Molecular mechanism of agonism and inverse agonism in ghrelin receptor

Jiao Qin1,2,3,4,5,9, Ye Cai2,9, Zheng Xu2,9, Qianqian Ming3,9, Su-Yu Ji1,3,4,5,9, Chao Wu2,9, Huibing Zhang1,3,4,5, Chunyou Mao6, Dan-Dan Shen1,3,4,5, Kunio Hirata7, Yanbin Ma8, Wei Yan2,9, Yan Zhang1,3,4,5 & Zhenhua Shao2

Much effort has been invested in the investigation of the structural basis of G protein-coupled receptors (GPCRs) activation. Inverse agonists, which can inhibit GPCRs with constitutive activity, are considered useful therapeutic agents, but the molecular mechanism of such ligands remains insufficiently understood. Here, we report a crystal structure of the ghrelin receptor bound to the inverse agonist PF-05190457 and a cryo-electron microscopy structure of the active ghrelin receptor-Go complex bound to the endogenous agonist ghrelin. Our structures reveal a distinct binding mode of the inverse agonist PF-05190457 in the ghrelin receptor, different from the binding mode of agonists and neutral antagonists. Combining the structural comparisons and cellular function assays, we find that a polar network and a notable hydrophobic cluster are required for receptor activation and constitutive activity. Together, our study provides insights into the detailed mechanism of ghrelin receptor binding to agonists and inverse agonists, and paves the way to design specific ligands targeting ghrelin receptors.
A n increasing number of GPCRs have been reported to exhibit high basal or constitutive activity in the absence of extracellular stimuli\textsuperscript{1-3}. The spontaneous manner of GPCRs is implicated in human physiological functions and various disorders\textsuperscript{4}. As a result, inverse agonists have been discovered as a new ligand category alongside agonists and neutral antagonists\textsuperscript{5}. In pharmacology, inverse agonists bind to the orthosteric site of receptors and reduce the constitutive activity of receptors, causing action exactly opposite to agonism and resulting in a paradigm shift in the field of GPCR pharmacology\textsuperscript{6,7}. Therefore, understanding the constitutive activity of GPCRs and the mechanism of inverse agonism would contribute to the development of new therapeutic drugs.

The ghrelin receptor\textsuperscript{8}, also known as the growth hormone secretagogue receptor (GHSR), belongs to the \(\beta\)-branch of class A GPCRs and displays constitutive activity\textsuperscript{9} (50% activity independent of the endogenous ligand ghrelin). The ghrelin receptor exerts a wide range of physiological functions, including appetite regulation, alcohol consumption, adipocyte metabolism, and glucose homeostasis\textsuperscript{10-13}. It is due to its broad distribution and multiple signaling pathways through divergent G-protein coupling or \(\beta\)-arrestin recruitment\textsuperscript{14}. The endogenous agonist ghrelin is a 28-amino acid peptide secreted primarily by the stomach, and an acylated modification, typically an octanoyl group, of the hydroxyl group of the Ser\textsuperscript{7+2} residue is required for the biological action of ghrelin as the unacylated form of ghrelin does not bind or activate the ghrelin receptor at all\textsuperscript{15,16}. In addition, the length of fatty acid modification can also produce diverse activation potency by comparison with the common octanoylated ghrelin of fatty acid modification, typically an octanoyl group, of the hydroxyl group of the Ser\textsuperscript{7+2} residue is required for the biological action of ghrelin as the unacylated form of ghrelin does not bind or activate the ghrelin receptor at all\textsuperscript{15,16}. In addition, the length of fatty acid modification can also produce diverse activation potency by comparison with the common octanoylated ghrelin in vivo\textsuperscript{17}. Previous studies have suggested that inhibition of the ghrelin–ghrelin receptor signaling axis and deacylation of ligands or deletion of receptors could potentially prevent obesity and type 2 diabetes (T2D); thus, blockade of the ghrelin receptor has been proven to be a great therapeutic approach for the treatment of related diseases\textsuperscript{18-23}.

Given the constitutive activity of the ghrelin receptor, inverse agonists would be the target of pharmacological agents for maximal efficacy\textsuperscript{24}. To date, several reported ligands from different pharmaceutical companies display consistent inverse agonism\textsuperscript{25,26} however, in some cases, preclinical research is confounding. PF-05190457 is the only reported small-molecule inverse agonist targeting the ghrelin receptor progressing to phase 1 clinical trial for T2D and alcoholism treatment\textsuperscript{27-29}. Despite a previous study revealing a bifurcated pocket in the neutral antagonist-bound ghrelin receptor structure\textsuperscript{30}, the molecular recognition of PF-05190457 by ghrelin receptors remains unclear, impeding effective drug development.

