Structural Insights into Ligand Recognition, Selectivity and Activation of the human Bombesin Receptor Subtype-3

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Abstract

Bombesin receptor subtype-3 (BRS3) is an important orphan G protein-coupled receptor that regulates energy homeostasis and insulin secretion. As a member of the bombesin receptor (BnR) family, which includes neuromedin B receptor (NMBR) and gastrin-releasing peptide receptor (GRPR), the lack of known endogenous ligands and high-resolution structure has impeded understanding of BRS3 signaling and function. Here, we present cryogenic electron microscopy (cryo-EM) structures of BRS3 in complex with heterotrimeric Gq protein in three states: apo, bound to the pan-BnR agonist, BA1, and bound to the synthetic BRS3-specific agonist MK-5046. These structures reveal the architecture of the orthosteric ligand pocket underpinning molecular recognition. Comparisons with BnR members provide insights into the structural basis for BRS3's selectivity and low affinity for bombesin peptides. Examination of conserved micro-switches suggests a shared activation mechanism among BnRs. Together our results enable deeper exploration of BRS3's ligand selectivity, signaling, and therapeutic targeting for diabetes and obesity.

Full Text

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Graphical Abstract
Molecular basis for recognition of exogenous agonists by the human bombesin receptor subtype-3 provides avenues for design of new modulators for diabetes and obesity.

Highlights
• Structures of BRS3-Gq complexes, both in the absence (apo-form) and presence of the ligands BA1 and MK-5046, were determined.
• The important residues in the ligand binding pocket for bombesin receptor subtype-3 activation were described.
• The basis for ligand selectivity of bombesin receptors was depicted in detail.
• Resolution of the binding pockets provides path for rational drug design against bombesin receptor subtype-3 for the treatment of diabetes and obesity.

SUMMARY

Bombesin receptor subtype-3 (BRS3) is an important orphan G protein-coupled receptor that regulates energy homeostasis and insulin secretion. As a member of the bombesin receptor (BnR) family, which includes neuromedin B receptor (NMBR) and gastrin-releasing peptide receptor (GRPR), the lack of known endogenous ligands and high-resolution structural information has impeded understanding of BRS3 signaling and function.

Here, we present cryogenic electron microscopy (cryo-EM) structures of BRS3 in complex with heterotrimeric Gq protein in three states: apo, bound to the pan-BnR agonist BA1, and bound to the synthetic BRS3-specific agonist MK-5046. These structures reveal the architecture of the orthosteric ligand pocket underpinning molecular recognition. Comparisons with BnR members provide insights into the structural basis for BRS3's selectivity and low affinity for bombesin peptides. Examination of conserved micro-switches suggests a shared activation mechanism among BnRs. Together, our results enable deeper exploration of BRS3's ligand selectivity, signaling, and therapeutic targeting for diabetes and obesity.

INTRODUCTION

G protein-coupled receptors (GPCRs), pivotal membrane proteins, orchestrate cellular responses to a variety of extracellular stimuli, including peptide ligands. Among these, bombesin, a 14-amino acid peptide originally isolated from the European fire-bellied toad (*Bombina bombina*)[1,2], stands out. In mammals, bombesin analogs such as neuromedin B (NMB) and gastrin-releasing peptide (GRP) interact with specific receptors in the bombesin receptor (BnR) family[3]. These receptors are integral to physiological processes in the gastrointestinal tract and central nervous system, influencing aspects like food intake[4], fear memory consolidation[5], and itch sensation[6,7].

Structural studies of NMBR and GRPR with various ligands have shed light on the interactions within the BnR family, enhancing our understanding of their structure-function relationships and aiding in the development of novel therapeutics for BnR-related conditions, including cancers and pruritus[8,9].

Bombesin receptor subtype-3 (BRS3), also a member of the BnR family, is an orphan GPCR primarily found in the brain. Unlike its counterparts NMBR and GRPR, BRS3 does not exhibit high affinity for known endogenous bombesin family peptides[3,10,11] (Table S1), and its natural ligand remains elusive. Despite this, specific ligands have been identified for BRS3. Among them, the synthetic bombesin analog [D-Phe^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]Bn(6–14) (where D and β indicate D- and β-conformations, respectively), referred to as BA1, exhibits high affinity, binding to all three bombesin family receptors. As a pan-BnR agonist, BA1 is frequently utilized in research as a BRS3 agonist[12,13]. Nonpeptide agonists like MK-5046 have shown selective BRS3 agonism, enhancing our ability to investigate BRS3's functions[14,15].

Additional agonists, such as DSO-5a and oridonin, have displayed potent and selective BRS3 activation, suggesting their potential in treating metabolic disorders[16,17]. Particularly, in comparison with MK-5046, DSO-5a activates peripheral BRS3 to control blood glucose homeostasis while circumventing central effects[18]. Additionally, oridonin, derived from *Isodon rubescens* and commonly employed in Traditional Chinese Medicine[17], exhibits pharmacological activities encompassing hypoglycemic and anti-inflammatory effects[19], as well as anti-cancer effects[20]. As a novel BRS3 ligand, oridonin holds promise as a lead compound for treating metabolic disorders, including type 2 diabetes and obesity.

Utilizing these synthetic ligands and animal models has revealed BRS3's critical roles in regulating energy homeostasis, glucose metabolism, and blood pressure. Its significant function in obesity was initially identified[21], followed by findings underscoring its necessity for energy balance and insulin secretion[22–24]. BRS3 neurons in the hypothalamus can be activated by cold exposure, refeeding, and certain agonists. Notably, activation of BRS3 neurons in the dorsomedial hypothalamus increased sympathetic nervous tone, elevating energy expenditure, body temperature, heart rate, and blood pressure via brown adipose tissue,

heart, and arteries, respectively, without affecting food intake or physical activity. Conversely, the activation of BRS3 neurons in the paraventricular nucleus of the hypothalamus reduced food intake, without affecting energy expenditure, body temperature, or physical activity[25] (Figure 1A).

Additionally, agonists of BRS3 can stimulate insulin secretion from pancreatic β cells (Figure 1A), making BRS3 a promising target for metabolic diseases such as obesity and diabetes[26]. However, the lack of structural data for BRS3, especially in different ligand-bound states, has hindered our understanding of its molecular mechanisms and the development of targeted treatments.

In this study, we present cryo-EM structures of BRS3 in active conformations, both in the absence and presence of ligands (BA1 and MK-5046). Together with mutational studies, these structures elucidate the mechanisms of ligand binding and receptor activation, offering crucial insights into this orphan receptor. This information is invaluable for structure-based drug design targeting BRS3, potentially revolutionizing treatment approaches for obesity, diabetes, hypertension, and related diseases.

