Biased Signaling is Structurally Encoded As An Autoproteolysis Event in Adhesion G Protein-Coupled Receptor Latrophilin-3/ADGRL3

Running title: Adhesion receptor signaling bias

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ABSTRACT

Adhesion G protein-coupled receptors (aGPCRs) possess a unique topology including the presence of a GPCR proteolysis site (GPS) which upon autoproteolysis generates two functionally distinct fragments that remain non-covalently associated at the plasma membrane. A proposed activation mechanism for aGPCRs involves the release of a tethered agonist which depends on cleavage at the GPS. However, this hypothesis has been challenged by the observation that non-cleavable aGPCRs exhibit constitutive activity, thus making the function of GPS cleavage widely enigmatic. In this study, we sought to elucidate the function of GPS-mediated cleavage through the study of G protein coupling with Latrophilin-3/ADGRL3, a prototypical aGPCR involved in synapse formation and function. Using BRET-based G protein biosensors, we reveal that an autoproteolysis-deficient mutant of ADGRL3 retains constitutive activity. Surprisingly, we uncover that cleavage deficiency leads to a signaling bias directed at potentiating the activity of select G proteins such as G_{i2} and $G_{12/13}$. These data unveil the underpinnings of biased signaling for aGPCRs defined by GPS autoproteolysis.

INTRODUCTION AND BACKGROUND

Adhesion G protein-coupled receptors (aGPCRs) form a distinct membrane protein subfamily mainly involved in the recognition of ligand molecules mediating cell-cell or cell-matrix contacts [1]. As aGPCRs support structural cell positioning within a given tissue, they are attributed biological functions as diverse as cell migration, synapse formation, angiogenesis, differentiation and apoptosis to name a few [2-6]. The importance of aGPCRs in sustaining physiological functions was highlighted by their association with a plethora of human disorders for which receptor dysfunctions are thought to account for most of the underlying etiological factors [7,8]. Their involvement in such a wide range of physiological or pathophysiological events posit aGPCRs as potential pharmacological targets amenable to therapeutic use hence the heightened interest in studying their function.

The reason why aGPCRs are set apart from other GPCR subfamilies is partly due to their peculiar domain topology consisting of the modular organization of adhesion motifs concentrated within their N-terminal extracellular region (NTF) followed by a hallmark autoproteolytic domain named GAIN which in most cases introduces a discontinuous attachment to the remaining C-terminal canonical membrane-anchored heptahelical structure (CTF)[9]. Thus, aGPCRs heterophilic interactions with other adhesion molecules or matricellular proteins through their N-terminal region induce intracellular signaling via the C-terminal seven transmembrane region [1]. However, ligand-mediated activation of aGPCRs does not constitute a strict pre-requisite to elicit intracellular signaling as these receptors possess an intrinsic constitutive activity [10]. Such activity has been linked to the presence of a tethered agonist embedded deep into the GAIN domain immediately downstream from the GPCR proteolytic site (GPS) [11-14]. It is thought that cleavage at the GPS removes the structural constraint brought on by the surrounding GAIN domain and therefore liberates the occluded N-termini of the tethered agonist, which in turn can be excised from the GAIN domain upon mechanosensitive signals or ligands acting on the adhesion motif-containing N-terminal region. The orthosteric activation model would have the tethered agonist dissociate from the GAIN and bind directly to the receptor binding pocket formed by the transmembrane region while the allosteric model describes a non-dissociative state for which the GAIN and adjacent adhesion domains would act as

conformational modulators of the transmembrane region [1]. Nonetheless, cleavage at the GPS constitutes a central factor for distinguishing between these two activation models. However, this hypothesis remains a contentious issue given the characterization of aGPCR members, which despite possessing a GAIN domain, do not undergo cleavage but display constitutive activity [1].

