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Downstream signalling of the disease-associated mutations on GPR56/ADGRG1

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Abstract

GPR56/ADGRG1 is an adhesion G protein-coupled receptor (GPCR) and mutations on this receptor cause cortical malformation due to the over-migration of neural progenitor cells on brain surface. At pial surface, GPR56 interacts with collagen III, induces Rho-dependent activation through $G\alpha_{12/13}$ and inhibits the neuronal migration. In human glioma cells, GPR56 inhibits cell migration through $G\alpha_{q/11}$-dependent Rho pathway. GPR56-tetraspanin complex is known to couple $G\alpha_{q/11}$. GPR56 is an aGPCR that couples with various G proteins and signals through different downstream pathways. In this study, bilateral frontoparietal polymicrogyria (*BFPP*) mutants disrupting GPR56 function but remaining to be expressed on plasma membrane were used to study receptor signalling through $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_{11}$ with BRET biosensors. GPR56 showed coupling with all three G proteins and activated heterotrimeric G protein signalling upon stimulation with *Stachel* peptide. However, *BFPP* mutants showed different signalling defects for each G protein indicative of distinct activation and signalling properties of GPR56 for $G\alpha_{12}$, $G\alpha_{13}$ or $G\alpha_{11}$. β-arrestin recruitment was also investigated following the activation of GPR56 with *Stachel* peptide using BRET biosensors. N-terminally truncated GPR56 showed enhanced β-arrestin recruitment; however, neither *wild-type* receptor nor *BFPP* mutants gave any measurable recruitment upon *Stachel* stimulation, pointing different activation mechanisms for β-arrestin involvement.

KEYWORDS

adhesion GPCR, ADGRG1, bilateral frontoparietal polymicrogyria (BFPP), biosensor, G protein, GPCR, GPR56, β-arrestin

1 | INTRODUCTION

Bilateral frontoparietal polymicrogyria (*BFPP*) is an autosomal recessive genetic disorder, caused by the mutations in the GPR56/ADGRG1 gene located on human chromosome 16q21.[1,2] The genotype–phenotype analysis of *BFPP* patients yielded a cobblestone-like cortical malformation, which is characterized as formation of neuronal ectopias on the surface of the brain and aberrant migration of neural progenitor cells.[3–5] At the pial surface, the interaction of GPR56 with extracellular matrix protein collagen III activates the Rho signalling pathway through $G\alpha_{12/13}$ and this results in inhibition of the neuronal migration.[6]

GPR56 is a member of the G protein-coupled receptor (GPCR) superfamily. In humans, over 800 members constitute this huge receptor superfamily, hallmarked by their 7-transmembrane (7TM) structure and ability to couple heterotrimeric G proteins upon activation. GPCRs are involved in almost all biological processes with their pivotal role as cellular signal transduction of immensely diverse extracellular stimuli (i.e., photons, ions, small molecules, neurotransmitters, hormones and even mechanical stimuli) inside the cell. Mutations and functional problems in GPCRs are linked to many important human diseases.[7] Hence, taking part in almost all physiological processes and possessing druggable sites accessible at the cell surface have already made GPCRs targets of almost 40% of the prescribed drugs.[8]

GPR56 is an adhesion GPCR (aGPCR) according to a further phylogenetic classification of GPCRs in the human genome.[9,10] With 33 members, aGPCRs constitute the second-largest GPCR family in humans. Almost all members in this receptor family have numerous splice variants and these receptor isoforms show tissue-dependent expression, various functions and different downstream signalling properties. All these features in aGPCRs add another layer of complexity and structural variety to this enigmatic family of receptors.[11–13] Arguably, the most intriguing characteristics of aGPCRs are their extremely large N-terminus, which consists of various protein domains that are involved in protein–protein, cell–cell and cell–matrix interactions, and these multi-domain structures point to the multifaceted roles of this receptor family in signal transduction, modulation and cell adhesion.[14,15] A very important characteristic feature of aGPCRs (except for GPR123/ADGRA1) is a highly conserved extracellular GPCR autoproteolysis-inducing (GAIN) domain.[16] In most aGPCRs, an autoproteolytic cleavage occurs at the GPCR proteolysis site (GPS) located within the GAIN domain, yielding non-covalently bound extracellular N-terminal fragment (NTF) and membrane-integrated C-terminal fragment (CTF) composed of 7TM structure and the *Stachel* peptide.[17–22]

GPR56 has a number of interacting partners or ligands such as collagen III in the developing brain,[6] tissue transglutaminase (TG2) in oligodendrocyte progenitor cells and melanoma cells,[23] laminin[24] and heparin in the brain[25]; the receptor was also previously reported to be activated by its *Stachel* peptide (*TYFAVLM*).[20] In neural progenitor cells, GPR56 couples with $G\alpha_{12/13}$ and induces Rho-dependent activation of the transcription mediated through the serum-response element (SRE) and NF-κB-responsive element.[26] GPR56-CD9-CD81 receptor-tetraspanin complexes were shown to couple with $G\alpha_{q/11}$ and Gβ subunits on the plasma membrane.[27] In glioma cells, activation of GPR56 through the receptor NTF leads to the stimulation of $G\alpha_q$-dependent Rho pathway.[28] In HEK293 cells, the exogenous expression of GPR56 induces Rho-dependent stimulation of SRE through $G\alpha_{12/13}$ coupling.[26] Another hallmark of GPCRs, the receptor desensitization through β-arrestin recruitment was also shown for GPR56 using co-immunoprecipitation and in NTF truncated receptors enhancement of arrestin recruitment was also reported.[22,29]

