are highly enriched in brain tissue. Among the three endophilin A proteins, endophilin A1 has the most brain-enriched expression pattern (Kjaerulff, Brodin, & Jung, 2011). To determine whether endophilin A1 can interact with endogenous BAI2 from brain tissue, we incubated GST-endoA1-SH3 domain with mouse brain lysates and probed pull-down fractions for BAI2. We observed a robust interaction with BAI2 (Figure 5D, $n = 3$).

4 | DISCUSSION

The aim of the present study was to investigate the signaling activity and regulation of the aGPCR BAI2 (ADGRB2) and assess the potential functional effects of a de novo disease-associated BAI2 mutation (p.R1465W) in a human patient. We found that this mutation in the

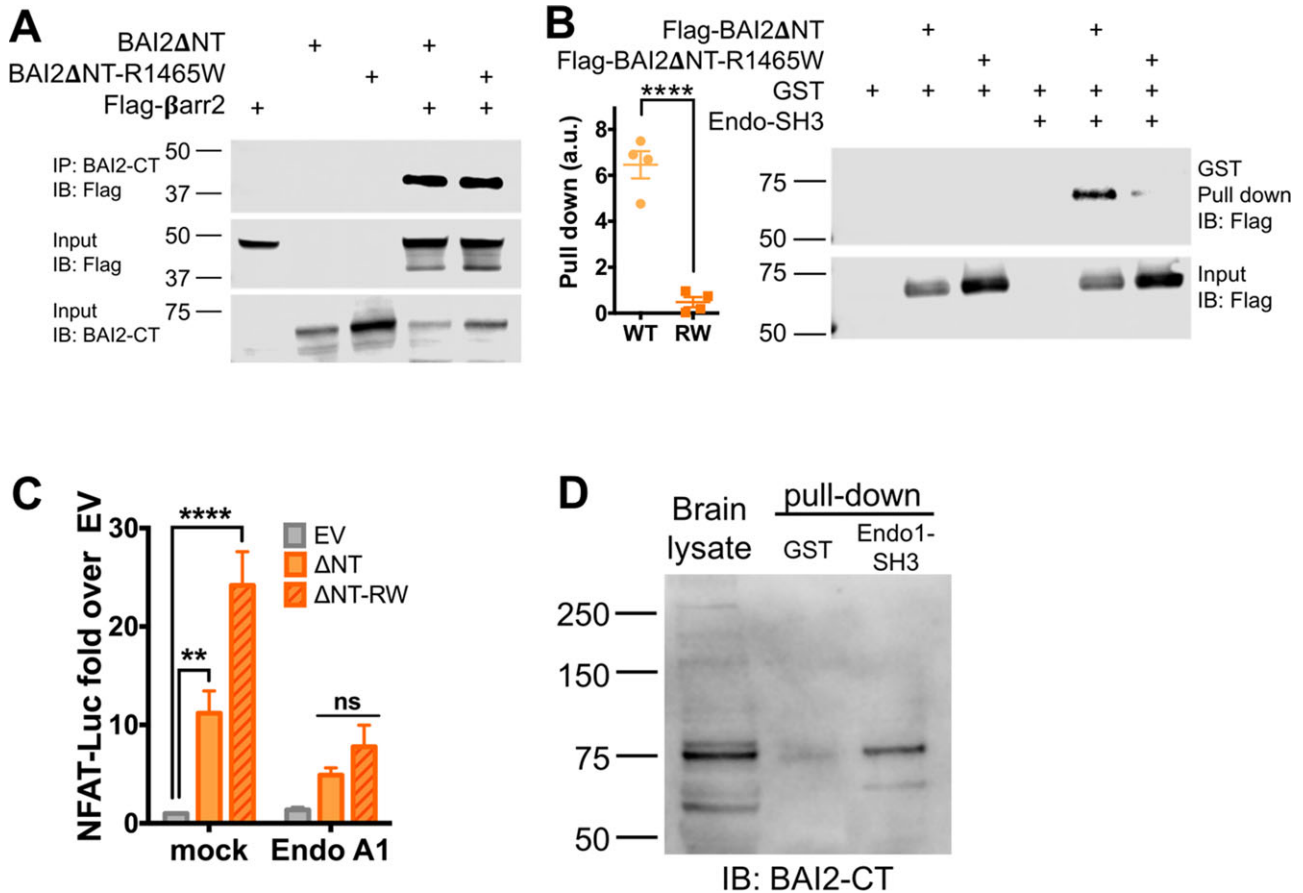


FIGURE 5 BAI2 interacts with endophilin A1. (A) BAI2ΔNT and BAI2ΔNT-RW co-immunoprecipitated equally well with β-arrestin2 ($n = 3$). (B) WT BAI2ΔNT robustly interacted with the endophilinA1 SH3 domain but interaction with the mutant BAI2ΔNT-RW was significantly attenuated (one sample t -test, **** $P < 0.0001$ vs. BAI2ΔNT, $n = 4$). (C) Co-transfection of 2 ng of Endo1 DNA inhibited BAI2ΔNT (second bar of each set) and BAI2ΔNT-RW (third bar of each set) signaling activity to NFAT luciferase (two-way ANOVA, ** $P < 0.01$ vs. EV, **** $P < 0.0001$ vs. EV, after Endo1 co-transfection no significant difference vs. EndoA1-EV, $n = 3$). (D) Endogenous BAI2 from mouse brain lysates interacts with the SH3 domain of endophilin A1 ($n = 3$)

C-terminal region of BAI2 potentiates the receptor's signaling activity and enhances receptor surface expression. In agreement with a previous study, we found that a truncated form of BAI2, corresponding to the predicted BAI2 polypeptide after GAIN domain autoproteolysis and NT shedding, robustly activates the NFAT luciferase