The aGPCR Latrophilin-3/ADGRL3 is part of a three-member subfamily and has been mostly characterized for its role in synapse formation and function due to its high expression in brain tissues where its genetically linked protein dysfunction has been associated with the development of neurological disorders [8,15]. Its presence in peripheral tissues albeit at lower expression levels has linked ADGRL3 to non-neuronal functions such as insulin release thus denoting its wide-ranging biological functions [16]. As a prototypical aGPCR that is cleaved at the GPS, ADGRL3 remains to date the most structurally characterized aGPCR with the recent revelations of its promiscuous coupling to members of multiple G protein families such as G_i, G_q, G_s and $G_{12/13}$ [17,18]. While these observations of wide-ranging signaling abilities offer a plausible way to explain how ADGRL3 might exert its function in multiple tissues it remains unclear how a selection between its different effectors may actually be achieved. Because of intrinsic agonism given by the tethered agonist present in aGPCRs due to GPS cleavage, we sought to investigate the role of autoproteolysis in modulating intrinsic receptor coupling to G proteins. Although previous studies have reported the impact of GPS cleavage on downstream effectors or second messengers, none had addressed the dynamics of direct receptor coupling to its upstream effectors, the G proteins [5,19]. Using a profiling strategy involving BRET-based biosensors of different G protein families, we provide evidence that GPS cleavage supports an intrinsically biased agonism towards specific G proteins within ADGRL3.

MATERIALS AND METHODS

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [20]

Cell culture and transfections

HEK293 cells (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle medium (DMEM; Corning) supplemented with 10 % Fetal Bovine Serum (FBS; Biowest), 2 mM GlutaMAX (Invitrogen) and 1000 U ml^{-1} Penicillin/Streptomycin (In Vitro) at 37 °C in a 5 % CO_2 humidified incubator. Cells were transfected following a 30 min incubation with a complex composed of 1:3 ratio between DNA and polyethylenimine (PEI, linear, MW 25,000; Polysciences Inc, Warrington, FL, USA) in DMEM which was then supplemented with 20 % FBS and 4 mM GlutaMAX. For immunoblotting assays, cells were seeded in 6-well plates and transfected 16 h later at a 80 % confluency with 4 µg of receptor DNA. For BRET assays, 10 ng of DNA encoding each G protein subunits from TRUPATH biosensors (Addgene kit #1000000163) [21] were co-transfected with indicated receptor DNA amounts per the following combinatorial scheme: subunits β3 and γ9 were mixed jointly with either αi1, αi3, α12, α13, αs (short splice variant) or αq; β3 and γ8 jointly with αi2; β3 and γ13 with α11.

Plasmids

Plasmid expressing human Lphn3/ADGRL3 N-terminally fused to a DYKDDDDK (FLAG) sequence with a YPYDVPDYA (HA) sequence insertion in extracellular loop 1 was previously described [22]. Amino acid composition as well numberings are based on ADGRL3 isoform 2 [NCBI accession number NP_001374458.1]. ADGRL3-T855G was generated by mutagenesis of the ADGRL3 template plasmid using the overlapping PCR method with the following forward and reverse oligonucleotides respectively: 5'-GTAATCACCTGGGAAACTTTGCTGTC-3'; 5'-GACAGCAAAGTTTCCCAGGTGATTAC-3'. Amplified fragments were then inserted into EcoRI and Bsu36I sites of the ADGRL3 original template. The presence of the correct mutation was confirmed by Sanger

sequencing on Applied Biosystems 3130 apparatus (Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental, Instituto Potosino de Investigación Científica y Tecnológica).

Immunoblotting procedures

Transfected HEK293 cells were harvested 48 h post-transfection, solubilized in Laemmli sample buffer and analyzed by electrophoresis on 8 % SDS-PAGE gels followed by transfer to nitrocellulose membranes. A membrane-blocking step was performed for 2 h at room temperature in a solution consisting of TBST (50 mM Tris pH 7.4, 150 mM NaCl, 0.05 % Tween 20) supplemented with 3 % BSA before adding primary antibodies, mouse monoclonal anti-HA (BioLegend, 901515) and rabbit polyclonal anti-FLAG (Sigma-Aldrich, F7425) both at a 1:1000 antibody:solution ratio which remained in contact with the membranes for 16 h at 4 °C. Primary antibody solution was replaced by a solution containing secondary antibodies, IRDye800CW-coupled anti-mouse antibody or IRDye680RD-coupled anti-rabbit antibody both at a 1:10,000 ratio for a 2 h incubation period at room temperature. Finally, fluorescent signals were quantified by scanning the membranes using the 700 nm and 800 nm channels on a LI-COR Odyssey Fc instrument and processing the resulting image with Image Studio 5.2 software.