RESULTS

Structure Determination of BRS3-Gq Complexes

For the cryo-EM analysis of BRS3-Gq complexes, we engineered human BRS3 by attaching a thermostabilized apocytochrome b562RIL (BRIL) to its N-terminus. This was done to enhance the stability and homogeneity of the complex, crucial for effective cryo-EM studies[27] (Figure S2). Additionally, we utilized the NanoBiT tethering strategy: fusing the large part of NanoBiT (LgBiT) to the C-terminus of BRS3 and a 13-amino acid NanoBiT peptide (HiBiT) to the C-terminus of the Gβ subunit[28]. We also employed an engineered Gq protein chimera, similar to the one used in prior structural studies of NMBR and GRPR[8]. Here, 'Gq' refers to this specific engineered chimera. We assembled the BRS3-Gq complexes for cryo-EM by co-expressing and purifying BRS3-LgBiT, Gq, G$\beta\gamma$-HiBiT, antibody scFv16, and nanobody-35 (Nb35)[29,30]. These components were processed both in the absence (apo-form) and presence of the ligands BA1 and MK-5046. This approach facilitated the efficient formation and enhanced stability of the apo and ligand-bound BRS3-Gq complexes.

Single-particle cryo-EM analysis of the collected datasets yielded density maps at nominal global resolutions of 3.5 Å, 2.9 Å, and 3.0 Å for the apo BRS3-Gq-scFv16, BA1-bound BRS3-Gq, and MK-5046-bound BRS3-Gq-scFv16-Nb35 complexes, respectively (Figures 1B-1G, S3-S5; Table S2). These maps clearly delineated the positions of the receptor, G-protein trimer, and scFv16/Nb35 in each complex, with most amino acid side chains being resolved (Figure S6). In the apo BRS3-Gq-scFv16 complex, the orthosteric binding pocket (OBP) did not exhibit any additional densities. However, for the BA1- and MK-5046-bound complexes, densities for BA1 and MK-5046 were well-resolved, allowing for the construction of atomic models with a high degree of confidence. All

three complexes displayed a similar overall structural arrangement, with root mean square deviation (RMSD) values for Cα atoms ranging between 0.3-0.6 Å, indicating minor variations among them (Figures S7A-S7C).

Recognition of BA1 by BRS3

The quality of the cryo-EM maps allowed for precise modeling of all nine amino acid residues of BA1. This finding aligns with our previous structures of NMB-NMBR and GRP(14-27)-GRPR, where only the C-terminal 9 residues of the longer bombesin agonists (33-residue peptide NMB[30] and 14-residue peptide GRP(14-27), respectively) were observed, despite the utilization of the full-length peptides during complex preparation (Figures 2A and 2B; Table S3). This suggests that a minimal 9-residue segment might be sufficient for full agonist potency. In the BRS3 structure, BA1 is positioned within the central OBP located in the upper transmembrane domain (TMD), interacting with all extracellular loops and transmembrane segments except TM1. The peptide's C-terminus penetrates deeply into the helical bundle, while the N-terminus of BA1 extends outward towards the extracellular ligand binding cavity. BA1 assumes a dumbbell-shaped conformation, reminiscent of NMB and GRP(14-27) in their respective complexes, underlining a conserved recognition pattern among bombesin receptor family agonists (Figure 2B). For clarity, all positions of BA1 are numerically designated from the amino terminus of NMB or NMC (Figure 2A).

The nine residues of BA1 (residues 2-10) can be categorized into three motifs: the C-terminal HFX(8-10)BA1 motif (where X is Nle), the middle AVβA(5-7)BA1 motif, and the N-terminal DFQW(2-4)BA1 motif (Figure 2A). The HFX(8-10)BA1 motif adopts an extended structure at the bottom of the OBP, with the side chain of X10BA1 penetrating the deepest. The side chains of X10BA1 and F9BA1 are encased in a hydrophobic cavity comprising L96$^{2\cdot53}$, L128$^{3\cdot33}$, V131$^{3\cdot36}$, C221$^{5\cdot42}$, F225$^{5\cdot46}$, W284$^{6\cdot48}$, A319$^{7\cdot42}$, and F320$^{7\cdot43}$ (Figure 2C). These hydrophobic interactions are crucial for BRS3 activation, as demonstrated by significantly reduced potency when L96$^{2\cdot53}$, L128$^{3\cdot33}$, and V131$^{3\cdot36}$ are substituted with alanine (Figures 2F and S8; Table S4). In addition, F9BA1 engages in stabilizing π-π packing interactions with the surrounding F225$^{5\cdot46}$, H288$^{6\cdot53}$ and Y291$^{6\cdot55}$ (Figure 2D). The H8BA1 residue forms hydrogen bonds with S124$^{3\cdot29}$ and S205^{2}, differing from the sandwiching of the corresponding H8 residues in NMB and GRP(14-27) by P$^{3\cdot29}$ and P^{2} in the NMB-NMBR and GRP(14-27)-GRPR structures (Figures 2D and S7F). The alanine mutations of S124$^{3\cdot29}$ or S205^{2} had a minimal effect on BRS3 activation by BA1, yet the proline substitution of S124$^{3\cdot29}$ or S205^{2} substantially lowered BRS3 activity coordinated by BA1 (Figures 2D and S8; Table S4). This highlights the pivotal roles of S124$^{3\cdot29}$ and S205^{2} in peptide BA1 recognition by BRS3. H8B also forms hydrogen bonds with R127$^{3\cdot32}$ and E182$^{4\cdot60}$ (Figure 2D). Furthermore, R127$^{3\cdot32}$ also forms a hydrogen bond with the backbone carbonyl of F9B, and R316$^{7\cdot39}$ forms a hydrogen bond with the

backbone carbonyl of H8B (Figure 2D). The carboxyl group of X10BA1 forms two hydrogen bonds with the side chains of R127$^{3\cdot32}$ and R316$^{7\cdot39}$ (Figure 2D). Mutation of R127$^{3\cdot32}$, E182$^{4\cdot60}$, C221$^{5\cdot42}$, H288$^{6\cdot52}$, Y291$^{6\cdot55}$ and R316$^{7\cdot39}$ in BRS3 results in a reduced BA1-induced signaling response (Figures 2F, 2G and S8; Table S4), confirming the essential roles of these residues in BA1 binding and receptor activation.

The AVβA(5-7)BA1 motif primarily engages in hydrophobic interactions with F191 ^{2} and F200 ^{2} from ECL2, and P304 ^{3} from ECL3 (Figure 2D). Indeed, alanine mutation of F200 ^{2} resulted in decreased Gq signaling (Figures 2G and S8; Table S4). In addition to the hydrophobic interactions, the backbone carbonyl group of βA7BA1 formed a hydrogen bond with the backbone amide group of S205 ^{2}, further stabilizing BA1 binding.

