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

Ryan H. Purcell[1], **Camilo Toro**[2], **William A. Gahl**[2], and **Randy A. Hall**[1,*]

[1]Department of Pharmacology, Emory University School of Medicine, Atlanta, GA USA 30322

[2]NIH Undiagnosed Diseases Program, Office of the Director, NIH, Maryland, USA 20892

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) underling this effect revealed that wild-type BAI2 primarily couples to $G\alpha_z$, 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

brain; activation; Gβγ; Gz; RGS20; NFAT

Introduction

Adhesion 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 et al., 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 et al., 2004), and a mutation in EMR2 (*ADGRE2*) has been associated with vibratory urticaria (Boyden et al., 2016).

*Corresponding author: rhall3@emory.edu, Telephone: 404-727-3699, Fax: 404-727-0365.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

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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 sub-family receptors, which are also known as Brain-specific Angiogenesis Inhibitors 1–3 (BAI1-3) (Stephenson et al., 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 et al., 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 a resilience to learned-helplessness behavior (Okajima et al., 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 et al., 2010), and at least six other aGCPRs from five different sub-families (Kishore and 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 NFAT luciferase reporter (Okajima et al., 2010), but this study did not elucidate the G protein alpha subunit (Gα) 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 et al., 2000; Paavola et al., 2011; Stephenson et al., 2013; Tobaben et al., 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 (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 $G\alpha_z$, with the mutation promoting enhanced $G\alpha_i$ coupling.

Materials and Methods

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) according to the manufacturer's recommended procedures. After phenol–chloroform DNA purification, samples underwent massively paralleled sequencing utilizing the TruSeqV2 Exome Kit on the Illumina HiSeq2000 (Illumina) 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). 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 (IGV) and compared against publicly available clinical or functional datasets in OMIM (Online Mendelian Inheritance in Man), HGMD (Human Gene Mutation Database), and PubMed. BAI2 snake plot (Fig. 2A) was constructed using Protter (Omasits et al., 2014).

DNA Constructs

Human *ADGRB2* wild-type and R1465W plasmids were synthesized in pcDNA3.1 vectors (Genscript, Piscataway, NJ). Sequences for BAI2 NT and BAI2 NT-RW (912–1585) 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 NT and BAI2 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-$G\alpha_z$ 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-βarrestin2 (Luttrell et al., 1999) was a gift from Robert Lefkowitz (Addgene plasmid # 14692).

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Cell culture

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

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 50mM NaCl, 10mM HEPES pH 7.3, 0.1% Tween-20 (Sigma)) 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) and visualized with Thermo Scientific SuperSignal West solutions on a Li-Cor Odyssey Imager.

Cell Surface Biotinylation

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

Co-immunoprecipitation

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

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

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CO_2 asphyxiation. Brain tissue was harvested and homogenized in ice-cold 150mM NaCl, 25mM HEPES, 10mM MgCl2, 1mM EDTA, 1× fresh protease and phosphatase inhibitor (HALT, Thermo) pH 7.4. Large debris were cleared by centrifugation at 2000 × *g* at 4C and supernatant proteins were solubilized with 1% Triton X-100 (Sigma) for 1hr at 4C. The insoluble fraction was cleared by centrifugation at 17,000 × *g* for 15 min at 4C and samples were normalized to 1mg/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 4C overnight. The following day, beads were washed thoroughly with 1% Triton buffer, eluted with an equal volume of 2× Laemmli buffer, and 20ul was loaded for SDS-PAGE as described above.

Luciferase assays

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

Results

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 (Fig. 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 Undiagnosed Diseases Program (UDP) performed whole exome sequencing on the trio of the patient (proband) and her parents. Sequencing revealed a *de novo* mutation in

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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]). This R1465W mutation is located in the middle of a highly-conserved region of the BAI2 C-terminus (Fig. 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.

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 (NT) forms of the both the WT and mutant receptors using a surface biotinylation approach. These studies revealed that the BAI2 NT p.R1465W mutant exhibited significantly increased surface expression over WT BAI2 NT (Fig. 3A, one-sample t test of surface expression normalized to WT BAI2 NT : BAI2 NT-RW=2.06 ±0.15, $p=0.0019$, $n=5$). Total expression of BAI2 NT-RW also appeared to be increased, but was not statistically significant compared to WT BAI2 NT ($p=0.0637$).