Detection of cell-surface receptor expression (DECS assays)

HEK 293 cells transfected 24 h prior in 6-well plates were resuspended and transferred onto poly-L-lysine-treated 96-well clear plates (NEST). Cells were cultured for an additional day until they were fixed with cold 4 % paraformaldehyde for 10 min and incubated in the blocking solution (3 % Bovine Serum Albumin [BSA] in Phosphate buffer saline [PBS] with 0.1 % azide) for one hour at room temperature. Solution containing polyclonal rabbit anti-FLAG antibody was added (1:5000 in blocking solution) and replaced one hour later by horseradish peroxidase-coupled anti-rabbit secondary antibody (1:7000 in blocking solution; Jackson ImmunoResearch, Baltimore, MD, USA; 111-035-003) for an additional hour. The colorimetric reaction was initiated by adding the

horseradish peroxidase substrate 3′,5,5′-tetramethylbenzidine (TMB; Invitrogen) to each well and subsequently stopped with 1 N H_2SO_4 resulting in a yellowish solution. Absorbance was quantified at 450 nm with a Cytation 5 multimodal microplate reader (Biotek, Winooski, VT, USA).

BRET assays

HEK293 cells (3.5 x 10^5 cells/transfection samples) were co-transfected in suspension using receptor cDNA (250, 500, 750 ng) along with 10 ng of each TRUPATH biosensor components Gα-RLuc8: Gβ: Gγ-GFP2 and pCMV empty vector DNA to reach 1000 ng total DNA in each case. The transfected cells were plated in 96-well flat bottom white plates (SPL) at a density of 35,000 cells per well. 48 h post-transfection, growth medium was replaced with BRET buffer (in mM: 10 Hepes, 1 $CaCl_2$, 0.5 $MgCl_2$, 4.2 KCl, 146 NaCl, 5.5 glucose, pH 7.4) and luciferase activity was monitored by the addition of 5 µM coelenterazine 400a (GoldBio, C-320) to generate $BRET^2$ signals. Plates were read using the end-point modality of the microplate reader Cytation5 (Biotek/Agilent, Winooski, VT, EE.UU.) after a 5 min equilibration period. Emission values obtained at 515/30 nm (GFP2) and 410/80 nm (RLuc8-coelenterazine 400a) were recorded simultaneously in a well-by-well fashion. $BRET^2$ ratio values were obtained by calculating the ratio between the emission of the acceptor GFP2 and emission of the donor RLuc8. The inverse BRET (iBRET) index reflecting direct proportionality between $BRET^2$ signals and biosensor activation was calculated with the following equation: $BRET^2{}_{(0)}/BRET^2{}_{(x)}$, where $BRET^2$ ratio detected at 0 ng of receptor plasmid is divided by $BRET^2$ ratio obtained at x ng of receptor plasmid (250, 500, 750 ng), values >1 indicate activation.

Statistical analysis

Data are shown as mean ± SEM and represent the results of at least three independent experiments. Statistical analysis consisted of unpaired Student´s t-test for DECS assays ($p \leq 0.05$) and two-way ANOVA followed by Sidak's multiple comparisons test ($p \leq 0.05$) for BRET assays using GraphPad Prism 6 software. Statistically significant differences are indicated by asterisks (*) or hashtags (#). The experimenter was not blinded to the groups

to be analyzed and sample size was not pre-determined as results were collected until significance was reached.

RESULTS

Autoproteolysis deficiency increases ADGRL3 cell-surface expression

Engineering of a cleavage-deficient ADGRL3 was required in order to test the relationship between G protein signaling and receptor autoproteolysis. Because the GPS site encompasses both the cleavage consensus sequence and part of the tethered agonist (TA) residues, targeting this motif meant mutating a key residue that would specifically abrogate the autoproteolysis without affecting the functionality of the TA (Figure 1A). Thus, we opted to generate a T855G mutation effectively abrogating the nucleophilic potential thought to be essential for bond breakage between the Leucine and Threonine residues as part of the $CNHL^{P1}$-$T^{P1'}$ motif that would normally create a free N-termini at the P1' position, located immediately downstream of cleavage (Figure 1A). A similar modification engineered into ADGRL3 mouse homolog was recently shown to result in a cleavage-deficient mutant receptor that maintained an intact TA activity [19]. Analysis of total cell lysates from receptor-expressing HEK293 cells using immunoblotting of NTF- and CTF-associated epitopes, FLAG and HA tag respectively, yielded signals as single bands for both ADGRL3-WT and T855G mutant receptor evidencing proper expression. Importantly, while NTF and CTF detection strategies resulted in the identification of the expected two protein fragments for ADGRL3-WT migrating as ~120 kDa and ~60 kDa bands respectively, this was not the case for ADGRL3-T855G since only one fragment migrating as a ~180 kDa band could be detected, thus suggesting the absence of autoproteolytic cleavage (Figure 1B). When cell-surface expression was assessed through the detection of extracellular epitope FLAG on intact cells, we observed an almost ~two-fold increase in the colorimetric signal for ADGRL3-T855G as compared to ADGRL3-WT, thus suggesting a higher membrane retention/stability for the mutant receptor (Figure 1C).