Mutations on GPR56 were shown to cause *BFPP*.[3,5,30–33] Almost all reported missense mutations are located at the extracellular domains of the receptor, either on N-terminus, including the GPS, or on the extracellular loops. Previous studies showed that these receptor mutants cause *BFPP* through different mechanisms such as disrupting the interaction with collagen III,[34,35] inhibition of the receptor autoproteolysis,[3] reduced surface receptor expression, reduced receptor shedding and differential distribution of the 7TM moiety in lipid rafts.[35,36]

Activation of GPCRs on the extracellular side causes conformational changes that facilitate the interaction of receptors with G proteins or β-arrestins at the cytosolic side of the plasma membrane and mediate the downstream signalling pathways. Elucidating the molecular mechanisms of receptor activation and signalling with high spatiotemporal resolution is very crucial for GPCRs due to their pivotal roles in many physiological processes, their related pathologies, therapeutic relevance and druggability. During the past recent years, there has been a considerable effort to develop bioluminescent or fluorescent biosensors working on the principles of resonance energy transfer (RET) for the real-time monitoring of the GPCR activation in live cells.[37–39] These systems were successfully applied to study the activation of numerous GPCRs, their coupling with G proteins and recruitment of β-arrestins.[40,41]

In this work, the effects of *BFPP* mutations on GPR56 signalling upon *Stachel* peptide activation with $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_{11}$ were studied using BRET biosensors. In principle, biosensors used are based on the Gβγ dissociation from the G protein α-subunit upon GPCR activation in the exogenous expression of G protein-coupled receptor kinase (GRK).[42,43] β-arrestin recruitment BRET biosensor was employed to investigate the effects of disease-associated mutations on the arrestin recruitment for *Stachel* peptide activated receptors.[44]

2 | MATERIAL AND METHODS

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[45]

2.1 | Chemical reagents and other materials

General laboratory chemicals were purchased from Sigma–Aldrich (Darmstadt, Germany) or Merck (Darmstadt, Germany). Cell culture media components, Lipofectamine™ LTX Reagent with PLUS™, GeneJET Plasmid Miniprep Kit and GeneJET PCR Purification Kit were purchased from Thermo Fisher Scientific (MA, USA) or Invitrogen (MA, USA). Q5 Hot Start High-Fidelity DNA Polymerase was purchased from New England Biolabs (MA, USA). Primers used in this study were purchased from IDT Europe (Munich, Germany). Protease inhibitor cocktail tablets were from Roche Diagnostics, (Mannheim, Germany). Antibodies were from Santa Cruz Biotechnology (TX, USA), anti-GPR56 (sc-390192), anti-β-actin (sc-47778) and secondary horseradish peroxidase-conjugated antibody (sc-516102). Immobilon-P membrane was from Millipore (Millipore Corporation, Bedford, MA). ECL reagent was from Pierce (MA, USA). White 96-well Lumitrac microplates were purchased from Greiner Bio-One (Kremsmünster, Austria); Nano-Glo® Luciferase Assay System, which includes Furimazine, was from Promega (Promega, USA); Coelenterazine was from Invitrogen (MA, USA). Berthold Mithras² LB 943 multimode plate reader was used in bioluminescence/fluorescence-based experiments.

2.2 | Synthesis of P7 *Stachel* peptide

The *Stachel* peptide for GPR56 P7, "*TYFAVLM*" was synthesized using solid phase peptide synthesis based on Fmoc strategy using HBTU and DIEA as the coupling agents. Twenty percent piperidine-DMF solution was used as deprotection solution for Fmoc group, and Rink amide resin was used as solid support. To cleave the side chain protecting groups and the peptide from resin, TFA-TIPS-H_2O (95:2.5:2.5) system was used. Analytical HPLC was performed on a Dionex instrument, with Thermo-Scientific ODS Hypersil, 5μ C18 column. 0.1% TFA-water as solvent A and 0.08% TFA-acetonitrile as solvent B were used. The gradient was 0%–90% B 20 min, and peptide was used without the necessity of any further purification. The purity of synthesized peptide was >90%.

2.3 | DNA constructs

The plasmid carrying the full-length human cDNA of GPR56/ADGRG1 was a generous gift from Assoc. Prof. Dr. Demet Araç (University of Chicago, USA). GRK-based G protein BRET biosensors (Venus 156-239-Gβ1, Venus 1-155-Gγ2 and masGRK3ct-NanoLuc) were kind gifts from Prof. Dr. Kirill Martemyanov (University of Florida, USA). Finally, β-arrestin BRET biosensors (Rluc8-Arrestin-3-Sp1 and mem-linker-citrine-SH3) were generously donated by Prof. Dr. Nevin A. Lambert (Augusta University, USA).