Following the middle motif, the DFQW(2-4)BA1 motif extends along the upper pocket, engaging in π-π interactions with Y108$^{2\cdot65}$, F200 ^{2}, H308$^{7\cdot31}$, and F309$^{7\cdot32}$ from BRS3 (Figure 2E). Alanine substitutions at these sites diminish receptor activation compared to the wild-type BRS3 (Figure 2G; Table S4). Anion-π interactions between DF2BA1 and D193 ^{2}/D303 ^{3} also contribute to BA1's binding stability, with alanine mutation of D303 ^{3} significantly reducing Gq signaling (Figure 2G; Table S4). Furthermore, Q3BA1 forms extensive hydrogen bonds with the BRS3 backbone, and its carbonyl group interacts with H107$^{2\cdot64}$ (Figure 2E). In summary, these intricate details reveal the comprehensive mechanism behind BRS3 activation by the synthetic peptide BA1, highlighting the specific interactions and critical residues involved in this process.

BRS3 Regulation by MK-5046

Continuing our exploration of BRS3, we examined its interaction with MK-5046, the first BRS3-specific nonpeptide receptor agonist (Figure 3). MK-5046 is characterized by distinct functional groups, including a hydroxyl group and a trifluoromethyl group in the central segment, an additional trifluoromethyl group accompanied by a cyclopropane group and an imidazole group on one side, and a benzene ring and a pyrazole group on the other (Figure 3B). The structure of MK-5046-bound BRS3 reveals that MK-5046 adopts a "V"-shaped conformation, fitting snugly at the bottom of the BRS3 pocket. Notably, MK-5046, being smaller in volume (475 Å^3) than BA1 (1089 Å^3), occupies a specific sub-pocket that corresponds to the C-terminal HFX(8-10)BA1 motif (Figure 3A).

The trifluoromethyl group connected to the cyclopropane group projects towards the upper portion of TM3, establishing extensive fluorine-based halogen bonding interactions with S124$^{3\cdot29}$, R127$^{3\cdot32}$, and E182$^{4\cdot60}$ (Figure 3C). Mutations in R127$^{3\cdot32}$ and E182$^{4\cdot60}$ lead to significantly reduced MK-5046-induced signaling (Figures 3E and S8; Table S4), emphasizing the critical roles of these residues in MK-5046 binding and BRS3 activation. Replacement of R127$^{3\cdot32}$ with Q$^{3\cdot32}$ in

both NMBR and GRPR receptors notably decreased the Emax level for mutated BRS3 (Figures 3E and S8; Table S4), underlining $R127^{3\cdot32}$'s significance in MK-5046's selectivity for BRS3.

Additionally, the imidazole, benzene, and pyrazole rings of MK-5046 engage in extensive π-π interactions with $W284^{6\cdot48}$ and $Y291^{6\cdot55}$ of BRS3 (Figure 3C). The interaction between MK-5046's imidazole group and $Y291^{6\cdot55}$ is further reinforced by a hydrogen bond with S205 ^{2} (Figure 3C). Mutating S205 ^{2} and $Y291^{6\cdot55}$ results in a considerable decrease in MK-5046-induced BRS3 signaling (Figures 3E and S8; Table S4), confirming their essential role in this interaction. The central hydroxyl group of MK-5046 forms a hydrogen bond with $C221^{5\cdot42}$, while the central trifluoromethyl group creates robust packing interactions with $H288^{6\cdot52}$, complemented by π-π packing between $W284^{6\cdot48}$ and $H288^{6\cdot52}$ (Figures 3C and 3D). The opposite side of MK-5046, featuring a benzene ring and pyrazole group, is accommodated within a hydrophobic pocket formed by $L96^{2\cdot53}$, $V131^{3\cdot36}$, $F225^{5\cdot46}$, $W284^{6\cdot48}$, $A319^{7\cdot42}$, and $F320^{7\cdot43}$ (Figure 3D).

Previous competitive assays involving MK-5046, Peptide-1 (another pan-BnR agonist), and Bantag-1 (a BRS3-specific peptide antagonist) suggested that MK-5046 functions as an allosteric agonist for BRS3, possibly binding to a distinct pocket from Peptide-1[31]. Our structures show that MK-5046's binding site and pose closely resemble the C-terminal HFX(8-10)BA1 motif of BA1 (Figure 3B). The upper trifluoromethyl group of MK-5046 corresponds to the binding site for BA1's H8B, while its central trifluoromethyl group and benzene ring align with the lower hydrophobic cavity, mirroring the FX(9-10)B side chains of BA1. The pyrazole group of MK-5046 assumes a position akin to the carboxyl group of BA1's X10B. In summary, MK-5046's binding pattern shares similarities with the C-terminal HFX(8-10)BA1 tripeptide of BA1, suggesting that MK-5046 is an orthosteric agonist. Interestingly, alanine substitution of $R316^{7\cdot39}$ enhances BRS3 activity induced by MK-5046, showing higher efficacy in our IP1 assay (Figure 3F and S8; Table S4). This finding further underscores the intricate molecular interactions that govern BRS3 regulation and activation by MK-5046.

Activation of BRS3 by Different Types of Agonists

Understanding how receptor occupation correlates with activation, particularly in the context of peptide and nonpeptide agonists for GPCRs, is crucial. Interestingly, BRS3 exhibits unique behavior in this regard. The binding affinities of the peptide agonist BA1 and the nonpeptide agonist MK-5046 are 2.0 nM and 17 nM[10],[32], respectively, indicating that BA1 has nearly 10-fold higher affinity for the receptor than MK-5046. Correspondingly, analysis using dr_{sasa} reveals that the interface areas for BA1 and MK-5046 are 1094.26 Å^2 and 429.02 Å^2, respectively[33]. This suggests a direct relationship between larger buried surface area and higher binding affinity.

However, the activation potency of these agonists presents an intriguing con-

trast. In our IP1 assays, MK-5046 activates BRS3 with an EC_{50} value of 0.5 nM, demonstrating 26-fold greater potency in activating Gq signaling than BA1, which has an EC_{50} of 13.0 nM. This is notable as most nonpeptide agonists typically exhibit lower potency than natural peptide agonists of their respective receptors in activating downstream signaling. Yet, MK-5046 surpasses the peptide ligand BA1 in terms of Gq signaling activation.

To further investigate the potency differences between MK-5046-BRS3 and BA1-BRS3, we conducted molecular dynamics (MD) simulations, each spanning 500 ns over three replicates. These simulations focused on the ligand-binding domain composed of TM3, TM5, and TM6, a region known to influence ligand potency[34]. The simulations revealed distinct hydrophobic interaction networks in the extracellular TM3-TM5-TM6 region for the two agonists. Specifically, in the MK-5046-BRS3 system, a hydrophobic network surrounding the trifluoromethyl group of MK-5046 was present in 62.9% of the trajectories, compared to only 23.7% in the BA1 system around F9BA1. Representative structures (Figure 4A) showed that the trifluoromethyl group in MK-5046 engages in electronic interactions with $H288^{6\cdot52}$ and facilitates hydrophobic interactions between $F225^{5\cdot46}$ and $V131^{3\cdot36}$. In contrast, the larger F9BA1 moiety in the BA1 system interacts primarily with $V131^{3\cdot36}$, disrupting the hydrophobic contact between $F225^{5\cdot46}$ and $V131^{3\cdot36}$ (Figure 4B).