reporter (Okajima et al., 2010). Most GPCRs that activate NFAT luciferase do so via coupling G_{α_q} (Hill et al., 2001), but the signaling to NFAT by BAI2 was found in the present study to be almost entirely dependent on $G_{\beta\gamma}$ -mediated signaling. In terms of the G_{α} subunit involved, WT BAI2 exhibited a preferential coupling to the $G_{\alpha_{i/o}}$ -family member G_{α_z} , whereas the p.R1465W mutant exhibited significant coupling to both G_{α_z} and G_{α_i} , which may be related to the increased surface expression of the mutant receptor. Thus, the data presented here suggest two potentially connected mechanisms by which the p.R1465W mutation increases signaling activity: enhancement of receptor surface expression and increased coupling to specific $G_{\alpha_{i/o}}$ -family proteins.

Gain-of-function mutations in other GPCRs have been informative in understanding critical residues involved in receptor activation. For example, any substitution at position 293 in the α_{1B} -adrenergic receptor results in constitutive activity (Kjelsberg, Cotecchia, Ostrowski, Caron, & Lefkowitz, 1992). There are clinical consequences to GPCR

activating mutations as well. For example, missense mutations in the retinal-binding lysine-296 of rhodopsin can result in a constitutively active receptor and lead to the deterioration of rod cells in retinitis pigmentosa (Robinson, Cohen, Zhukovsky, & Oprian, 1992), and the substitution of aspartate for glycine at residue 578 in the luteinizing hormone receptor imparts constitutive activity, which can induce precocious puberty (Shenker et al., 1993). Thus, the investigation of GPCR-activating mutations is important for understanding human disease as well as for shedding light on basic receptor biology (Thompson, Hendy, Percy, Bichet, & Cole, 2014).