2.4 | Site directed mutagenesis

Five missense mutations from *BFPP* patients, which were shown not to fully block the surface expression of GPR56,[46] were introduced using site-directed mutagenesis with the double primer method. For this, both forward and reverse primers were designed to carry the targeted base change and to prevent the primer-primer dimers, and eight non-overlapping bases were introduced at the 3′ end of each primer. All primers used in the mutagenesis are shown in Table 1 (see Supporting Information for more). All constructs were verified by sequencing.

2.5 | Cell culture and transient transfection of the cells

Human embryonic kidney cells (HEK293) were maintained in a humidified incubator with 5% CO_2 and 90% humidity at 37°C. Cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) with high glucose (25 mM) and pyruvate (1 mM) supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin and 0.1 mg/mL streptomycin. Cells were regularly tested for mycoplasma contamination (EZ-PCR™ Mycoplasma Detection Kit, Kibbutz Beit-Haemek, Israel). For experiments, cells were seeded at a density of 2×10^5 to each well of the clear 24-well plate. Before transient transfection cells were grown to 60%–80% confluence. Transfection was performed using Lipofectamine™ LTX with PLUS™ transfection reagent according to the manufacturer's instructions. Biosensors were used in transfection as explained in the previous reports: expression constructs (total 0.5 μg/well) were transfected into the cells using Lipofectamine LTX (5 μL/well) and PLUS (5 μL/well) reagents. For $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_{11}$ BRET biosensor assays GPR56 (71 ng/well), Gα of interest (213 ng/well), Venus-156–239-Gβ1 (71 ng/well), Venus-1–155-Gγ2 (71 ng/well) and

TABLE 1 List of primers used to construct *BFPP* mutants of GPR56.

Change in amino acid	Change in base	Primer sequence (5′ > 3′)
R38W	112C > T	tgcagccag**t**ggaaccagacacacaggagc
		ctggttcc**a**ctggctgcagaagcgaaagtc
Y88C	263A > G	aggggcctct**g**ccacttctgcctctactggaac
		gcagaagtgg**c**agaggcccctggggtcagg
C91S	272G > C	ctctaccacttct**c**cctctactggaaccgacatgctggg
		ccagtagagg**g**agaagtggtagaggcccctggg
R565W	1693C > T	gtgctggatc**t**gggactccctggtcagctac
		cagggagtccc**a**gatccagcacatggaagg
L640R	1919 T > G	cttgtcgtcc**g**ctaccttttcagcatcatcacc
		gaaaaggtag**c**ggacgacaagctggaaggtgcc

Note: For each mutation, first lane corresponds forward primer and second lane gives reverse primer. All primers are given in 5′ > 3′.

masGRK3ct-Nluc-HA (71 ng/well) constructs were used.[42,43] For β-arrestin BRET biosensor assays, GPR56 (71 ng/well), Rluc8-Arrestin-3-Sp1 (8 ng/well), mem-linker-citrine-SH3 (178 ng/well), GRK2 (178 ng/well) and pCDNA3.1 (65 ng/well) were used.[44] Assays were performed 24 h after transfection as confluency reaches 80%.

2.6 | Membrane preparation and Western blot analysis

Cells were washed with ice-cold PBS buffer and lysed with RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40 [NP-40], 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 50 mM Na β-glycerophosphate, 1 mM NaF and 1× protease inhibitor cocktail tablet). Extracts were centrifuged for 30 min at 16000 × g at 4°C. Protein concentration was measured with Pierce™ BCA (Bicinchoninic acid) protein assay kit. Membrane proteins were solubilized in SDS sample buffer, and 20 μg of total protein from cells expressing GPR56 constructs was resolved by SDS-PAGE and transferred to Immobilon-P PVDF membrane (Millipore Corporation, USA). Blotting membranes were blocked with 5% non-fat dry milk in TBST for 1 h at room temperature and followed by three washes with TBST. Blotting membranes were incubated with 1:500 diluted anti-GPR56 raised against amino acids 289–381 mapping within an internal region of GPR56 of human origin (sc-390192) or anti-β-actin (sc-47778) primary antibodies overnight at 4°C. The next day, blotting membranes were washed three times with TBST and incubated with 1:7500 diluted HRP conjugated secondary antibody (sc-516102) for 1 h at room temperature. After the secondary antibody incubation, blotting membranes were washed with TBST and briefly incubated with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Pierce, USA) according to the manufacturer's instructions. Blots were imaged using ChemiDocTM MP imaging system (BioRad, USA).