Moreover, in the MK-5046 system, the trifluoromethyl group acts as a hydrophobic bridge between $H288^{6\cdot52}$ and $F225^{5\cdot46}$, stabilizing the activated extracellular TM3-TM5-TM6 conformation. This is evidenced by the fact that direct contact between $H288^{6\cdot52}$ and $F225^{5\cdot46}$ is less frequent (19.9% versus 78.1% in the BA1 system). Consistent with this, the H288A mutation significantly diminishes MK-5046's potency (from 0.53 nM to 126.9 nM), while having a less pronounced effect on BA1's binding (from 11.8 nM to 68.7 nM) (Figures 2G, 3F, and 4C). In summary, these findings illustrate the complex and distinct mechanisms by which different types of agonists, both peptide and nonpeptide, activate BRS3. They also highlight the nuanced molecular interactions that govern the receptor's response to these agonists, contributing to our understanding of GPCR activation and signaling.

Basis for Ligand Selectivity of Bombesin Receptors

In the BnR family, BA1, a synthetic analog, was found to have high affinity for BRS3, as well as for NMBR and GRPR receptors[10,35,36] (Table S1). BRS3 shares approximately 50% amino acid sequence identity with NMBR and 47% with GRPR[11,37,38]. A closer examination of BA1 binding in BRS3 and GRPR indicates that this synthetic peptide uniquely coordinates with all three bombesin receptors.

Structural alignment of the BA1-GRPR-Gq complex (PDB: 7W40)[9] with our BA1-BRS3-Gq structure showed a high degree of similarity, with an overall Cα RMSD of 0.75 Å (Figure 5A). Both the DFQW(2-4)B and HFX(8-10)BA1 mo-

tifs of BA1 align well between BRS3 and GRPR, with notable differences in the side chains of DF2BA1, W4BA1, and H8BA1 (Figure 5B). The key residues in BRS3's upper pocket, including Y108$^{2\cdot65}$, F200 ^{2}, H308$^{7\cdot31}$, and F309$^{7\cdot32}$, involved in π-π interactions with DF2BA1 and W4BA1, are highly conserved in GRPR without substitutions (Figure 5C), suggesting a preserved interaction pattern despite conformational differences in these aromatic amino acids. However, in BRS3, the H8BA1 side chain deviates from TM3, causing a shift of the middle AVβA(5-7)BA1 motif towards TM6, compared to its position in GRPR (Figure 5D). This shift is stabilized by two hydrogen bonds between H8BA1 and S124$^{3\cdot29}$ and S205$^{45\cdot52}$ in BRS3. In contrast, BA1's H8 is sandwiched by P$^{3\cdot29}$ and P$^{45\cdot52}$ in GRPR, resembling the coordination seen in NMB-NMBR and GRP(14-27)-GRPR structures (Figure S7F). The significant sequence homology and specific flexibility, especially in the ligand-binding pockets, explain BA1's ability to bind all three bombesin receptors.

The selectivity of natural bombesin-related peptide agonists for individual bombesin-related receptors is an interesting aspect. GRP, the natural ligand for GRPR, has an affinity for GRPR over 650-fold higher than for NMBR and more than 15,000-fold higher than for BRS3. NMB, the natural ligand for NMBR, shows over 650-fold higher affinity for NMBR compared to GRPR and more than 20,000-fold higher than for BRS3 (Table S1). Prior studies have clarified the determinants of selectivity between NMB30 and GRP(14–27) for NMBR and GRPR[8],[39],[40]. In our sequence and structure analyses of these receptors bound with different peptides, we observed five amino acids in the peptide agonist binding pockets that are conserved in both GRPR and NMBR but differ in BRS3: T106$^{2\cdot63}$, H107$^{2\cdot64}$, R127$^{3\cdot32}$, S205 ^{2}, and H294$^{6\cdot58}$ in BRS3 are substituted by S$^{2\cdot63}$, R$^{2\cdot64}$, Q$^{3\cdot32}$, P ^{2}, and R$^{6\cdot58}$, respectively (Figure S1), in NMBR and GRPR. A structure comparison of BA1-BRS3 with NMB-NMBR shows a clash between T6NMB of NMB and S202 ^{2} of BRS3, which is absent in the equivalent interaction in the BRS3 structure due to a shift of BA1's middle motif towards TM6 (Figure 5E). In the BRS3 structure, the equivalent V6BA1 residue of BA1 shifts away from S202 ^{2}, due to the shift of the middle AVβA(5-7)BA1 motif towards TM6. This shift of the BA1 middle motif may be a structural characteristic of BRS3-specific ligands, which is favored by the residue combination of the R127$^{3\cdot32}$ and H294$^{6\cdot58}$ substitutions in BRS3 (Figure 5E).

Through structural analysis, it is evident that BA1 presents a binding pose of the middle motif nearly identical to the natural agonist GRP in the GRPR structures[8],[9], located close to TM6 (Figure 5F). Interestingly, the NMB middle motif exhibits a closer alignment with TM2 (Figure 5F). Consequently, these ligand binding poses of NMB and GRP are favored by the conserved residue combination of the Q$^{3\cdot32}$ and R$^{6\cdot58}$ substitutions in NMBR and GRPR receptors. Thus, the Q$^{3\cdot32}$ and R$^{6\cdot58}$ substitutions contribute to the structural characteristics of ligand specificity for individual Bn-related receptors, as suggested in prior mutation studies[41]. For NMB, it has been reported that T6NMB forms a hydrogen bond with the main carboxyl group of L3NMB, playing a piv-

otal role in NMB binding and selectivity by NMBR[8],[9]. The clash of T6NMB here possibly contributes to the weak binding of NMB by BRS3.

In addition, structure comparison of BA1-BRS3 with our previously reported GRP(14-27)-GRPR structure (PDB code: 8H0Q) reveals that the T106$^{2\cdot63}$ substitution in BRS3 induces an inward push of the side chain of W113 ^{1} (Figure 5G). This movement is further stabilized by the packing between the side chain of W113 ^{1} and the H107$^{2\cdot64}$ substitution in BRS3, along with the conserved disulfide bond formed by C120$^{3\cdot25}$ and C203 ^{2} (Figure 5G). Together with the main chain carboxyl group of E201 ^{2}, the inward shift of W113 ^{1} subsequently results in an inward movement of the EGW(111-113) ^{1} of BRS3, causing a clash with the crucial residue H3GRP of GRP(14-27) (Figure 5H). When we compare BA1-BRS3 with the other GRP-GRPR structure (PDB code: 7W3Z), a clash between H3GRP and the carboxyl group of E201 ^{2} can also be observed (Figure S7G), despite H3GRP here adopting a rotamer conformation due to the similar ECL1 positions between the BRS3 and GRPR structures. Interestingly, H3GRP was also reported to accommodate the featured ECL2 of GRPR, and contribute to the weak binding of GRP to NMBR[8],[39], suggesting its pivotal role in selectivity for GRP(14-27) in the BnR family. Overall, these structural insights highlight the unique features of BRS3 that contribute to its low affinity for bombesin-related natural ligands like NMB and GRP.