To investigate the role of the p.R1465W mutation on BAI2 signaling activity, we over-expressed full-length and cleavage-mimicking (NT) forms of the wild-type (WT) and mutant receptors and assessed activation of a panel of luciferase reporters. Serum response factor (SRF) and nuclear factor of activated T cells (NFAT) were the most likely outputs based on homology to 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 (Fig. 3B, SRF data not shown). However, BAI2 NT 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 BAI2 NT, $n=4$).

BAI2 NT signals to NFAT luciferase via Gβγ and calcium channel activation

To shed light on the signaling pathway by which BAI2 NT activates NFAT luciferase, a number of different inhibitors were deployed. The BAI2 NT signal to NFAT luciferase was strongly inhibited by the Gβγ subunit inhibitor gallein (Lehmann et al., 2008) (Fig. 3C; Two-way ANOVA, main effect of inhibitor treatment, $F (3, 56) = 10.06$, Holm-Sidak *post-hoc* test BAI2 NT $p<0.001$ vs vehicle for gallein, BAI2 NT-RW $p<0.0001$ vs vehicle for gallein, $n=4$). Neither WT- nor mutant BAI2 NT-induced signaling was inhibited by the PLCβ inhibitor U73122, which blocks signaling downstream of $G\alpha_q$-coupled receptors, and only signaling by the mutant receptor BAI2 NT-RW showed statistically significant sensitivity to pertussis toxin (PTX) (Fig. 3C; BAI2 NT-RW $p<0.01$ vs vehicle, $n=4$), which inhibits $G\alpha_{i/o}$-mediated signaling.

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To further assess the importance of Gβγ liberation on BAI2 NT signaling to NFAT luciferase, we co-expressed GRK2-CT (βARKct), which can bind to and inhibit the activity of Gβγ subunits (Koch et al., 1993). Like the gallein treatment, GRK2-CT inhibited the activity of BAI2 NT-RW (Fig. 3D; two-way ANOVA, main effect of receptor × GRK2-CT interaction F (3, 9) = 7.378, Sidak *post-hoc* test BAI2 NT-RW+GRK2-CT **p=0.0016 vs.vehicle). Furthermore, we observed that the activation of NFAT luciferase by both WT and p.R1465W mutant BAI2 NT was almost completely blocked by the calcium channel inhibitor SKF96365 (Fig. 3D; two-way ANOVA, mian effect of SKF96365 F(1, 32) = 29.95, Holm-Sidak *post-hoc* test BAI2 NT **p = 0.0011 vs vehicle, BAI2 NT-RW ****p<0.0001 vs vehicle). These results indicate that the NFAT reporter activation by BAI2 NT is almost entirely due to Gβγ liberation and activation of a calcium channel.

BAI2 couples to $G\alpha_z$

NFAT-luciferase is a common readout downstream of $G\alpha_q$ signaling (Hill et al., 2001) but can also report activity from $G\alpha_{12/13}$ (Nishida et al., 2007). In addition, Gβγ-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 Gαq, $G\alpha_{12/13}$ and $G\alpha_i$ as the most likely candidates to be the cognate G protein for BAI2 (Kishore et al., 2016). However, we observed no interactions between BAI2 NT and $G\alpha_q$ or $G\alpha_{13}$ in co-immunoprecipitation experiments (Fig. 4A). In contrast, we observed that the $G\alpha_{i/o}$-family member $G\alpha_z$ robustly co-immunoprecipitated with BAI2 NT, with WT and p.R1465W mutant BAI2 NT immunoprecipitating $G\alpha_z$ to a similar extent (Fig. 4B).

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

Based on the findings described above showing that signaling by the R1465W mutant but not WT BAI2 NT was significantly inhibited by the $G\alpha_{i/o}$-inhibitor pertussis toxin (PTX), we hypothesized that R1465W mutant BAI2 NT 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 BAI2 NT and BAI2 NT-RW interactions with $G\alpha_{i1}$. No interaction was observable between WT BAI2 NT and $G\alpha_{i1}$, but substantial co-immunoprecipitation of $G\alpha_{i1}$was observed with the BAI2 NT p.R1465W mutant (Fig. 4D).