4.1 | Effects of the p.R1465W mutation on BAI2 signaling

In our model (Figure 6), removal or rearrangement of the 911-amino acid N-terminus of BAI2 allows the receptor to adopt its active conformation and associate with heterotrimeric G proteins. The data reported in the present study indicate that BAI2ΔNT signaling to the NFAT luciferase reporter is almost entirely mediated by $G_{\beta\gamma}$ subunits, as co-expression of the GRK2-CT or treatment of cells with gallein strongly attenuated the signal. We also found that BAI2 can be

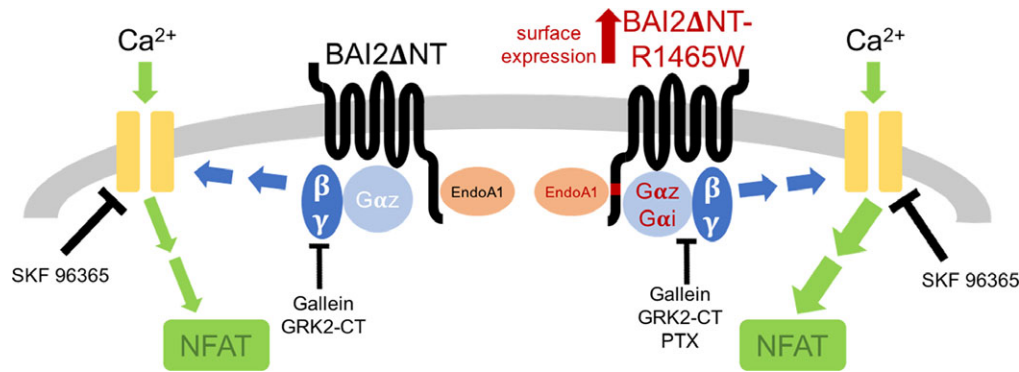


FIGURE 6 BAI2 signaling model. Shedding of the N terminus results in a constitutively active BAI2 (BAI2 Δ NT). BAI2 Δ NT couples to $G\alpha_z$, which liberates $G\beta\gamma$ subunits leading to calcium influx and activation of the NFAT luciferase reporter. However, the R1465W mutation in BAI2 Δ NT results in additional coupling to $G\alpha_i$. The mutation also reduces interaction with endophilin A1 (EndoA1). BAI2 Δ NT-RW is found at higher levels on the cell surface and is significantly more active than the WT receptor

co-immunoprecipitated with $G\alpha_z$, and furthermore observed that the $G\alpha_z$ -specific RGS protein, RGS20, significantly increased BAI2 Δ NT signaling. Thus, these data implicate $G\alpha_z$ as a mediator of BAI2 signaling, although the RGS20 findings are somewhat paradoxical in that a $G\alpha_z$ -specific RGS protein should inhibit $G\alpha_z$ -mediated signaling rather than potentiate it. However, it is important to point out that BAI2 Δ NT is a highly constitutively active receptor and thus is presumably highly desensitized. It may be the case that toning down the $G\alpha_z$ -mediated signal downstream of BAI2 results in less desensitization and therefore more sustained signaling, resulting in a paradoxical increase in the 48-hr luciferase reporter assay. Without an identified ligand or any other tool to activate BAI2 in a temporally controllable manner, overexpression of the cleavage-mimetic Δ NT form of BAI2 is the most effective way we have at present to study its signaling activity, so this represents a limitation of the present study. Alternatively, it is possible that activated $G\alpha_z$ may have unknown functions that limit signaling to NFAT, such that RGS20 relieves this inhibition and thereby potentiates the $G\beta\gamma$ -mediated NFAT activation. We demonstrated that nearly all of the activity that we observed to the NFAT reporter was dependent on calcium influx. A previous study found that $G\alpha_z$ can modulate ion channel function, including that of N-type calcium channels, in a PTX-insensitive manner (Jeong & Ikeda, 1998). Therefore, inhibiting $G\alpha_z$ with RGS20 may relieve inhibition on calcium channels and increase signaling activity to NFAT luciferase.