2.7 | $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{11}$ BRET biosensor assay

For Gα BRET biosensor assay, 24 h post-transfection, HEK293 cells were washed with BRET buffer (Dulbecco's phosphate-buffered saline containing 0.5 mM $MgCl_2$ and 0.1% glucose)[43] and gently detached from 24-well plate on the day of the assay. Cells were centrifuged at 900 rpm for 5 min, and 7.5×10^4 HEK293 cells were seeded into each well of a white opaque 96-well microplate. Triplicate wells were loaded with P7 *Stachel* peptide (*TYFAVLM*) with a final concentration of 1mM and 1:1000 ratio of Furimazine. After 5 min of incubation in the dark, bioluminescence and fluorescence signals were measured using Berthold Mithras² LB 943 multimode plate reader using MicroWin 2010 software (Berthold Technologies, Germany) equipped with high-efficiency BRET filters. For Nluc emission, BRET filter (460 nm/70) and for Venus emission, eYFP (540 nm/40) filters were used in biosensor-based BRET experiments. Data acquired represents at least from three independent experiments done in triplicates. The statistical evaluation of quiescent receptor versus *Stachel* stimulation within each construct was done by multiple *t*-tests. The comparison of each *BFPP* mutant with the *wild-type* GPR56 was done using ordinary one-way ANOVA and Dunnett's multiple comparison tests.

2.8 | β-Arrestin BRET biosensor assay

For β-arrestin recruitment BRET biosensor assay, 24 h post-transfection, HEK293 cells were washed with BRET buffer (Dulbecco's phosphate-buffered saline containing 0.5 mM $MgCl_2$ and 0.1% glucose) and gently detached from 24-well plate on the day of the assay. Cells were centrifuged at 900 rpm for 5 min, and 7.5×10^4 HEK293 cells were seeded into each well of a white opaque 96-well microplate. Wells were loaded with P7 *Stachel* peptide (*TYFAVLM*) to give a final concentration of 1 mM and with Coelenterazine native to yield a final concentration of 5 μM per well. After 5 min of incubation in the dark, bioluminescence and fluorescence signals were measured using Berthold Mithras² LB 943 multimode plate reader using MicroWin 2010 software (Berthold Technologies, Germany) equipped with high-efficiency BRET filters. For Citrine emission, eYFP filter (540 nm/40) and for RLuc8 emission, Coelenterazine filter (480 nm/20) were used in experiments. Data acquired represents at least from three independent experiments done in triplicates. The statistical evaluation of quiescent receptor versus *Stachel* stimulation within each construct was done by multiple *t*-tests. The comparison of each *BFPP* mutant with the *wild-type* GPR56 was done using ordinary one-way ANOVA and Dunnett's multiple comparison tests.

3 | RESULTS

3.1 | Expression of *BFPP* mutant receptors

GPR56 or GPR56R38W, GPR56Y88C, GPR56C91S, GPR56R565W and GPR56L640R *BFPP* mutants were expressed in HEK293 cells by transient transfection (Figure 1). It was previously reported that GPR56 is fully cleaved from the GPS in HEK293 cells.[47] The *BFPP* mutations studied in this work were also shown not to effect receptor autoproteolysis.[36] Under the SDS PAGE conditions reported herein, the non-covalent interactions between NTF and CTF were lost and only the receptor NTF was detected with the expected size around 55 kDa using anti-GPR56 antibody, which recognizes the NTF of the receptor. A decrease in the expression for all *BFPP* mutant receptors was observed compared with the *wild-type* GPR56 (Figure 2). All *BFPP* mutants used in this study were previously reported to be expressed on the cell surface.[46]

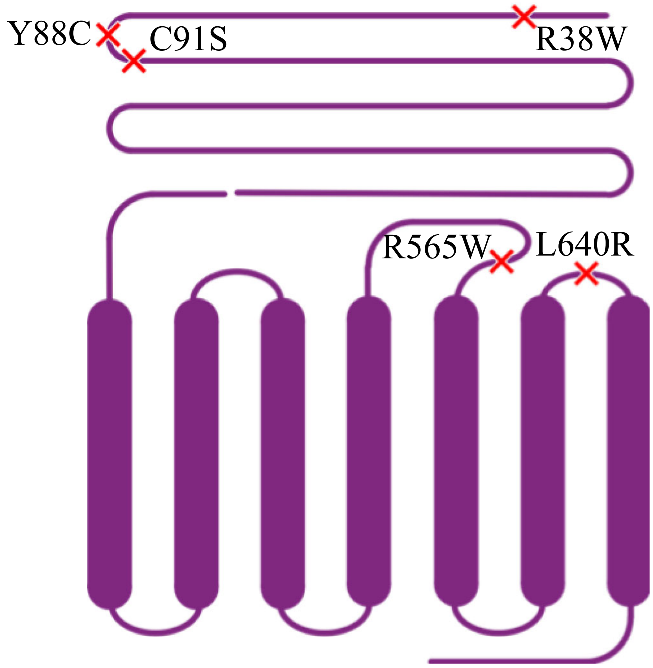

FIGURE 1 The schematic representation of GPR56 and positions of *BFPP* mutations on the receptor.

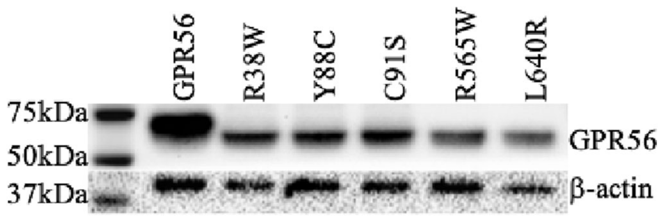

FIGURE 2 Western blot showing the expression of GPR56 and the *BFPP* mutants in transiently transfected HEK293 cells. The first row corresponds to the expression of GPR56 and the mutant receptors detected by anti-GPR56 antibody targeting the NTF. The lower row corresponds to β-actin expression detected by anti-β-actin antibody.