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 and

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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 NT robustly co-immunoprecipitates with β-arrestin2, with the R1465W mutation having no effect on this interaction (Fig. 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 NT. We found that WT BAI2 NT robustly interacted with the endophilin A1 SH3 domain, whereas interaction with BAI2 NT-RW was significantly reduced relative to WT (Fig. 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 NT. Indeed, we found that in 96-well format NFAT luciferase assays, as little as 2ng of endophilin A1 DNA significantly reduced the signaling of BAI2 NT and BAI2 NT-RW (Fig. 5C;Two-way ANOVA, main effect of endo1 transfection $F(1, 12)=22.45$, $p=0.0005$, Sidak *post-hoc* test BAI2 NT and BAI2 NT-RW not significantly different from EV with endo1 co-transfection, n=3). Both BAI2 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 et al., 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 (Fig. 5D, n=3).

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 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 $G\alpha_q$ (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 $G\alpha$ subunit involved, WT

BAI2 exhibited a preferential coupling to the $G\alpha_{i/o}$-family member $G\alpha_z$, whereas the p.R1465W mutant exhibited significant coupling to both $G\alpha_z$ and $G\alpha_i$, 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 et al., 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 et al., 1992), and the substitution of aspartate for glycine at residue 578 in the luteinizing hormone receptor imparts constitutive activity, which can induce precocious puberty (Shenker et al., 1993). Thus, the investigation of GPCR-activating mutations is important for understanding human disease as well as for shedding light on basic receptor biology (Thompson et al., 2014).

Effects of the p.R1465W mutation on BAI2 signaling

In our model (Fig. 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 BAI2 NT 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 co-immunoprecipitated with $G\alpha_z$, and furthermore observed that the $G\alpha_z$-specific RGS protein, RGS20, significantly increased BAI2 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 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-hour luciferase reporter assay. Without an identified ligand or any other tool to activate BAI2 in a temporally-controllable manner, overexpression of the cleavage-mimetic 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 pertussis toxin-insensitive manner (Jeong and Ikeda, 1998). Therefore, inhibiting $G\alpha_z$ with RGS20 may relieve inhibition on calcium channels and increase signaling activity to NFAT luciferase.

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Additional experiments will be required to more fully understand the mechanisms underlying the Gβγ-dependent Ca^{2+} influx observed in the present study to be downstream of BAI2 NT in HEK cells. SKF96365 is most commonly used as an antagonist of transient receptor potential canonical type (TRPC) channels, but at the concentration we utilized (50μM) it can also block several other types of calcium channels (Singh et al., 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 Gβγ subunits.

Effects of the R1465W mutation on BAI2 trafficking

We observed that expression of BAI2 NT-p.R1465W is significantly higher than WT BAI2 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 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.

Potential clinical importance of *ADGRB2*

Mutations to GPCRs that increase constitutive activity are frequently toxic (Parnot et al., 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-exisiting 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

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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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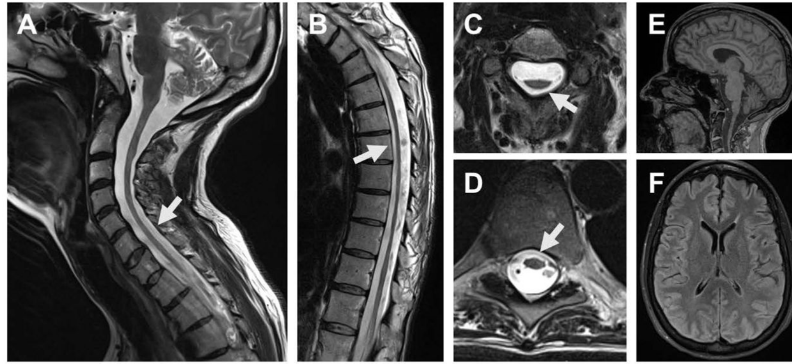

Figure 1. Clinical Presentation

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.