In this work, we determine the crystal structure of the ghrelin receptor in complex with the inverse agonist PF-05190457 and the cryo-electron microscopy (EM) structure of the active ghrelin receptor bound to endogenous ghrelin coupled to the Go heterotrimer. Our study provides an opportunity to comprehensively understand the distinct conformations of ghrelin receptors bound to different types of ligands. Moreover, our structures reveal a distinct binding mode of inverse agonists with receptors, define cavities for ligand recognition, and decipher the action mechanism of inverse agonists and agonists for ghrelin receptors.

**Results**

**Overall structures of the ghrelin receptor in complex with inverse agonist and endogenous agonist.** The ghrelin receptor is activated by endogenous ghrelin peptide and contains constitutive activity as \(~50%\) of the maximal activity in the absence of ghrelin peptide\textsuperscript{31}. Antagonist compound 21 had no effect on the constitutive activity of the ghrelin receptor\textsuperscript{32}, whereas the constitutive activity of the ghrelin receptor was significantly reduced upon the addition of the inverse agonist PF-05190457 (Fig. 1a).

To describe the molecular mechanism of the ghrelin receptor in binding with PF-05190457, the initial construct of the ghrelin receptor was truncated by eliminating the first N-terminal 34 amino acid residues and the C-terminus after residue 342. To facilitate crystallization, a thermostabilized apocytochrome bRIL was fused to the third intracellular loop (ICL3). Furthermore, mutations of T130\textsuperscript{3.39K} and N188\textsuperscript{ECL2Q} were introduced to improve the thermostability and homogeneity of the receptor as described in our previous study\textsuperscript{32} (Supplementary Fig. 1a-d). This construct was crystallized in a complex with PF-05190457 and determined at a resolution of 2.94 Å (Fig. 1b). In the resolved crystal structure, the PF-05190457-bound ghrelin receptor is packed in a P2\textsubscript{1} monoclinic lattice with two complex molecules per asymmetric unit (Supplementary Fig. 1e). The two receptor molecules display high identity with an RMSD value of 0.3 Å (root-mean-square deviation of \(C\textsubscript{x}\)) (Supplementary Fig. 1f).

In addition, we determined the ghrelin-bound ghrelin receptor-Go complex using a single-particle cryo-EM technique. The full-length of wild-type human ghrelin receptor, the thermostabilized miniGao1\textsuperscript{32}, G\textsubscript{\beta}1, and Gy\textsubscript{2} were co-expressed in insect cells. We used the NanoBiT tethering strategy\textsuperscript{34} to
stabilize the complex, in which the C-terminus of ghrelin receptor was attached to LgBiT subunit and Gb1 was fused with a C-terminal HiBiT subunit. An antibody fragment, scFv16, was also added. The final cryo-EM map has a nominal resolution of 2.8 Å after refinement, and a clear density map allows us to build the ghrelin peptide, receptor, Go, Gβ, Gγ, and scFv16 (Fig. 1c and Supplementary Figs. 2 and 3). The ghrelin receptor-Go complex displays similar conformations compared with previously solved activated GPCR-Go structures (Supplementary Fig. 4), suggesting that the ghrelin receptor bound to ghrelin was in an activated state.

Compared with the active structure, the extracellular portion of PF-05190457-bound ghrelin receptors, especially TM6 and TM7, displayed notable outward movement, enlarging the orthosteric pocket of the receptor (Fig. 1d). In addition, distinct conformational changes were also found in the cytoplasmic region of the receptor, in which TM6 swing inwards by ~10 Å to hinder the coupling of G proteins (Supplementary Fig. 5a–c).