3.2 | The effect of *BFPP* mutations on $G\alpha_{12}$ coupling

GPR56 or GPR56R38W, GPR56Y88C, GPR56C91S, GPR56R565W and GPR56L640R *BFPP* mutants were co-expressed with $G\alpha_{12}$ and GRK-based Gβδ biosensors to assess the effect of each mutation on the G protein coupling, upon receptor activation with the *Stachel* peptide. For this, HEK293 cells were treated with 1 mM P7 *Stachel* peptide and $G\alpha_{12}$ coupling and heterotrimeric G protein activation was measured as BRET signal. The most severe coupling defect between the receptor and $G\alpha_{12}$ was observed for GPR56L640R ($p < 0.01$ compared with GPR56). $G\alpha_{12}$ coupling defects were also measured for GPR56R565W ($p < 0.01$ compared with GPR56) and GPR56C91S ($p < 0.01$ compared with GPR56). GPR56R38W and GPR56Y88C *BFPP* mutants showed $G\alpha_{12}$ coupling upon *Stachel* peptide stimulation with no significant change compared with the *wild-type* receptor (Figure 3).

3.3 | The effect of *BFPP* mutations on $G\alpha_{13}$ coupling

GPR56 or *BFPP* mutants of the receptor were co-expressed with $G\alpha_{13}$ and GRK-based Gβδ biosensors to assess the effect of each mutation on the $G\alpha_{13}$ protein coupling, upon receptor activation with the *Stachel* peptide. For this, HEK293 cells were treated with the 1 mM P7 *Stachel* peptide and $G\alpha_{13}$ coupling and heterotrimeric G protein activation was measured as BRET. Under the biosensor conditions reported in this work, all *BFPP* mutants showed disrupted $G\alpha_{13}$ coupling while the most severe was observed for GPR56R38W (Figure 4).

3.4 | The effect of *BFPP* mutations on $G\alpha_{11}$ coupling

GPR56 was previously shown to couple with $G\alpha_{q/11}$ as a GPR56-CD81-$G\alpha_{q/11}$ complex.[27] In human glioma cells, stimulation of GPR56 with its agonistic monoclonal antibodies resulted in signalling through $G\alpha_q$-dependent Rho pathway.[28] GPR56 or *BFPP* mutants of the receptor were co-expressed with $G\alpha_{11}$ and GRK-based Gβδ biosensors to assess the effect of each mutation on the G protein coupling when the receptor is stimulated with its *Stachel* peptide. For this, HEK293 cells were treated with the

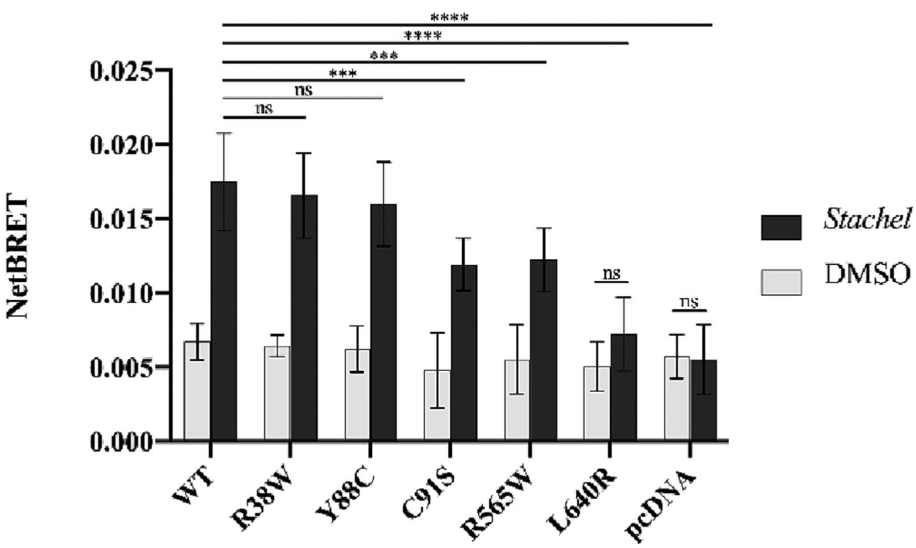

FIGURE 3 The coupling of Stachel-activated GPR56 and disease associated mutations of the receptor with $G\alpha_{12}$. Data represents three independent experiments done in triplicates.