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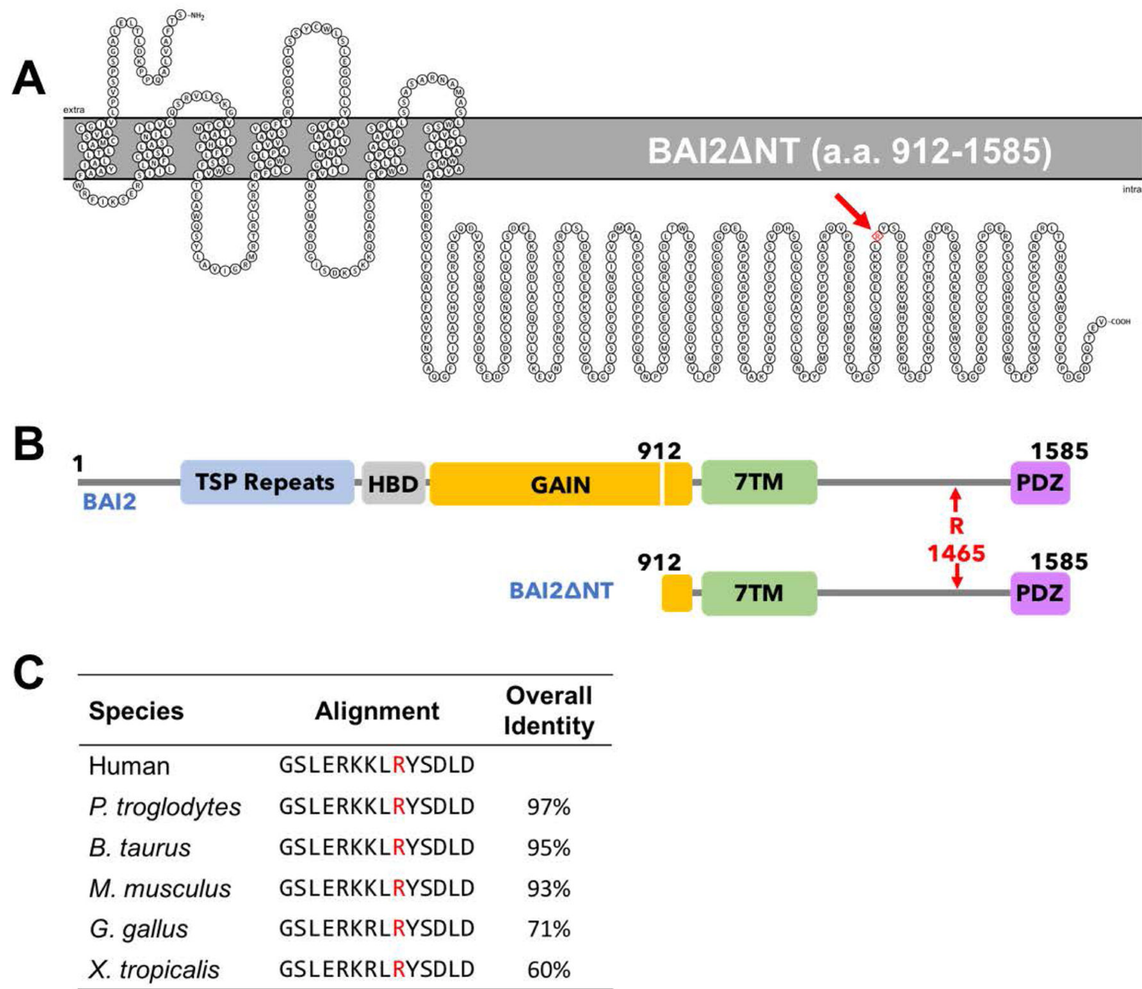

Species	Alignment	Overall Identity
Human	GSLERKKLRYSDLD	
P. troglodytes	GSLERKKLRYSDLD	97%
B. taurus	GSLERKKLRYSDLD	95%
M. musculus	GSLERKKLRYSDLD	93%
G. gallus	GSLERKRLRYSDLD	71%
X. tropicalis	GSLERKRLRYSDLD	60%

Figure 2. BAI2 p.R1465W

A) Topological amino acid diagram of BAI2 NT with an arrow indicating the location of R1465. B) Domain structure schematics of full-length BAI2 and BAI2 NT. 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).