Common Activation Features of the Bombesin Receptor Subfamily

Examining the significant structural similarities between the BA1- and MK-5046-bound BRS3 complexes, we chose the BA1-BRS3 complex for detailed analysis of BRS3 activation. Comparing the active BA1-bound BRS3-Gq complex with both the active structure of GRPR bound to BA1 (PDB: 7W40) and the inactive structure of GRPR bound to a synthetic antagonist, PD176252 (PDB: 7W41), we observe that BRS3 exhibits classic activation features typical of Class A GPCRs in response to BA1.

Notably, the cytoplasmic end of TM6 in BRS3 displays a pronounced outward movement while the cytoplasmic part of TM7 shifts inward. This rearrangement accommodates the C-terminal $ 5*helix of the G* $q subunit, a key feature of Class A GPCR activation (Figures 6A-6C). Additionally, the extracellular end of TM1 in the BA1-bound BRS3 shifts 5.3 Å towards TM7, similar to movements observed in NMBR and GRPR structures (Figures 6B and S7H).

In comparison to PD176252, BA1 binds deeper within the orthosteric pocket, surrounded by TM3, TM6, and TM7, and forms hydrophobic contacts with W284$^{6\cdot48}$ (Figure 6D). BA1's binding causes a significant deflection of the W284$^{6\cdot48}$ indole ring, initiating a cascade of conformational changes linked to receptor activation[42] (Figure 6D). This process mirrors the classic activation mechanism seen in Class A GPCRs, where the displacement of W284$^{6\cdot48}$ exerts a downward force on F280$^{6\cdot44}$ in the conserved P$^{5\cdot50}$V$^{3\cdot40}$F$^{6\cdot44}$ core triad, leading to changes in the D$^{3\cdot49}$R$^{3\cdot50}$Y$^{3\cdot51}$ motif (Figures 6D-6F). Ad-

ditionally, the conserved $N^{7 \cdot 49}P^{7 \cdot 50}xxY^{7 \cdot 53}$ motif is replaced with a $N^{7 \cdot 49}P^{7 \cdot 50}xxL^{7 \cdot 53}$ motif in BRS3. In the structure, BA1's X10BA1 carboxyl group forms a hydrogen bond with $R316^{7 \cdot 39}$, contributing to activation of the $N^{7 \cdot 49}P^{7 \cdot 50}xxL^{7 \cdot 53}$ motif in BRS3. Typically, the conserved $Y^{7 \cdot 53}$ in this motif undergoes a significant rotation towards TM3, leading to TM7's inward movement[43]. However, in BRS3, the leucine substitution at $Y^{7 \cdot 53}$ undergoes a lateral shift towards TM1 and TM2, highlighting a unique yet conserved activation mechanism shared among bombesin receptors.

During BRS3 activation, the $P^{5 \cdot 50}V^{3 \cdot 40}F^{6 \cdot 44}$ and $N^{7 \cdot 49}P^{7 \cdot 50}xxL^{7 \cdot 53}$ motifs, which link the ligand-binding pocket to the G protein-coupling interface, undergo rearrangements. These involve movements of $F^{6 \cdot 44}$ and $L^{7 \cdot 53}$, triggering significant outward shifts of TM5 and TM6 (3.4 Å at $R^{5 \cdot 63}$; 8.1 Å at $R^{6 \cdot 30}$), thereby facilitating engagement of the Gαq subunit's C-terminus. This feature is consistent with activation mechanisms observed across GPCRs (Figure 6C; Table S5). These structural insights from our BRS3 studies enhance our understanding of the activation mechanisms within the bombesin receptor subfamily, demonstrating how specific molecular changes lead to receptor activation.

Comparison with the AlphaFold2-Predicted Structure

We compared our cryo-EM resolved structures with the structure predicted by AlphaFold2, specifically focusing on the receptor domain (Figure S7I). Given the notable structural similarities between the BA1- and MK-5046-bound BRS3 complexes, we selected the BA1-BRS3 complex for further analysis. The resolved BA1-BRS3 structure and the AlphaFold2-predicted structure exhibited somewhat dissimilar overall arrangements, with a Cα RMSD of 1.5 Å. Despite AlphaFold2 capturing the general backbone contours of the receptor, substantial discrepancies existed between the predicted models and experimental structures regarding the assembly of the extracellular and transmembrane domains. Compared to the BA1-BRS3 structure, the AlphaFold2 structure represents an inactive conformation, with the intracellular part of TM5 tilting outward and the intracellular part of TM6 shifting inward. Specifically, the key residues in the OBP in the AlphaFold2 BRS3 structure, including $H107^{2 \cdot 64}$, $S124^{3 \cdot 29}$, $R127^{3 \cdot 32}$, and $W284^{6 \cdot 48}$ (Figure S7J), adopt distinct positions that cannot favor BA1 binding. In conclusion, while the AlphaFold2-predicted model serves as a valuable reference point, obtaining additional empirical structural information is imperative to accurately guide drug design.

DISCUSSION

Our study on BRS3, an orphan member of the bombesin receptor subfamily, offers significant insights into the complex dynamics and mechanisms of GPCRs. As orphan receptors, entities like BRS3, without identified endogenous ligands, present challenges in understanding their roles, particularly in physiological and pathological contexts like metabolic diseases and cancer[44–46].

The structural analysis of BRS3 in its apo form and when bound to the synthetic analog BA1 and the nonpeptide agonist MK-5046 reveals a high degree of structural conservation (Figure S7A). This similarity, despite the evolutionary loss of binding to natural ligands like NMB and GRP in placental mammals, underscores the evolutionary adaptability of BRS3[47]. Unlike other orphan receptors that drive constitutive activation through their ECL2s, such as GPR12 and GPR21[48–50], BRS3 does not exhibit this feature, indicating unique structural and functional characteristics.

Our investigation reveals unique aspects of ligand recognition and activation in BRS3. The complex interplay of residues within the receptor's binding site, including those contributing to agonist-induced activation, highlights the receptor's intricate regulation mechanisms. These findings provide a foundation for future exploration of BRS3 as a potential therapeutic target, particularly in diseases linked to dysfunctional GPCR signaling.

In the bombesin receptor family, the synthetic analog BA1 exhibits high affinity for BRS3, NMBR and GRPR. Our structural analysis comparing the BA1 complexes with BRS3 and GRPR shows that the overall binding mode of BA1 to the two receptors is highly similar despite some differences in their amino acid side chains. This is largely attributable to the high homology of key residues in the ligand binding pockets of these receptors. These findings explain BA1's ability to bind all members of the BnR family.