Inverse agonist binding pocket of ghrelin receptor. The compound PF-05190457 was synthesized from a spiro-azetidinopiperidine analog to improve the selectivity and inverse agonism. Like most inverse agonists, the PF-05190457-bound ghrelin receptor complex reflects an inactive state conformation that differs from the complex bound with agonists or antagonists. Strikingly, unambiguous electron density at the orthosteric pocket placed PF-05190457 at an unusual site toward TM2, TM3, and TM6 (Fig. 2a), contrasting with the site occupied by the antagonist in the ghrelin receptor (Fig. 2b). The antagonist compound 21-bound ghrelin receptor complex structure demonstrates a bifurcated ligand-binding pocket separated by a salt bridge between E1243.33 and R2836.55, which is referred to as cavity I and II (Fig. 2b). Whereas PF-05190457 adopts an extended conformation (Fig. 2c–g), the arm-1 ((6-methylpyrimidin-4-yl)-2,3-dihydro-1 H-inden-1-yl moiety) (Fig. 2c) of the ligand projects into the cleft between TM2 and TM3 and is covered by ECL1 and ECL2 regions, making van der Waals contacts and hydrophobic interactions with R1022.63, Q1203.29, and F1193.28 as well as ECL2 regions, making van der Waals contacts and hydrophobic interactions with R1022.63, Q1203.29, and F1193.28 as well as disulfide-bound C1163.25 and C198ECL2 (Fig. 2d). The cleft between TM2 and TM3 accommodates arm-1 of PF-05190457 as defined as cavity III. Two residues, F1193.28A and Q1203.29A substitution, were found to be essential for the inverse agonism potency of PF-05190457 (Supplementary Figs. 6a and 7a, b).

The diazaspiro core (2,7-diazaspiro [3,5] nonan-7-yl moiety) of PF-05190457 is adopted in cavity I, forming direct interactions with D992.60 and S3087.38 by hydrogen bonding (Fig. 2e, c). Alanine replacement with D992.60 significantly reduced the potency of inverse agonism induced by PF-05190457 (Fig. 2g and Supplementary Fig. 7a, b), indicating that D992.60 may be a key factor for inverse agonist recognition or receptor activation, which is consistent with a previous report that the diazaspiro core moiety was typically required for inverse agonism. In addition, mutation of S3087.38 to alanine decreased the inverse agonism potency of PF-05190457, which may affect the recognition of the ligand and reduce the stability of the inactive conformation (Fig. 2g and Supplementary Fig. 7a, b). Both the D992.60A and S3087.38A mutants retained the expression level of the inactive ghrelin receptor by normalization (Supplementary Figs. 6a and 7b).

Arm-2 (2-methylimidazo moiety) penetrates deeply into the helical core of the receptor, and it packs against the side chains of residues F2796.51, W2766.48, and F3127.42 in TM6 and TM7, opening another hydrophobic pocket defined as cavity IV (Fig. 2f). In addition, arm-2 is also observed to form hydrogen bonds with residues W2766.48 and S3087.38 (Fig. 2f). Disrupting the hydrophobic pocket by replacing F2796.51, W2766.48, and F3127.42 with alanine significantly reduced the inverse agonistic activity (Fig. 2g). Moreover, these mutations also impaired the potency of the agonist ghrelin peptide and antagonist compound 21 (Fig. 2g and Supplementary Fig. 6b), indicating that aromatic residues are essential for the activation transition of the ghrelin receptor.

Structural comparison of PF-05190457-bound with compound 21-bound ghrelin receptor complex reveals a notable difference. The salt bridge between E1243.33 and R2836.55 formed in the antagonist receptor structure is disrupted upon receptor binding with PF-05190457 (Fig. 2h). Residue R2836.55 swings away from E1243.33, leading to the disappearance of the boundary between cavity I and cavity II. In contrast to the obvious change in the extracellular portion, the cytoplasmic ends of TM5 and TM6 in the PF-05190457-bound structure display conformations similar to those of the inactive ghrelin receptor, suggesting that the inverse agonist stabilizes the receptor in an inactive conformation (Supplementary Fig. 5d–f).

Agonist binding pocket of ghrelin receptor. Distinct from the small-molecule inverse agonist binding mode, the endogenous ghrelin peptide is well folded and stably anchored into its binding site through an extensive network of contacts with the receptor. The synthetic ghrelin peptide contains 28 residues, and an octanoyl modification was introduced at the side chain of Ser2+2 (the superscript +n indicates the amino acid position of ghrelin peptide). The first sixteen residues are clear in our density map (Supplementary Fig. 3). The N-terminal portion of ghrelin from Gly1 to Pro2+2 penetrates into the conserved orthosteric pocket (Fig. 3a), where Gly2+1 and Ser2+2 fill cavity I and are stabilized by a hydrogen-bound network formed by S1233.32, N3057.35, and R2836.55 in the ghrelin receptor (Fig. 3b). Meanwhile, R2836.55 is stabilized via a salt bridge with E1243.33 and a hydrogen bond with S2175.43 (Fig. 3b).