G protein coupling selectivity is modulated by ADGRL3 autoproteolysis

In previous reports, the use of functional assays to monitor downstream signaling pathways of aGPCRs provided a divergent portrait of the importance of GPS

autoproteolysis for receptor functions [12,23,24]. Moreover, aGPCRs have been shown to couple to various G protein families, in particular ADGRL3 being able to form stable active complexes with G_i, G_s, G_q and $G_{12/13}$ proteins families [17,18]. In this study, we sought to address the impact of autoproteolysis by monitoring the direct functional coupling of ADGRL3-T855G with G proteins, some of the receptor's most upstream effectors (Figure 2A). The coupling promiscuity displayed by ADGRL3 prompted us to adopt a profiling strategy that would allow the dissection of coupling yields with individual G protein isoforms (Figure 2B). For this, we implemented the use of TRUPATH G protein biosensors based on bioluminescence resonance energy transfer (BRET) which consisted in monitoring the activity-dependent proximity between a $G\alpha$ subunit being fused to luciferase Rluc8 and a $G\gamma$ subunit fused to the green fluorescent protein GFP2 [21]. Thus, G protein activation levels can be quantified by measuring the efficacy of resonance energy transfer to GFP from light emitted by luciferase-mediated conversion of the substrate coelenterazine: high transfer corresponding to low G protein activation, and conversely, low energy transfer equating high G protein activation (Figure 2B). Receptor activation protocols relied on the intrinsic constitutive activity described for ADGRL3. Different levels of constitutive activity were generated by co-transfecting increasing DNA amounts of each receptor separately with BRET-based G protein biosensors, therefore leading to increasing receptor expression and consequently promoting the formation of a higher number of active G protein-receptor complexes [8,19]. Monitoring constitutive receptor coupling to Gi family members confirmed the activation of G_{i1}, G_{i2}, and G_{i3} by ADGRL3-WT (Figure 2C-E). Interestingly, while the ADGRL3-T855G mutant receptor maintained similar activation patterns for G_{i1} and G_{i3} as its WT counterpart (Figure 2C, E), a selective potentiation of G_{i2} activation was detected, as observed by higher $BRET^2$ values for all receptor expression levels tested (Figure 2D). Going forward, constitutive coupling to $G_{12/13}$ family members was further confirmed for ADGRL3-WT. A significant potentiation from ADGRL3-WT $BRET^2$ values could be observed when assaying distinct biosensors of the G_{12} and G_{13} family members with ADGRL3-T855G (Figure 2F-G). In contrast, testing $G_{q/11}$ family biosensors revealed that the coupling between ADGRL3-WT and G_q biosensor, but not G_{11} biosensor, was slightly dampened by the presence of the T855G mutation as shown by % $BRET^2$ ratio signals,

although the difference did not reach statistical significance (Figure 2 I-J). Notably, G_q biosensor $BRET^2$ signals detected that a higher expression level was required of the mutant receptor to reach the threshold for achieving constitutive activity in comparison to the wild-type receptor (Figure 2 I-J). Finally, probing receptor coupling to G_s protein unveiled that both ADGRL3-WT and ADGRL3-T855G displayed robust activation of the G_s biosensor with $BRET^2$ values reaching comparable levels (Figure 2H).