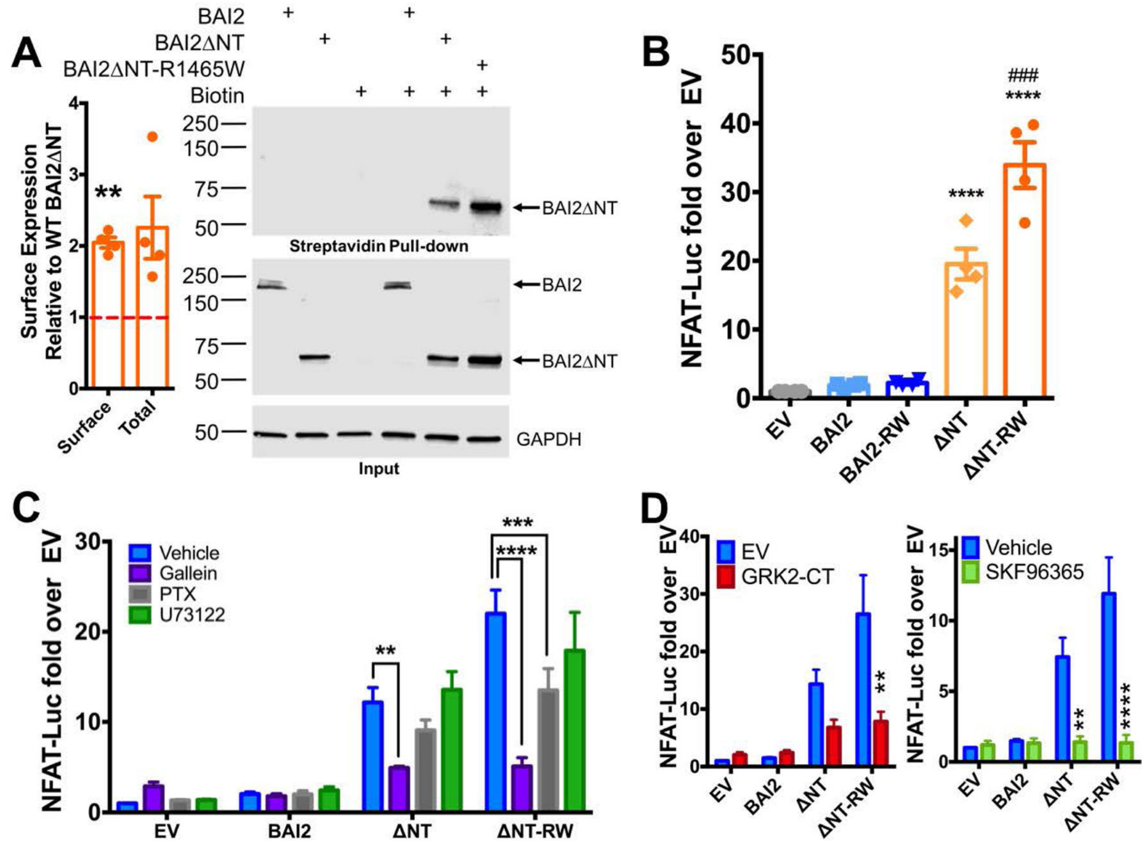

Figure 3. BAI2 p.R1465W increases signaling activity and surface expression

A) The R1465W mutation increased surface and total expression of BAI2 NT, as assessed by surface biotinylation (top, total protein expression bottom). Inset: one sample t test compared to WT=1.00 (Surface **p<0.01 vs B2 NT, n=5). B) BAI2 NT activated NFAT luciferase (one-way ANOVA, Sidak multiple comparisons BAI2 NT ****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 BAI2 NT (### p=0.0001 vs BAI2 NT, n=4). C) The NFAT luciferase signal from BAI2 NT and BAI2 NT-RW was abolished by the Gβγ inhibitor gallein (second bar of each set; **p<0.01 vs B2 NT-vehicle condition, ****p<0.0001 vs BAI2 NT-RW vehicle n=4). The mutant receptor was sensitive to pertussis toxin (PTX, 100ng/ml) but the wild-type receptor was not (third bar of each set; ***p=0.001 vs BAI2 NT-RW vehicle condition, n=4). Neither receptor was inhibited by 10μM U73122 (fourth bar of each set). D) A transfectable Gβγ inhibitor, GRK2-CT (left), significantly inhibited the NFAT signal from BAI2 NT-RW (GRK2-CT-treated