Notably, the interaction between S2175.43 and R2836.55 was found only in the agonist-bound structure and not in the antagonist-bound structure (Supplementary Fig. 8a). These observations, particularly for the rotamer change of R2836.55, reveal that rearrangement of the residues in the polar network appears to tether those key residues on TM3, TM5, TM6, and TM7, contracting the agonist binding pocket of the receptor. In agreement with our structural comparison, alanine substitution of residues E1243.33 and N3057.35 in the receptor markedly reduced the activation potency induced by the ghrelin peptide, while alanine substitution of residue R2836.55 almost abolished the efficacy (Fig. 3c), suggesting that the polar interactions of E1243.33, R2836.55, S2175.43, and N3057.35 in the receptor and Gly2+1 and Ser2+2 in the ghrelin peptide play significant roles in receptor activation. In accordance with previously published Gq-coupled ghrelin receptors, Gly2+1 and Ser2+2 in ghrelin are engaged in similar contacts with receptors (Supplementary Fig. 8b).

In the GPCR activation transition, rearrangement of the polar network is required for conformational propagation. The activation process of β2AR, for example, is the best-characterized member of the GPCR family; residues D3.32, S5.42, and S5.46 from TM3 and TM5 constitute a key polar motif for distinguishing different types of ligands and trigger signaling pathways (PDB: 4LDO40, and a polar interaction manner was also found in the D1 receptor (PDB: 7CKZ41 (Supplementary Fig. 8d–f). Despite the divergent nature of the endogenous ligand between the ghrelin receptor and amnergic receptors, the ghrelin receptor appears to have a similar activation process as amnergic receptors.
Although residue Ser\textsuperscript{+3} of ghrelin could be modified with different lengths of acylated modification\textsuperscript{42,43}, octanoyl modification is optimal for its activation and biological function\textsuperscript{16,17}. The octanoyl group in our structure shows a well-defined density through the cryo-EM map and is observed to bind to hydrophobic cavity II, which comprises residues I178\textsuperscript{4.60}, L181\textsuperscript{4.63}, L210\textsuperscript{5.36}, M213\textsuperscript{5.39}, V214\textsuperscript{5.40}, and F286\textsuperscript{6.58} from TM4, TM5, and TM6 (Fig. 3d). As seen from the cut view of cavity II, the octanoyl group definitely fits well in the pocket rather than the other type of fatty acid modification (Supplementary Fig. 8g, h).

The importance of these hydrophobic contacts was validated by single-mutation and cell-based function assays, and our results showed that mutations of I178\textsuperscript{4.60}A, L210\textsuperscript{5.36}A, and F286\textsuperscript{6.58}A decreased the receptor activation induced by ghrelin (Fig. 3d). This finding indicates the critical role of the octanoylation of...
Mechanism of ghrelin-induced activation. The comparison of the ghrelin-bound complex structure with the neutral antagonist-bound structure shows that the TM bundles at the extracellular portion are similar, except that the helix end of TM7 is tilted outward by ~5.5 Å (Fig. 4a). Moreover, the conformation of the intracellular end of TM bundles is widely changed. The ends of TM3, TM5, TM6, and TM7 shifted by ~2.8, 2.3, 13.4, and 2.9 Å (referring to the Ca of each TM terminal residue: C146$^{3.55}$, L239$^{5.65}$, L253$^{6.25}$, and L322$^{7.52}$), respectively (Fig. 4b). The obvious rearrangement of TM6 as well as the movement of other ends of TMs, which was likewise observed in the Gq-coupled ghrelin receptor structure, make a suitable binding cavity available for the G protein (Fig. 4b and Supplementary Fig. 9c).

The N-terminal part in ghrelin stretches on top of an aromatic cascade formed by W276$^{6.48}$, F279$^{6.51}$, and F312$^{7.42}$, which is referred to as the WFF cluster hereafter. In the neutral antagonist-bound structure, F312$^{7.42}$ was positioned closely and tightly packed against F279$^{6.51}$ and W276$^{6.48}$. In the ghrelin-bound structure, the side chains of F312$^{7.42}$, W276$^{6.48}$, and F279$^{6.51}$ display significant rotation and displacement toward the extracellular end (Fig. 4c).