On the other hand, natural peptides like NMB and GRP demonstrate high selectivity for individual BnR receptors. Our sequence and structural comparisons of apo and peptide-bound BRS3 identified five key residues that differ from NMBR and GRPR, which may account for its low affinity for NMB and GRP. This provides evidence to understand the loss of binding to these natural peptides by BRS3. Our study offers insights into the structural basis of ligand selectivity, aiding efforts to design BRS3 peptide ligands with high selectivity.

In addition to these findings, our research aligns with the classical activation features observed in Class A GPCRs, including the outward movement of TM6 and the inward shift of TM7 in response to BA1 binding. This conformational change, particularly the deflection of the $W284^{6 \cdot 48}$ indole ring, echoes activation mechanisms seen in other GPCRs. Such structural insights into BRS3, in tandem with its evolutionary trajectory, contribute to a deeper understanding of its functions and potential therapeutic implications.

Our comprehensive study, therefore, not only unravels the unique structural and functional attributes of BRS3 but also enhances the overall understanding of GPCR activation mechanisms. This knowledge is crucial for developing targeted interventions for diseases associated with GPCR dysfunction. Future research is needed to delve deeper into BRS3's self-activation mechanism and explore its potential as a therapeutic target within the bombesin receptor family.

COMPETING INTERESTS

The authors declare no competing interests.

DATA RESOURCES

Materials are available from the corresponding authors upon reasonable request. Density maps and structure coordinates have been deposited in the Electron Microscopy Data Bank (EMDB) and the Protein Data Bank (PDB) with accession codes EMD-38927 and 8Y51 for apo BRS3-Gq-scFv16 complex; EMD-38928 and 8Y52 for BA1-bound BRS3-Gq complex; and EMD-38929 and 8Y53 for MK-5046-bound BRS3-Gq complex. Source data are provided with this paper.

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METHODS

Constructs

Human BRS3 (residues 1-361) was cloned into pFastBac with an N-terminal haemagglutinin (HA) signal peptide followed by thermostabilized apocytochrome b562RIL (BRIL)[1]. LgBiT was also inserted at the C-terminus of BRS3 using homologous recombination (CloneExpress One Step Cloning Kit, Vazyme). After LgBiT, a tobacco etch virus (TEV) protease cleavage site and a double maltose binding protein (MBP) tag were added to BRS3 constructs for improved expression and purification. The engineered Gαq construct was generated based on mini-Gs/q71[2], which carries two dominant-negative mutations (G203A and A326S)[3] to decrease nucleotide binding affinity and facilitate G$\alpha\beta\gamma$ complex stability. The N-terminal 1-18 amino acids and the α-helical domain of the mini-Gs/q71 were substituted with the corresponding sequences of human Gαi1 for binding to the antibody fragments scFv16[4],[5]. Rat G$ $1 was connected with a C-terminal HiBiT by a 15-residue linker.

The engineered Gαq, G$ $1 - $HiBiTandbovineG$ $2 were cloned independently into pFastBac vectors (Invitrogen). The antibody fragment scFv16 was cloned into a modified pFastBac vector containing an N-terminal GP67 secretion signal peptide.

Expression and Purification of Nb35

Nanobody-35 (Nb35) with an N-terminal pelB signal peptide and a C-terminal His6 tag was expressed in the periplasm of *Escherichia coli* BL21(DE3) bacteria (NEB)[6]. Cultures were grown at 37°C in Luria-Bertani media containing 50 g/mL ampicillin to an OD_{600} of 1.0 and induced with 0.1 mM IPTG at 28°C, 180 r.p.m. for another 8 h. Cells were harvested by centrifugation (5316 × g, 30 min) and lysed in ice-cold buffer (20 mM HEPES pH 7.4, 500 mM NaCl), then centrifuged to remove cell debris. Nb35 was first purified by nickel affinity chromatography, followed by size-exclusion chromatography using a HiLoad 16/600 Superdex 200 prep grade column with a buffer of 20 mM HEPES pH 7.4 and 100 mM NaCl. Peak fractions were concentrated to 2 mg/mL with 15% glycerol and kept frozen at -80°C for later use. Purified protein quality was assessed by SDS-PAGE.

Expression and Purification of Complexes

High Five cells (Expression Systems) were cultured in ESF921 serum-free medium (Expression Systems) and co-infected with baculoviruses expressing the receptor, Gαq, G$ 1, G 2, and scFv16 at a 1 : 1 : 1 : 1 : 1 ratio for 48 hat 27°C using Bac−to−Bac baculovirus system. The cell pellets were lysed by dounce homogenization i$ 0.1 mM TCEP (Sigma-Aldrich), 10% glycerol, and EDTA-free protease inhibitor cocktail (TargetMol).

The supernatant was isolated by centrifugation at 65,000 × g for 40 minutes to collect the membranes. The washed membranes were resuspended in buffer containing 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, agonists (10 M BA1, and 10 M MK-5046, Synpeptide and MedChemExpress, respectively), 25 mU/mL apyrase (Sigma-Aldrich), 20 g/mL Nb35, 0.1 mM TCEP and EDTA-free protease inhibitor cocktail, and incubated at 4°C for 8 hours. After incubation, cell membranes were solubilized with 1% (w/v) n-dodecyl-β-d-maltopyranoside (DDM, Anatrace) and 0.2% (w/v) cholesteryl hemisuccinate TRIS salt (CHS, Anatrace) at 4°C for 2 hours. The supernatant was collected by centrifugation at 65,000 × g for 35 minutes and incubated with dextrin resin (Dextrin Beads 6FF, Smart Life Sciences) at 4°C for 5 hours. The resin was washed, eluted, and the protein complexes were further purified by size-exclusion chromatography on a Superdex 200 increase 10/300 GL column (GE Healthcare) in buffer containing 20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM TCEP, agonists (10 M BA1, 10 M MK-5046, and 1 mM oridonin), 0.00075% (w/v) LMNG, 0.00025% (w/v) GDN and 0.0002% (w/v) CHS.

Fractions containing monomeric protein complex were collected and evaluated by SDS-PAGE (Figure S2), and then concentrated 30-fold for cryo-electron microscopy experiments.

Cryo-EM Data Collection

Cryo-EM grids were prepared with the Vitrobot Mark IV plunger (FEI) set to 8°C and 100% humidity. Three microliters of the BRS3-Gq protein complex in apo state was applied to glow-discharged copper R1.2/1.3 holey carbon grids. The sample was incubated for 10 s on the grids before blotting for 3.5 s (double-sided, blot force 1) and flash-frozen in liquid ethane immediately. The same condition was used for the BA1-BRS3-Gq complex and MK-5046-BRS3-Gq complex.