is second bar of each set; two-way ANOVA, BAI2 NT-RW **p=0.001 vs EV, n=4). The calcium channel inhibitor SKF96365 (right, 50μM) completely blocked the NFAT signal from both receptors (SKF96365-treated is second bar of each set; two-way ANOVA, BAI2 NT **p>0.01, BAI2 NT-RW ****p<0.0001, n=5).

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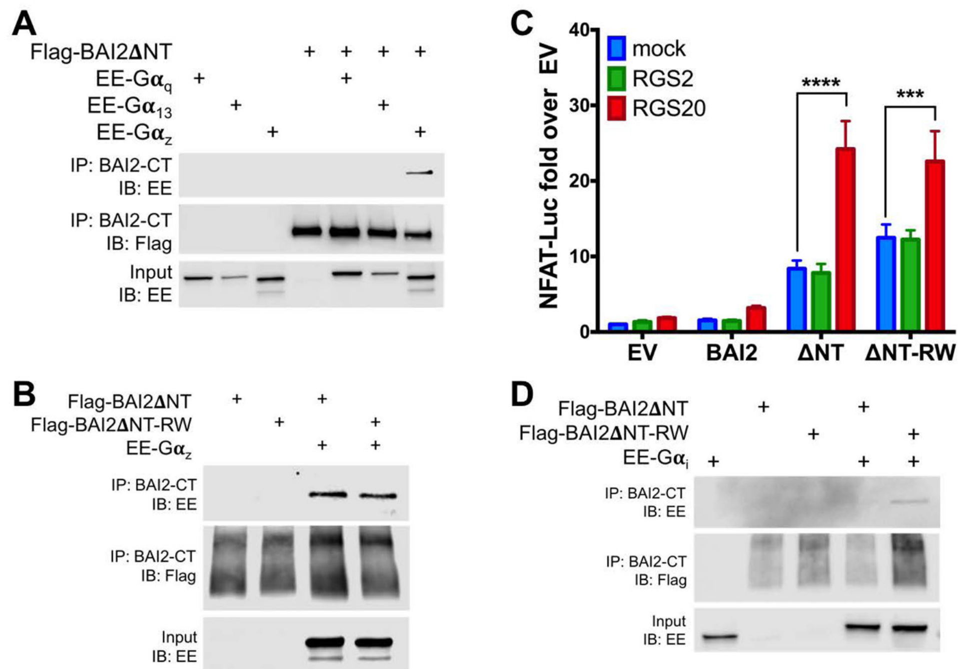

Figure 4. Coupling of BAI2 to G proteins

A) Among a panel of G protein α subunits, only Gαz detectably co-immunoprecipitated with BAI2 NT (n=4). B) Both WT and R1465W forms of BAI2 NT co-immunoprecipitated with Gαz and there was no significant difference in their ability to interact (n=5). C) The Gαq-specific RGS protein RGS2 (second bar of each set) had no effect on WT or RW signaling, but the Gαz-specific RGS20 (third bar of each set) increased both BAI2 NT and BAI2 NT-RW activity (two-way ANOVA, Tukey test BAI2 NT-RGS20 ****p<0.0001 vs BAI2 NT-mock, BAI2 NT-RW ***p=0.001 vs BAI2 NT-RW-mock, n=4). D) Mutant BAI2 NT-RW interacted with Gαi1 but no interaction with the WT receptor was detected (n=3).