DISCUSSION

The activation mechanism of adhesion GPCRs remains elusive despite recent advances in the elucidation of their active structure depicting a NTF-free tethered agonist embedded into the seven transmembrane domain in a complex with G proteins [25]. Given its strategic location in the topology of all aGPCRs, the GAIN domain appears to be structurally instrumental in regulating the activation process due to its engulfing of both the tethered agonist sequence and the GPS site cleavage. In this study, we focused on the role of the GPS as a potential conformational modifier using a cleavable aGPCR and rendering it non-cleavable to probe the dynamics of direct G protein coupling. Our data point to an autoproteolytic cleavage-dependent modulatory role of select G protein pathways supporting biased signaling. In this regard, ADGRL3-G protein coupling selectivity has been recently attributed to changes in the exposure of specific determinants at the receptor-G protein interface in addition to transmembrane domain rearrangements, thus resulting in a conformational selection [17]. Here, our data suggest that these different conformations could originate in part from a spectrum of structural variations brought by the different modes of association between the N-terminal region and the transmembrane C-terminal region which occur in either case of cleavable or non-cleavable aGPCRs but could be more persistent in a non-dissociative model such as a non-cleavable receptor. The nature of such conformational changes is currently unknown although the study of naturally-occurring non-cleavable aGPCRs with constitutive activity might provide important clues [14,26-28]. Thus, GPS cleavage provides a structural encoding of such conformational biases that may be propagated to the transmembrane region and cytoplasmic interface either directly or via intermediate domains [29]. Additionally, hierarchical factors are known to dictate GPCR signaling bias among them affinity for G protein isoforms which can be differentially affected by their cognate ligands. While we describe an intrinsically-generated G protein coupling bias, future studies will address whether or not endogenous ADGRL3 ligands can elicit such bias.

The efficiency and productivity of receptor-G protein coupling is an important issue that is raised by our observations. Indeed, we detected comparable levels of activation for select G protein biosensors by both receptors despite describing an increase in cell-

surface expression for the cleavage-deficient receptor mutant. The efficiency aspect aimed at quantitatively monitoring the stoichiometry of receptor-G protein coupling is further complicated by the ability of GPCR to signal in different cellular compartments [30]. The productivity aspect is lacking in our approach since the goal of this study was to look at direct G protein coupling dynamics, thus downstream effectors were not assayed in parallel experiments giving the many limitations emanating from monitoring multiple convergent and divergent pathways due to ADGRL3 promiscuous coupling with many G proteins but also with G protein-independent signaling pathways [5,8,17,22,31]. On one hand, our data on $G_{12/13}$ potentiation conceptually depart from other studies which reported either no changes or a significant decrease in the activity of a reporter biosensor bearing a promoter sensitive to $G_{12/13}$ downstream signaling induced by an ADGRL3 cleavage-deficient mutant [19]. However, on the other hand, our data on unaffected G_s coupling support the effect of the same cleavage-deficient mutant on cAMP-dependent synapse formation events [5]. While we recognize the need to reconcile these data, the concept of unproductive GPCR-G protein coupling should be taken into account given that it has recently been highlighted to describe the signaling properties of GPCRs with promiscuous G protein coupling in particular the vasopressin V2 receptor for which complexes formed with G_{12} proteins did not lead to downstream signaling [32]. Interestingly, we observed that coupling with the $G_{12/13}$ protein family was potentiated upon ADGRL3 cleavage deficiency and only G_{i2} from the G_i protein family but not with other G protein families or members tested thus indicating that mutant receptor expression levels alone cannot account for these diametrically opposed results. In any case, the validity of our conclusion would remain whether coupling efficiency or productivity are assessed because only select G proteins are affected in their activation profile hence, the basis for biased signaling.

The investigation into the role of GPS cleavage hinted in its inception that autoproteolysis was required for aGPCR trafficking to the plasma membrane, but this suggestion was later discarded [9,33]. These conclusions were based on the modification in ADGRL1 of the P1' Threonine residue that was first substituted with a Proline known to induce changes in the angle of amino acid chains, which resulted in GPS cleavage impairment but also blocked trafficking of ADGRL1 as it was found to be retained in the endoplasmic reticulum

and improperly inserted into the plasma membrane [33]. This assumption was reversed when a less drastic substitution of the P1' Threonine with a Glycine or Alanine residue was engineered into ADGRL1 and restored receptor trafficking to the plasma membrane [9]. However, our data unveiling the cell-surface expression level of the T855G mutant receptor reignites this now discarded debate by introducing a different angle. Indeed, our results suggest that the persistent association between the N-terminal and C-terminal regions promotes receptor stability by either aiding overall receptor folding or protecting it from degradation. It is however possible that the phenotypes observed for ADGRL3-T855G may be dependent on the precise nature of the substituting amino acid. It is noteworthy that aGPCRs mutants for which the N-terminal region was deleted displayed an alteration in their cell-surface exposure, an effect that might stem from a compound effect of chronic tethered agonist exposure thereby inducing internalization or degradation machineries and absence of stabilizing interactions from the N-terminal region [34]. Further studies will have to be conducted to determine the underlying cause of this membrane-stabilizing effect.

Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest: The authors declare no conflict of interest

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Authors contributions: EYOM, BRH, PLSL and AAB designed the study; EYOM, BRH, PLSL and KGCB performed experiments and analyzed the data; all authors contributed to the drafting, and revision of the manuscript; all authors agree to their accountability of the data presented in the manuscript.

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FIGURE LEGENDS

Figure 1. Increased cell-surface expression of cleavage-deficient ADGRL3 mutant receptor. A) Schematic representation of ADGRL3 and cleavage-deficient mutant ADGRL3-T855G, depicting the Thr to Gly substitution at the GPCR proteolysis site (GPS) located within the hallmark GPCR autoproteolysis inducing (GAIN) domain. Additional domains part of the N-terminal fragment (NTF) are identified as: lectin (LEC), olfactomedin (OLF), hormone-binding domain (HBD), serine/threonine-rich region (S/T); the C-terminal fragment (CTF) comprises a heptahelical transmembrane region with interconnecting extracellular and intracellular loops capped with a cytoplasmic tail exhibiting a PDZ domain binding motif (PDZ BM). **B)** Immunoblotting of total cell lysates obtained from transfected HEK293 cells expressing the ADGRL3-WT and ADGRL3-T855G receptor. CTF (~60 kDa) was detected with a mouse anti-HA antibody, while NTF (~120 kDa) with a rabbit anti-FLAG antibody, both immunolabeled with respective fluorescent secondary antibodies (green: IRDye800W-labeled anti-mouse antibody; red: IRDye680RD-labeled anti-rabbit antibody). ★ indicates bands representing uncleaved receptor molecules. **C)** Detection of cell-surface expression levels of FLAG-tagged ADGRL3 and ADGRL3-T855G using anti-Flag primary and HRP-coupled anti-rabbit secondary antibodies followed by an HRP-based colorimetric assay. Data were normalized to ADGRL3-WT values and displayed as means of percentages for at least three independent experiments done in triplicates; error bar indicates ± SEM of at least three independent experiments. Statistical analysis was performed with Student's t test (**** $p< 0.0001$).

Figure 2. Autoproteolysis deficiency selectively accentuates constitutive coupling of ADGRL3 with G proteins G_{i2}, G_{12}, and G_{13}. A) Representation of the working hypothesis supporting the dissection of G protein profiling for ADGRL3 by assessing coupling efficiency to various classes of G proteins to probe the effect of autoproteolysis deficiency. **B)** BRET-based G protein biosensors in this study detect low levels of G protein activity by generating a high $BRET^2$ signal whereas high activity levels result in a

low $BRET^2$ signal. **C-J)** $BRET^2$ ratio curves obtained at given receptor-encoding DNA amounts (250, 500 and 750 ng) expressed as a percentage of basal $BRET^2$ value in absence of receptor expression (left panels) alongside with iBRET index graphs (right panels) comparing ADGRL3-WT (grey label) to ADGRL3-T855G (blue label) for BRET-based biosensors encompassing various G protein families: G_{i1} (**C**), G_{i2} (**D**), G_{i3} (**E**), G_{12} (**F**), G_{13} (**G**), G_s (**H**), G_q (**I**) and G_{11} (**J**)**.** Data are represented as the mean values of at least three independent experiments comprising four technical replicates each (n = 3) and error bars indicate SEM. Statistical analysis was conducted using two-way ANOVA with the Sidak multiple comparison test. *p*-values are indicated as: * or # $p < 0.05$, ** or ## $p < 0.01$, *** or ### $p < 0.001$, **** or #### $p < 0.0001$; for % $BRET^2$ ratio curves * represents the comparison between a given receptor DNA amount and 0 ng receptor DNA whereas the same symbol represents cross-receptor comparison for the same DNA amount in iBRET index graphs and # describes the significance from the reference value of 1 (dotted line) in iBRET index graphs.