R283$^{6.55}$ is situated above F279$^{6.51}$ and connects the WFF cluster with the polar network associated with the binding of ghrelin. Compared with the neutral antagonist-bound structure, the side chain of R283$^{6.55}$ has an obvious swing toward TM5 in the ghrelin-bound structure and appears to trigger local rearrangement of the TM6 bundle. Residues F279$^{6.51}$ and W276$^{6.48}$ shift significantly along with the outward movement of TM6. Notably, F279$^{6.51}$ interacts directly with ghrelin and involves cation–π interactions with R283$^{6.55}$, which also contributes to the movement of F279$^{6.51}$. The swing of W276$^{6.48}$ subsequently switches F272$^{6.44}$ (Fig. 4d), resulting in a cascade of relocations of highly conserved activation motifs such as the P5.50-I (V)$^{3.40}$, P6.44 motif, D (E)$^{3.49}$-R3.50-Y3.51 motif, and N7.49-P7.50-xx-Y7.53 motif (Supplementary Fig. 9d–f), leading to the change in TM6 on the intracellular side and enabling the receptor to bind to the G protein. Mutagenesis and functional analyses further verify our hypothesis. Site-directed mutants E124$^{3.33}$A, R283$^{6.55}$A, F279$^{6.51}$A, or W276$^{6.48}$A almost abolished the activity of the ghrelin receptor, while F312$^{7.42}$A impaired ghrelin-induced G-protein signaling, implying the critical role of the polar network and the WFF cluster in activation (Fig. 4e).

Aromatic amino acids also contribute to the activation of several other peptide receptors. For instance, W321$^{6.48}$, F358$^{7.42}$, and Y324$^{6.51}$ in NT5R1, which belongs to the ghrelin receptor family (PDB ID: 4XEE), display a similar significance in
Mechanism of inverse agonist action on the ghrelin receptor.

The comparison of the inverse agonist-bound ghrelin receptor structure with agonist-bound and neutral antagonist-bound structures enabled us to visualize the plasticity of the TM domain, reflecting different conformational states of the ghrelin receptor (Supplementary Fig. 5). As observed, the inverse agonist PF-05190457 appears to stabilize the receptor in an inactive conformation with respect to the binding of the G protein (Supplementary Fig. 5b, e). PF-05190457 has distinct chemical moieties with neutral antagonist compound 21, displaying extensive contacts with orthosteric sites. Arm-2 (2-methylimidazo moiety) of PF-05190457 penetrates deeply into the helical bundle and is sandwiched by the WFF cluster (Fig. 5a). As the basal activity of the ghrelin receptor is blocked significantly by inverse agonists, the WFF cluster from the hydrophobic pocket exhibits significant displacement relative to that in agonist-bound and antagonist-bound receptor structures (Fig. 5b). Notably, the side chain of W2766.48 rotates nearly 180° and tilts ~3 Å toward the extracellular end of TM6 and enlarging the ligand-binding pocket, subsequently impeding the formation of a salt bridge by dragging R2836.55 away from E1243.33 (Fig. 5c). These conformational changes also impair the rearrangement of the PIF motif, ERY motif, and NPxxY motif (Supplementary Fig. 9d–f), which is distinct from the conformational changes in agonist-bound and antagonist-bound structures.

In addition to the change in the hydrophobic portion in the orthosteric site, the rearrangement of the polar network occurs as inverse agonist binding. Through structural comparison with ghrelin-bound, the PF-05190457-bound complex structure shows breaking of the salt bridge between R2836.55 and E1243.33, breaking of the weak polar interaction between R1022.63 and Q1203.29, and newly established polar interactions between the inverse agonist and residues S3087.38 and D992.60 (Fig. 5d). Notably, E1243.33A and R2836.55A mutants impaired the inverse agonism potency of PF-05190457 (Fig. 5e and Supplementary Fig. 7a), suggesting that both E1243.33 and R2836.55 (referred to as the E-R motif) should play an important role in the constitutive activity of the ghrelin receptor and the action of the inverse agonist.
The ghrelin receptor is reported to have high constitutive activity. 