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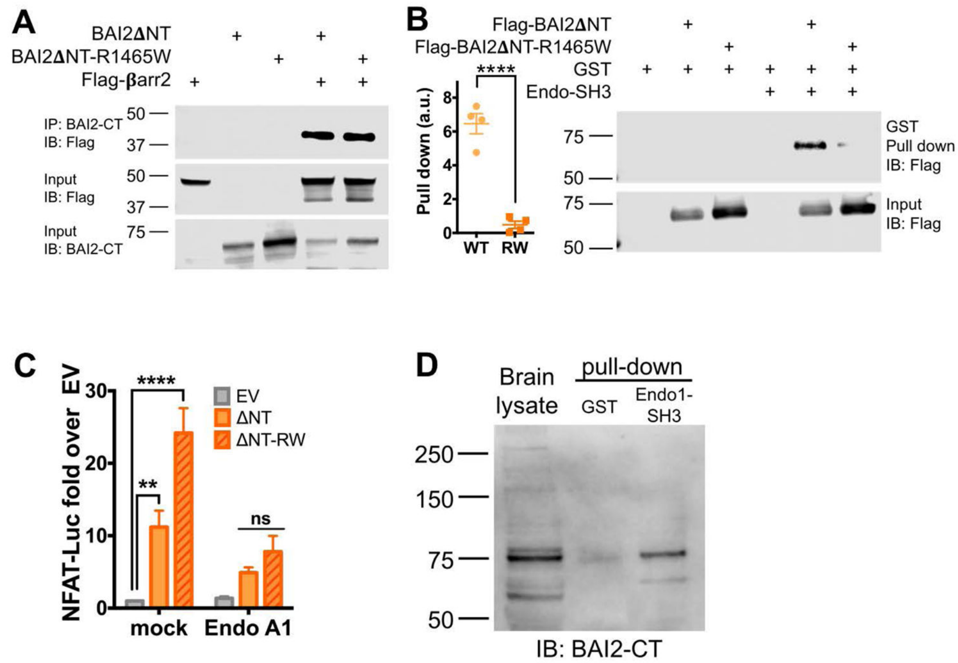

Figure 5. BAI2 interacts with Endophilin A1

A) BAI2 NT and BAI2 NT-RW co-immunoprecipitated equally well with β-arrestin2 (n=3). B) WT BAI2 NT robustly interacted with the endophilinA1 SH3 domain but interaction with the mutant BAI2 NT-RW was significantly attenuated (One sample t test, ****p<0.0001 vs BAI2 NT, n=4). C) Co-transfection of 2ng of Endo1 DNA inhibited BAI2 NT (second bar of each set) and BAI2 NT-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).

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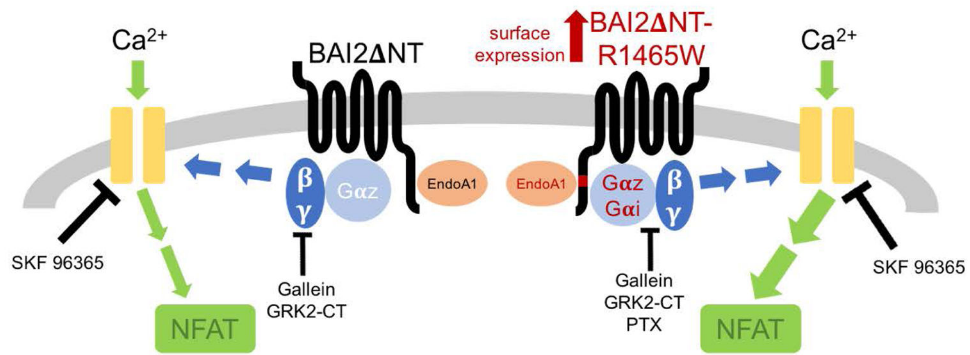

Figure 6. BAI2 Signaling Model

Shedding of the N terminus results in a constitutively active BAI2 (BAI2 NT). BAI2 NT couples to Gαz, which liberates Gβγ subunits leading to calcium influx and activation of the NFAT-luciferase reporter. However, the R1465W mutation in BAI2 NT results in additional coupling to Gαi. The mutation also reduces interaction with endophilin A1 (EndoA1). BAI2 NT-RW is found at higher levels on the cell surface and is significantly more active than the WT receptor.