Additional experiments will be required to more fully understand the mechanisms underlying the $G\beta\gamma$ -dependent Ca^{2+} influx observed in the present study to be downstream of BAI2 Δ NT in HEK cells. SKF96365 is most commonly used as an antagonist of transient receptor potential canonical type channels, but at the concentration we utilized (50 μ M) it can also block several other types of calcium channels (Singh, Hildebrand, Garcia, & Snutch, 2010). Interestingly, it was recently reported that the *Drosophila* homolog of the adhesion GPCR ADGRL1/latrophilin (*dcIRL*) modulates the action of a TRP channel to influence mechanosensation (Scholz et al., 2015). Further studies will be required to determine how exactly BAI2 activation impacts calcium channel function and whether this regulation depends on direct channel association with $G\beta\gamma$ subunits.

4.2 | Effects of the R1465W mutation on BAI2 trafficking

We observed that expression of BAI2 Δ NT-p.R1465W is significantly higher than WT BAI2 Δ NT on the cell surface, with the magnitude of this effect being comparable to the extent by which the mutation increases receptor signaling activity. Additionally, we found that association with β -arrestins was unchanged by the p.R1465W mutation, which suggested other mechanisms may play a role in dictating BAI2 surface expression. Endophilin A1 was recently found to bind to a number of GPCRs and mediate their internalization via a pathway independent of β -arrestins, ubiquitination or clathrin coat proteins (Boucrot et al., 2015). We found that the SH3 domain of endophilin A1 avidly interacts with WT BAI2 Δ NT, but interacts less robustly with the BAI2-RW mutant. However, the mutation does not completely abrogate binding, and overexpression of endophilin A1 in heterologous cells appeared in our studies to overcome the binding deficit and inhibit signaling by the RW mutant to an extent that was not significantly different from the effect on the WT receptor. Thus, the present studies identify endophilin A1 as a novel binding partner of BAI2, although it is uncertain whether the reduced binding of endophilin A1 by the BAI2 RW mutant contributes to the altered trafficking and activity of this receptor. A complicating factor in these studies is that there are three closely-related endophilins (A1-3) that are all widely expressed, so further studies will be needed to dissect the potential regulation of BAI2 by the various members of the endophilin family.

4.3 | Potential clinical importance of ADGRB2

Mutations to GPCRs that increase constitutive activity are frequently toxic (Parnot, Miserey-Lenkei, Bardin, Corvol, & Clauser, 2002). Our data indicate that the BAI2 R1465W mutation, which was discovered in a patient with a progressive neuromuscular disorder, significantly increases BAI2 signaling activity. Given the preferential expression of BAI2 in the nervous system, it is conceivable that heightened and/or prolonged activity from BAI2 could lead to neuromuscular disease. However, because these studies have been focused on a

mutation found in a single patient to this point, it is impossible to make any definitive statements about causality. Still, given the lack of other explanations for the patient's pathology, combined with our data revealing that this mutation induces constitutive over-activity in a receptor expressed in the diseased areas, it is plausible that this mutation either underlies the pathology or acts as a modifier to exacerbate a pre-existing condition, such as multiple sclerosis, to result in an atypical presentation. In either case, BAI2 dysfunction would be implicated in neurodegeneration, and it would therefore be desirable to develop therapeutic strategies to normalize BAI2 activity. Deletion of *Bai2* in mice has been reported to have no obvious negative consequences, with *Bai2* null animals actually displaying increased hippocampal neurogenesis and exhibiting an antidepressant phenotype that includes resistance to learned helplessness behavior (Okajima et al., 2011). Together, these data suggest that BAI2 may be an attractive target for the development of antagonists that might have therapeutic value, and a recent report about the development of a small molecule antagonist for another adhesion GPCR (ADGRG1/GPR56) demonstrates the feasibility of this approach (Stoveken et al., 2016). Thus, the pharmacological targeting on BAI2 will be of interest to pursue in future work, as will future studies, building on the findings presented here, aimed at shedding further light on the function(s) of BAI2 in vivo and the mechanisms by which BAI2 signaling is regulated.

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DISCLOSURE STATEMENT

The authors declare no conflict of interest.

ORCID

Ryan H. Purcell  <http://orcid.org/0000-0002-6652-2747>

Randy A. Hall  <http://orcid.org/0000-0002-8318-8728>

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