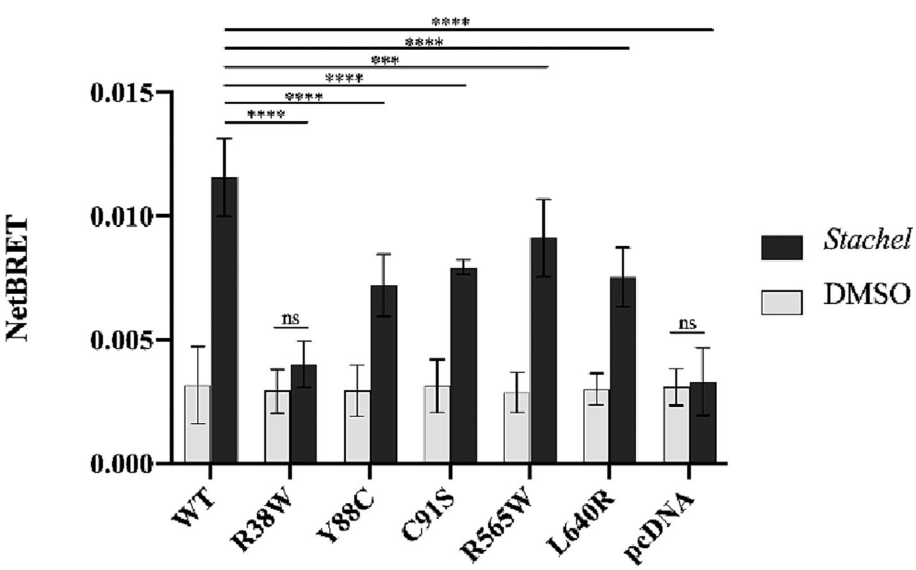

FIGURE 4 The coupling of Stachel-activated GPR56 and disease associated mutations of the receptor with $G\alpha_{13}$. Data represents at least from three independent experiments done in triplicates.

1mM P7 *Stachel* peptide and Gα_{11} coupling and heterotrimeric G protein activation was measured as BRET signal. GPR56C91S gave statistically indistinguishable BRET compared with *wild-type* receptor. A decrease in BRET indicative of G protein coupling defect was measured in all other *BFPP* mutants; however, GPR56R565W did not show any Gα_{11} coupling (Figure 5).

3.5 | The effect of *BFPP* mutations on β-Arrestin recruitment

It was previously reported that NTF truncated human GPR56 from amino acids 1–342 gave enhanced interactions with β-arrestin compared with the full-length receptor.[29] In this work, GPR56 or *BFPP* mutants of the receptor were co-expressed with β-arrestin (Rluc8-arrestin3-Sp1) and GRK-based biosensors[44] to assess the effect of each mutation on the β-arrestin recruitment, upon receptor activation with the *Stachel* peptide. For this, HEK293 cells were treated with 1 mM P7 *Stachel* peptide and β-arrestin recruitment was measured as a BRET signal. The arrestin biosensor assay utilized in this study gave increased plasma membrane recruitment for the NTF truncated GPR56; however, stimulation of *wild-type* receptor or *BFPP* mutants with the *Stachel* peptide did not lead to any measurable recruitment (Figure 6).

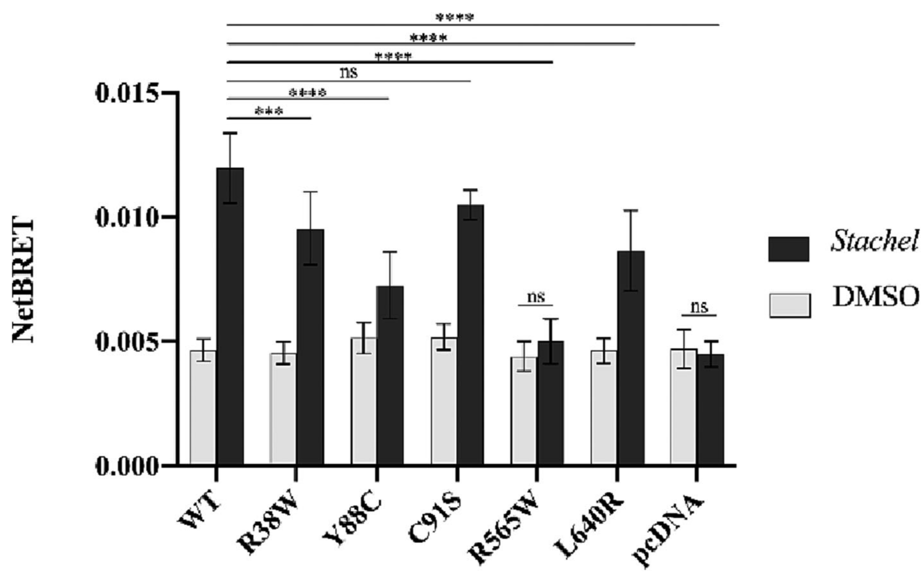

FIGURE 5 The coupling of Stachel-activated GPR56 and disease associated mutations of the receptor with Gα_{11}. Data represents at least from three independent experiments done in triplicates.