For the BRS3-Gq complex dataset, 3,098 movies were collected on a Titan Krios equipped with a Gatan K3 direct electron detection device at 300 kV with a magnification of 64,000, corresponding to a pixel size of 1.08 Å. Image acquisition was performed with EPU Software (FEI Eindhoven, Netherlands). We collected a total of 36 frames accumulating to a total dose of 50 e^{-} $Å^{-2}$ over 2.5 s exposure.

For the BA1-BRS3-Gq complex dataset, 5,016 movies were collected on a Titan Krios equipped with a Gatan K3 direct electron detection device at 300 kV with a magnification of 105,000, corresponding to a pixel size of 0.824 Å. Image acquisition was performed with EPU Software (FEI Eindhoven, Netherlands). We collected a total of 36 frames accumulating to a total dose of 50 e^{-} $Å^{-2}$ over 2.5 s exposure.

For the MK-5046-BRS3-Gq complex dataset, 8,738 movies were collected on a Titan Krios equipped with a Falcon4 direct electron detection device at 300 kV with a magnification of 165,000, corresponding to a pixel size of 0.73 Å. Image acquisition was performed with EPU Software (FEI Eindhoven, Netherlands). We collected a total dose of 50 e^{-} $Å^{-2}$ over 2.5 s exposure on each EER format movie[8]. Each movie was divided into 36 frames during motion correction.

Cryo-EM Image Processing

MotionCor2 was used to perform frame-based motion correction to generate drift-corrected micrographs for further processing, and CTFFIND4 provided estimation of the contrast transfer function (CTF) parameters[9],[10].

For the BRS3-Gq complex dataset, approximately 2,000 particles were manually picked and two-dimensional (2D) classes were calculated and used as references for automatic picking. All subsequent steps including particle picking and extraction, 2D classification, three-dimensional (3D) classification, 3D refinement, CTF refinement, Bayesian polishing, post-processing and local resolution estimation were performed using Relion3.0[11].

A total of 3,294,877 particles were extracted from the cryo-EM micrographs and subjected to two rounds of reference-free 2D classification, yielding 3,270,779 particles after clearance. Three rounds of 3D classification were used to separate out 233,381 particles, which were refined to a structure at 3.93 Å global

resolution. After CTF refinement, polishing, and postprocessing, the particles were reconstituted to a 3.29 Å structure (Figure S3; Table S2).

For the BA1-BRS3-Gq complex dataset, subsequent steps were performed with CryoSPARC[12]. Blob-pick was used for particle picking. 6,245,406 particles were automatically picked and extracted from 5,016 cryo-EM micrographs. After 2 rounds of 2D classification, 799,895 particles were selected from 2,116,883 particles and subjected to generate 5 "good" references and 1,040,145 particles were selected and subjected to generate 5 "bad" references. Two rounds of heterogeneous refinement using "good" and "bad" references yielded 675,060 particles after clearance. We then continued processing in CryoSPARC. After homogeneous refinement and non-uniform refinement, the particles were reconstituted to a 2.90 Å structure (Figure S4; Table S2).

For datasets of MK-5046-BRS3-Gq complex, a similar strategy was used. After non-uniform refinement, 196,746 particles were reconstituted to a 2.93 Å structure corresponding to the MK-5046-BRS3-Gq complex (Figure S5; Table S2).

Model Building

The BRS3 structure predicted from Alphafold2 was used as the starting reference model for receptor building[13]. Structures of Gαq, Gβ, Gγ, the NB35 nanobody, and scFv16 derived from PDB entry 8H0Q[14] were rigid body fit into the density. All models were fitted into the EM density map using UCSF Chimera[15] followed by iterative rounds of manual adjustment and automated rebuilding in COOT[16] and PHENIX[17], respectively.

The models were adjusted in ISOLDE[18] followed by refinement in PHENIX. The final model statistics were validated using Comprehensive validation (cryo-EM) in PHENIX[17] and provided in Table S2. All structural figures were prepared using Chimera[15], Chimera X[19], and PyMOL (Schrödinger, LLC.).

Functional Assay

AD293 cells (Agilent) were cultured in high glucose DMEM medium (GE Healthcare) supplemented with 10% (v/v) fetal bovine serum (FBS, Gemini) and 1% penicillin/streptomycin at 37°C in 5% CO_2 incubator. Inositol phosphate 1 (IP1) production was measured using the IP-One HTRF kit (Cisbio, 621PAPEJ)[20]. Briefly, cells were seeded onto 12-well plates 16 hours before transfection. Cells were then transiently transfected with different BRS3 constructs using FuGENE HD transfection reagent.

After 24 hours, cells were harvested and resuspended at 7×10^5 cells/mL in IP1 stimulation buffer. Cells were then plated onto 384-well assay plates at 4,900 cells/7 L/well. 7 L IP1 Stimulation Buffer 2 containing ligand was added and incubated for 1 hour at 37°C. Intracellular IP1 was measured using the IP-One HTRF kit and an EnVision multiplate reader according to the manufacturer's

protocol. The HTRF ratio was converted to a response (%) using: response (%) = (ratio of sample/WT) × 100.

Data presented are mean ± S.E.M. of at least three independent biological experiments.

Cell-Surface Expression Assay

Cell-surface expression for each BRS3 mutant was monitored by fluorescence-activated cell sorting (FACS). The mutants were cloned into pcDNA6.0 vector (Invitrogen) with an N-terminal FLAG tag. Cells were seeded and transfected as described for the functional assay. After 24 hours of transfection, cells were washed with PBS and detached with 0.2% (w/v) EDTA in PBS. The expressed cells were then incubated with monoclonal anti-FLAG M2-FITC (Sigma-Aldrich) at 1:100 dilution for 15 minutes at 4°C, followed by a 9-fold excess of PBS. Cells were resuspended and fluorescence intensity was quantified using a BD Accuri C6 flow cytometer (BD Biosciences) with 488 nm excitation and 519 nm emission. FACS data were analyzed with BD Accuri C6 software 1.0.264.21 and normalized to wild-type BRS3.

Computational Methods

The simulation systems were derived from MK-5046-BRS3-G protein complex and BA1-BRS3-G protein complex. G proteins were removed before simulations. Protonation states of residues were determined using Propka3 software[21]. The CHARMM-GUI platform facilitated embedding of these structures into a 75×75 *POPC lipid bilayer*^{22}$.

The lipid bilayer was then surrounded by a 15 Å aqueous layer. Systems were then adjusted to a 0.15 mol/L NaCl concentration, supplemented with counterions. We applied the CHARMM36m force field for amino acids and lipids, while CHARMM general force field was applied for ligands[23],[24]. The systems underwent a 7-step equilibration process, with gradual minimization and relaxation of constraints, as outlined by CHARMM-GUI. We conducted 3×500 *ns independent production runs for each system using pmemd.cuda in Amber* 20^{25} under the NPT ensemble at 303.15 K and 1 atm. Long-range electrostatics were managed via the Particle Mesh Ewald method, while short-range electrostatic and van der Waals interactions used a 12 Å cutoff, with a smooth transition between 10 and 12 Å. The hydrophobic network was evaluated using the "nativecontact" command in CPPTRAJ. The interacting interface area was calculated using dr_{sasa}[26].