Discussion

The ghrelin receptor is reported to have high constitutive activity. To better understand the constitutive activity mechanism of the ghrelin receptor, we compared ghrelin receptors with different states as well as with previously described receptors with high basal activation, such as GPR52. The structure of GPR52 contains a built-in ECL2 region that is inserted into the orthosteric binding pocket (Supplementary Fig. 10a). In particular, Y185ECL2 and H186ECL2 act as built-in "agonist" for activating GPR52, thus resulting in a high level of basal activity of the receptor (Supplementary Fig. 10b). The structural superposition of GPR52 with the ghrelin receptor indicates that the ECL2 region of ghrelin receptor sits over the ligand-binding pocket (Supplementary Fig. 10c). Together with the structural comparison with ghrelin receptors with different types of ligands, our results suggest that polar networks such as the E-R motif and WFF cluster may contribute to their basal activity.

Numerous GPCRs have been reported to contain constitutive activity, and several structures of inverse agonist-bound GPCRs have been determined so far. As shown in Supplementary Fig. 12b, inverse agonists in DRD2, 5-HT2aR, 5-HT2cR, and ghrelin receptor appear to extend deeply into the orthosteric pocket, forming directly hydrophobic interactions with the indole ring of the "switch" residue W6.48. We note that the aromatic moiety of inverse agonists packs against either the left or right of W6.48 in the receptors mentioned above (Supplementary Fig. 12b). Methiothepin, RIT, and risperidone were observed to bind with the hydrophobic cavity on the left of W6.48 in the ghrelin receptor and ETB receptor. Compared with the activated 5-HT2cR and DRD2 structures, the hydrophobic W6.48, p.51/6.52, f.547 cluster also notably displaced the structures of inverse agonist-bound receptors (Supplementary Fig. 12d–f). The deep binding conformation of the inverse agonist impeded the side-chain rotations of W6.48 and p.6.44, restricting the conformational change of TM6 from inactive to active state.

In addition, sequence alignment and structural comparison of ghrelin receptors with 5-HT2aR, 5-HT2cR, DRD2 and ETB receptors revealed that the E3.33-R5.55 motif is not conserved (Supplementary Fig. 12a, c), and the distance between TM3 and TM6 appears to play a role in ligand selectivity. Collectively, in
the ghrelin receptor family (including ghrelin receptor, NTSR1, NTSR2, and GPR39), the conserved E3-R5-R6-55 motif together with the WFF cluster is suggested to be essential for inverse agonist binding as well as agonist activation.

When our manuscript was under review, another study about the Gq-coupled ghrelin receptor was published, and it reported the structure of the ghrelin-bound receptor complex39. Obviously, Gly+1 and Ser+2 of ghrelin inserted into the same orthosteric site of the receptor, whether binding to either Go or Gq. The backbone of the ghrelin peptide resembled the identical conformation. However, a subtle difference was the position of the octanoyl groups at Ser+3 in the two ghrelin peptides (Supplementary Fig. 8b). The acyl chain of Ser+3 extends towards TM5 in the Gq-bound receptor, while the octanoyl tail stretches horizontally toward the gap between TM4 and TM5 and five carbons of the octanoyl group can be placed in the density in the Gq-bound state, reflecting a presumptive dynamic character of acyl modification in the ghrelin receptor (Supplementary Fig. 8b). Coincidentally, previous literature has proven the dynamic characteristics of ghrelin receptors using NMR spectroscopy52. Compared with the available structure of the ghrelin peptide, the disordered C-terminus of the peptide folds into α-helix when ghrelin binds to its receptor (Supplementary Fig. 8c), displaying the dynamic conformation of the peptide. Therefore, it is not hard to speculate that the octanoyl group of ghrelin could behave in variable conformations. In agreement with this speculation, molecular simulation courses and NMR data indicated some degree of conformational and local dynamics of the ghrelin peptide in the ligand-binding pocket53.

In this study, we present the structures of ghrelin receptors in complex with the inverse agonist PF-05190457 as well as in complex with endogenous ghrelin and Go protein coupling. Combined with the inverse agonist- agonist- and antagonist-bound structures, the molecular mechanism of the activation of ghrelin receptor has gradually emerged, and it can be summarized as follows: The binding of ghrelin peptide to the receptor contracts the extracellular TM bundles and may change the orientation of the polar network and hydrophobic core including E-R motif and WFF cluster, further expanding the intracellular end of TM6 and allowing the ghrelin receptor to interact with G proteins by rearranging the cascade motifs (including P-F motif, E-R-Y motif, and NPxxY motif) (Fig. 6a). In addition, the binding of the antagonist occupies the orthosteric site and maintains the conformation of the E-R motif and WFF cluster (Fig. 6b). However, the binding of PF-05190457 will break the tight conformation of the WFF cluster, push against the extracellular TM6 bundle, and contract the intracellular end of the TM6 helix to close the G-protein-binding pocket (Fig. 6c).