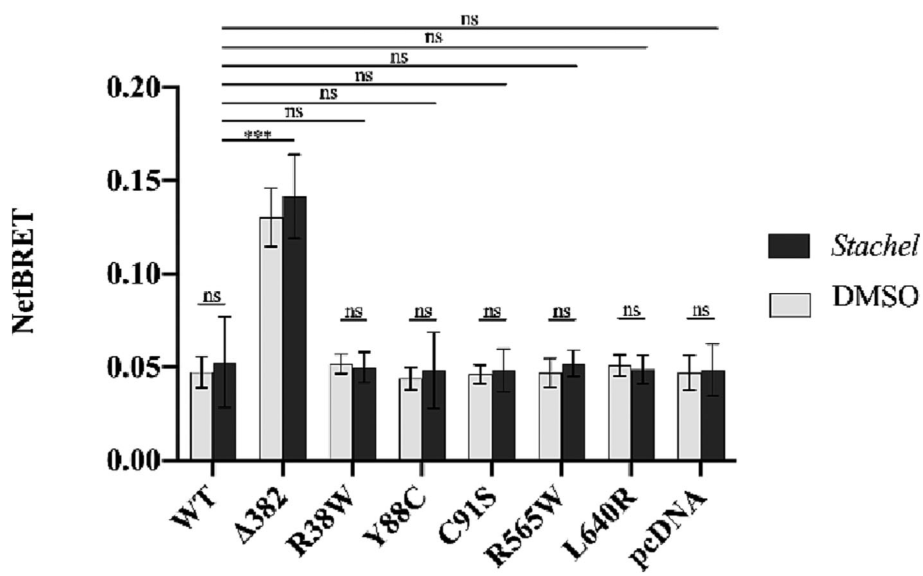

FIGURE 6 β-arrestin recruitment of the Stachel-activated GPR56 and *BFPP* associated mutants of the receptor. Data represents three independent experiments done in triplicates.

4 | DISCUSSION

GPR56 is known to be involved in a wide range of physiological processes in the nervous system, immune system, male reproductive organs, haematopoietic stem and progenitor cells; functional problems with the receptor result in pathologies such as *Bilateral frontoparietal polymicrogyria (BFPP)*, depression and arguably GPR56 is the most studied aGPCR in cancer.[48] *BFPP* is a monogenetic disease resulting in a severe brain malformation and several mutations in GPR56 were found in patients diagnosed with *BFPP*.[2] In this work, five missense *BFPP* mutations on GPR56 were chosen that were previously reported to be expressed on the plasma membrane.[35,36,46] From those, R38W, Y88C and C91S are found on the ligand binding domain of the receptor NTF and previously reported to impair collagen III binding[34] R565W is located on the 2nd extracellular loop and L640R is on the 3rd extracellular loop (Figure 1). The two GPS mutations C346S and W349S were excluded from the study since they were shown not to traffic out of the ER[46] and hence reported to fully block the surface expression of GPR56.[36] All five GPR56 mutants showed reduced total expression compared with the *wild-type* receptor (Figure 2). Regarding the surface expression, the NTF of GPR56R38W, GPR56Y88C, GPR56C91S, GPR56R565W and GPR56L640R were previously shown to be detected from the medium of cells expressing these mutants.[36] In the same study, it was further reported that the amount of soluble NTF was correlated positively with the surface expression levels of receptors when compared with the flow cytometry data and surface expression for all these *BFPP* mutants was observed with confocal immunofluorescence staining.[36] In a separate study, R565W and L640R mutations were shown not to affect the surface expression of the receptor and these *BFPP* mutants were detected with comparable levels with the *wild type*; however, R38W, Y88C and C91S mutants were reported to have decreased surface expression.[35]

GPR56 is known to inhibit neural progenitor cell migration by coupling with $G\alpha_{12/13}$ and inducing Rho-dependent activation of the transcription mediated through the serum-response element (SRE) and NF-κB-responsive element resulting in an actin fibre reorganization.[26] GPR56 is also known to form a complex with tetraspanin proteins CD9 and CD81 on the plasma membrane and this complex couples with $G\alpha_{q/11}$ and Gβ subunits.[27] Agonistic monoclonal antibodies generated towards the NTF of GPR56 resulted in the inhibition of human glioma cell migration through the $G\alpha_q$-dependent Rho pathway.[28] GPR56 was also reported to promote myoblast fusion through SRE and nuclear factor of activated T-cell (NFAT)-mediated signalling.[49] Expression of GPR56 into HEK293 cells is known to lead $G\alpha_{12/13}$ activation and induce Rho-dependent stimulation of SRE[26]; however, the Rho-, SRE-, or NFAT-mediated downstream signalling studies fail to discriminate between $G\alpha_{12}$ and $G\alpha_{13}$ signalling, which were known to have distinct physiological roles.[50–52] Furthermore, Gβγ and $G\alpha_{q/11}$ were also reported to induce Rho activation.[53,54]

GPR56 has numerous interacting partners such as collagen III,[6] tissue transglutaminase (TG2),[23] and laminin[24]; the receptor was also previously reported to be activated by its *Stachel* peptide (*TYFAVLM*).[20] In the current work, the coupling of GPR56 with $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_{11}$ upon the receptor activation with its *Stachel* peptide and the effects of *BFPP* mutants on G protein coupling was studied. For this, BRET based biosensor system was chosen that is suitable for untagged receptor, untagged Gα, Venus tagged Gβ1γ2 and masGRK3ct-Nluc, which is composed of G protein-coupled receptor kinase 3 carrying a myristic acid plasma membrane attachment peptide tagged with Nluc.[42] In this biosensor design, upon receptor activation, the Venus fluorescent protein-tagged Gβ1γ2 heterodimer dissociates from the Gα resulting an increased proximity towards membrane-bound masGRK3ct-Nluc and hence an increased BRET readout.