SUPPLEMENTARY INFORMATION

Figure S1. Sequence alignment of BRS3, NMBR, and GRPR. Secondary structure elements are annotated underneath the sequences based on the structure of the BRS3-Gq complex. The ligand-binding pockets of NMB-NMBR,

GRP(14-27)-GRPR, BA1-BRS3, and MK-5046-BRS3 are displayed in different colors (purple, orange, green, and violet, respectively). Additionally, the non-conserved residues including $S^{2\cdot63}$, $R^{2\cdot64}$, $Q^{3\cdot32}$, $P^{45\cdot52}$, and $R^{6\cdot58}$ are circled in dotted boxes.

Figure S2. Purification and characterization of BA1-BRS3–Gq complex and MK-5046-BRS3-Gq complex. (A) Schematic diagram of the protein engineering of BRS3, engineered Gαq, Gβ, Gγ, scFv16, and Nb35 used in this study. (B) Gel filtration (Superdex 200 Increase 10/300 column, GE Healthcare) profile of the apo BRS3–Gq complex. The black arrow indicates the sharp peak for apo BRS3–Gq complex. (C) Coomassie-stained SDS-PAGE analysis of the purified apo BRS3–Gq complex, showing balanced ratios for each subunit. (D) Gel filtration (Superdex 200 Increase 10/300 column, GE Healthcare) profile of the BA1-BRS3–Gq complex. The black arrow indicates the sharp peak for BA1-BRS3–Gq complex. (E) Coomassie-stained SDS-PAGE analysis of the purified BA1-BRS3–Gq complex, showing balanced ratios for each subunit. (F) Gel filtration (Superdex 200 Increase 10/300 column, GE Healthcare) profile of the MK-5046-BRS3-Gq complex. The black arrow indicates the sharp peak for MK-5046-BRS3-Gq complex. (G) Coomassie-stained SDS-PAGE analysis of the purified MK-5046-BRS3-Gq complex, showing balanced ratios for certain subunits, except for the Gγ subunit.

Figure S3. Cryo-EM data processing of apo BRS3–Gq complex. (A) Flowchart of computational sorting of cryo-EM data. (B) A representative cryo-EM micrograph of apo BRS3–Gq complex with 50 nm scale bar included as a size reference. (C) Twelve representative reference-free 2D cryo-EM class averages. Scale bar, 5 nm. (D) 'Gold-standard' Fourier shell correlation curve of the reconstruction. The resolution was reported at 3.30 Å using the Fourier shell cutoff at 0.143. (E) Local resolution map of apo BRS3–Gq complex.

Figure S4. Cryo-EM data processing of BA1-BRS3–Gq complex. (A) Flowchart of computational sorting of cryo-EM data. (B) A representative cryo-EM micrograph of BA1-BRS3–Gq complex with 50 nm scale bar included as a size reference. (C) Twelve representative reference-free 2D cryo-EM class averages. Scale bar, 5 nm. (D) 'Gold-standard' Fourier shell correlation curve of the reconstruction. The resolution was reported at 2.90 Å using the Fourier shell cutoff at 0.143. (E) Local resolution map of BA1-BRS3–Gq complex.

Figure S5. Cryo-EM data processing of MK-5046-BRS3-Gq complex. (A) Flowchart of computational sorting of cryo-EM data. (B) A representative cryo-EM micrograph of MK-5046-BRS3-Gq complex with 50 nm scale bar included as a size reference. (C) Twelve representative reference-free 2D cryo-EM class averages. Scale bar, 5 nm. (D) 'Gold-standard' Fourier shell correlation curve of the reconstruction. The resolution was reported at 2.93 Å using the Fourier shell cutoff at 0.143. (E) Local resolution map of MK-5046-BRS3-Gq complex.

Figure S6. Cryo-EM density maps of TM1-7 in receptors, αH5 and αHN in Gq proteins, and ligands in apo BRS3-Gq complex structure (A), BA1-BRS3–Gq

complex structure (B), and MK-5046-BRS3-Gq complex structure (C).

Figure S7. Structural comparison of bombesin receptors family. (A-C) Different views of the structural superposition of apo-state BRS3 (forest green), BA1-bound BRS3 (medium aquamarine), MK-5046-bound BRS3 (dark khaki). (D and E) Side view of the structural superposition of BA1-BRS3, NMB-NMBR, and GRP(14-27)-GRPR (D), and three ligands (E). (F) The H8B residue forms hydrogen bonds with $S124^{3\cdot29}$ and $S205^{2}$, differing from its analogs in NMB and GRP(14-27). (G) The structural superposition of BRS3 in BA1-BRS3, NMBR in NMB-NMBR, GRPR in GRP(14-27)-GRPR, and GRPR in PD176252-GRPR. (H and I) The structural comparison of BRS3 in BA1-BRS3 and AlphaFold2 website (H) and the clashes between BA1 and OBP in the AlphaFold2 BRS3 structure (I).

Figure S8. IP1 response curves of BA1 and MK-5046. BRS3 mutations on agonists BA1 (A-E) and MK-5046 (F-I) induced signaling effects. Dose-response curves for ligands were performed in transiently transfected AD293 cells. The results are expressed as percentage of the maximal effect observed in BRS3 wild type. Wild-type and mutated BRS3 data are shown by different colors as indicated. Each point represents the mean ± S.E.M. of 3 to 7 independent experiments with triplicate determinations.

Figure S9. Sequence alignment of BRS3 of different species including placental mammals (human, macaca mulatta, rabbit, rat, and mouse) and non-placental vertebrates (chicken, turtle, snake, and frog).

Table S1. The affinity (pKi) data of Bn-related ligands for human bombesin receptor family.

Table S2. Cryo-EM data collection, model refinement and validation statistics.

Table S3. Sequence of Bn-related peptides used or mentioned in this study.

Table S4. Ligand binding affinities and expression levels of WT and mutated BRS3. The wild type (WT) and mutants of BRS3 discussed in this manuscript were individually analyzed. The affinities are derived from at least 3 independent experiments using IP1 function assay. The expression level of mutant BRS3 was normalized to wild-type BRS3 as 100%, respectively. Each data point represents mean ± standard error of the mean (S.E.M.). All data were analyzed by two-sided Student's t test. *P<0.05,* ***P<0.01,*** P<0.001 vs. WT. Source data are available online. Definitions: NA – not applicable; NT, not tested.

Table S5. Sequence alignment of the key residues in sodium site, DRY motif, PV(I)F motif, toggle switch and NPxxY motif, as well as residues involved in disulfide bond formation in bombesin receptors.

Note: Figure translations are in progress. See original paper for figures.