Taken together, the discovery of constitutive receptor activity and inverse agonism extends the knowledge of GPCR pharmacology, which may help pharmacologists to achieve a greater degree of control over receptor function. Our study provides insights into the detailed mechanism of ghrelin receptor binding to inverse agonists and agonists, and it may pave the way for designing specific ligands targeting ghrelin receptors.

**Methods**

**Construct design and expression.** For crystallization construct ghrelin receptor-bRIL, the wild-type human ghrelin receptor cDNA gene (UniProt accession: Q92847) was cloned into a modified pFastBac1 (Invitrogen) baculovirus expression vector with the haemagglutinin (HA) signal sequence followed by a Flag epitope tag at the N-terminus and a 10× His tag at the C-terminus. To facilitate receptor expression and crystallization, the N-terminal 34 residues and C-terminal residues after P342 were removed, TEV protease recognition sites were introduced before the residue Leu35 at N-terminus and before the residue P343 at C-terminus. Residues R244-T252 and T258-Q262 of the intracellular loop 3 (ICL3) were replaced with the thermostabilized apocytochrome b562RIL (bRIL) (PDB: 1M6T). Furthermore, mutations T1303.39K and N188ECL2Q were introduced to improve thermostability and homogeneity. The final construct of the ghrelin receptor was transfected into DH10Bac® Escherichia coli to produce a recombinant baculovirus with the Bac-to-Tac system (Invitrogen). The recombinant baculovirus was used to infect Sf9 insect cell culture at a cell density of 2.5 × 10⁶ cells per ml -1. Insect cells were grown for 48 h at 27 °C before harvesting, and the cell pellets were stored at −80 °C for future use.

For cryo-EM constructs, the full-length of wild-type human ghrelin receptor was subcloned into pFastBac1 vector with an N-terminal FLAG tag and C-terminal 10×His tag. We used the NanoBiT tethering strategy, in which the C-terminus of the ghrelin receptor was directly attached to the LgBiT subunit followed by a TEV protease cleavage site and a double MBP tag. Gq was fused with a C-terminal HiBiT, together with G2y were cloned into pFastBac dual vector. An engineered human Gaq1 with Gaq1 H domain deletion, named miniGaq1 was cloned into pFastBac1 according to published literature53. The ghrelin-induced ghrelin receptor activity was measured by NanoBiT-G-protein dissociation assay using a chimeric Gaq protein according to the previous publication except for replacement Gaq with Gaq54. The Gaq were generated by replacing the six amino acids of the C-terminal of Gaq with those from GaqA1. Other constructs including the full-length and various site-directed mutagenesis human ghrelin receptors were cloned into pcDNA3.1 vector for NanoBiT-G-protein dissociation assay. Ghrelin receptor, miniGaq1, and Gqβ1y2 were co-expressed in Sf9 insect cells (Expression System) using the Bac-to-Bac baculovirus expression system (ThermoFisher). The cell pellets were collected by centrifugation 48 h post infection and stored at −80 °C until use.