Under the biosensor conditions reported in this study, stimulation of GPR56 with its *Stachel* peptide showed G protein coupling and heterotrimeric G protein activation for $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_{11}$. However, GPR56 *BFPP* mutants showed different coupling defects for each G protein α-subunit. Coupling between GPR56 and $G\alpha_{12}$ was disrupted in both extracellular loop *BFPP* mutations (GPR56L640R and GPR56R565W); on the other hand, receptor $G\alpha_{13}$ coupling and heterotrimeric G protein activation were measured for the extracellular loop receptor mutants. These results indicate the distinct signalling roles of GPR56 with $G\alpha_{12}$ or $G\alpha_{13}$. *Stachel* peptide stimulation of the receptor also showed measurable $G\alpha_{11}$ activation in the biosensor assay reported herein. GPR56 activation was shown to lead Gβγ subunit dissociation from $G\alpha_{11}$ and liberated Gβγ activates the calcium channels.[47] The working principle of the biosensor in this study relies on the change in BRET upon dissociation of Gβγ from Gα as a result of receptor activation. While the results confirmed the previous findings, studying *BFPP* mutants provided further insights into $G\alpha_{11}$ signalling. The most severe disruption was observed between GPR56R565W and $G\alpha_{11}$ coupling. All *BFPP* mutants showed disrupted coupling with $G\alpha_{11}$ and heterotrimeric G protein activation except for GPR56C91S. In sum, stimulation of GPR56 with its *Stachel* peptide resulted in measurable activation in $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_{11}$ signalling; however, *BFPP* mutations resulted in distinctly different effects for each signalling pathway. It is noteworthy to mention at this point that the data supplied in this study reflect the activation of GPR56 with its *Stachel* peptide, collagen III or TG2 treatment will provide more insights about the signalling of this receptor.

In the second part of the study, β-arrestin recruitment of GPR56 or *BFPP* mutants was measured upon activation of the receptor with its *Stachel* peptide. For this, a BRET-based biosensor suitable for untagged receptors that measure the translocation of β-arrestin to the plasma membrane upon receptor activation was utilized. In the biosensor design, Rluc8 tagged β-arrestin and membrane-bound citrine fluorescent protein were used and the recruitment of β-arrestin was measured as a BRET increase in the exogenous expression of G protein-coupled receptor kinase 2.[44] In this biosensor design, receptor activation leads to the recruitment of Rluc8 tagged β-arrestin to the plasma membrane resulting an increased proximity towards membrane-bound citrine fluorescent protein and hence an increased BRET readout. Activated and GRK phosphorylated GPCRs are known to complex with β-Arrestins. This blocks receptors' coupling with G proteins and results in their desensitization and internalization via clathrin-coated pits.[55] It was previously reported that NTF truncated GPR56 showed higher basal activity and enhanced binding with β-arrestin.[29,47] In accordance with the previous findings, NTF truncated GPR56 gave enhanced β-arrestin recruitment to the plasma membrane; however, *Stachel* peptide stimulation of neither GPR56 nor *BFPP* mutants showed any significant change in β-arrestin recruitment. These results point to a different receptor activation mechanism for β-arrestin possibly through the activation of GPR56 with other ligands/interacting partners and leading to receptor desensitization.

The complex architecture of aGPCRs arises from their extremely large NTFs. These structures carry various protein domains that are involved in diverse array of protein interactions including the extracellular matrix proteins, cell–cell interactions and cell adhesion. Some members of this enigmatic family of receptors were also shown to be involved in sensing the mechanical stimuli at the cellular level.[18,56–58] Adhesion GPCRs also function like classical GPCRs, signalling canonically through their CTF domains that couple with and activate heterotrimeric G proteins. Hence, our current knowledge points to the multifaceted and multi-functional roles of this receptor family in signal transduction and modulation.[14,15] The results reported in this study measured the direct coupling of three G proteins, $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_{11}$ with GPR56 and the heterotrimeric G protein activation. Previous reports on GPR56 signalling rely mostly on the Rho-dependent activation of further downstream elements rather than the direct G protein activation. Studying the downstream signalling defects of the disease-associated mutations of GPR56 indicated that the receptor has distinct activation and signalling properties for each G protein and mutations located in various compartments of the receptor showed distinctly different coupling disruptions. In β-arrestin recruitment assays, NTF truncated receptor showed enhanced β-arrestin translocation to the plasma membrane; however, stimulation of *wild-type* GPR56 or *BFPP* mutants with the *Stachel* peptide did not result in any recruitment. These results might indicate that β-arrestin recruitment possibly requires different activation mechanisms.

The BRET-based biosensors used in this study showed the direct coupling of GPR56 with $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_{11}$ and the heterotrimeric G protein activation. Also, BRET-based β-arrestin biosensor measured an enhanced recruitment of the NTF truncated receptor as previously reported. Considering the rich physiology and related pathologies of GPR56, the biosensors utilized in this work can further be applied for studying the mechanisms of receptor activation through different interacting partners and applied for drug screening studies.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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