**Purification of ghrelin receptor-bRIL protein.** The cell pellets were lysed using a Dounce homogenizer in a hypotonic buffer containing 10 mM HEPES (pH 7.5), 20 mM KCl, 10 mM MgCl2, 160 µg/ml benzamidine, and 100 µg/ml leupeptin. The cell membranes were isolated by ultracentrifugation at 100,000×g for 30 min at 4 °C. Washing of the membranes was performed by two rounds of Dounce homogenization and centrifugation in a high-osmolarity buffer containing 10 mM
HEPES (pH 7.5), 1.0 M NaCl, 20 mM KCl, and 10 mM MgCl2, 160 μg/ml benzamidine, and 100 μg/ml leupeptin. Purified membranes were incubated with 10 μM PF-05190457 (Tocris) and 2 mg/ml iododeoxyxenonide (Iod) at 4°C. The membranes were then solubilized in a solubilization buffer containing 10 μM PF-05190457, 50 mM HEPES (pH 7.5), 500 mM NaCl, 10% (v/v) glycerol, 1% (w/v) n-dodecyl-β-D-maltopyranoside (DDM), 0.2% cholesteryl hemisuccinate (CHS), and 0.2% (w/v) sodium cholate, 160 μg/ml benzamidine, 100 μg/ml leupeptin for 2.5 h at 4°C, followed by ultracentrifugation at 125,000 × g for 30 min at 4°C. The supernatant was incubated with TALON IMAC resin (TaKaRa) in a batch over- night at 4°C. After binding, the resin was collected by centrifugation at 500 × g for 5 min, resuspended with wash buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 0.1% lauryl maltoside neopentyl glycol (LMNG, Anatrace), 0.02% sodium cholate, 0.02% CHS, 5% (v/v) glycerol) supplemented with 160 μg/ml benzamidine, 100 μg/ml leupeptin, 10 mM imidazole, 1 μM PF-05190457 and was repeated one more time. Then the resin was loaded on a glass column and slowly washed with wash buffer containing 160 μg/ml benzamidine, 100 μg/ml leupeptin, 15 mM imidazole, and 1 μM PF-05190457 for gradually exchanging to LMNG. The protein was eluted with 10 ml wash buffer containing 200 mM imidazole, 10 μM PF-05190457. The N-terminal FLAG tag and C-terminal His tag were cleaved by TEV protease for 6 h at 4°C. Finally, the receptor was run on a Superdex 200 size-exclusion column (GE Healthcare) with buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.02% LMNG, 0.004% CHS, and 10 μM PF-05190457.

Crystallization data collection, and structure determination. The purified receptor-PF-05190457 complex was concentrated to >60 mg/ml using a 100-kDa cutoff Vivaspin concentrator (Sartorius). The initial crystallization screen was set up using the lipidic cubic phase (LCP) method.55 The sample of the complex was mixed with the lipid (monolein and cholesterol at 1:0.1 by mass) at a weight ratio of 2:3 using a syringe mixing apparatus at room temperature.56 The mesophase was dripped onto glass bed plates by using a micropipette and flash-cooled with an experience cryoprotectant solution using a Gryphon LCP robot (Art Robbins Instruments). The full-size crystals of ghrelin receptor were grown over 1 week at 20°C in the following overlying precipitant condition: 25–6% PEG300, 100 mM HEPES pH 7.0, 100–150 mM NaCl for 30 min at 4°C. Finally, the crystals were frozen quickly in liquid nitrogen after being cryoprotected with a solution containing 20% glycerol, 30% sitting drop buffer, and 50% glycerol. The X-ray diffraction data were collected at beamline 32XU at Spring-8, Hyogo, Japan, using a beam size of 10 μm and a Pilatus 6 M detector (X-ray wavelength 1.0000 Å). The data-collection strategy was designed and performed on the basis of initial rater results as described previously.57 Full datasets of PF-05190457-bound ghrelin receptor were collected from 23 crystals assigned to the radiation-sensitive dataset 509 crystalline crystals. Diffraction images were indexed, integrated, and scaled using XDS58 and merged using SCALA. The resolution limit was set to 2.94 Å. Data-collection statistics are shown in Supplementary Table 1. The structure of the PF-05190457-bound ghrelin receptor was determined by molecular replacement with Phaser59 using the ghrelin receptor (receptor only, PDB: 6KO5) as an initial model for model rebuilding with Phenix60 and COOT61, respectively. The refinement statistics were validated using the module validation (cryo-EM) in Phenix. The model was then subjected to iterative rounds of manual adjustment and automated refinement in Coot62 and Phenix63, respectively.

Cyto-EM model building, refinement, and validation. The crystal structure of the ghrelin receptor30 (PDB: 6KO5) was used as an initial model for model rebuilding and refinement against the electron microscopy map of ghrelin receptor-miniGαo1-Gβ1γ2 complex. The BCM–GPR97–Goless complex33 (PDB: 7D7E) was used to generate the initial models of Go, Gβγ, and scFv16. Ligand and lipid coordinates and geometry restraints were generated using phenix.elbow. Models were docked into the EM density map of the receptor-ligand complex using phenix.helixmap64. The receptor-ligand complex was subjected to rigid-body refinement and automated refinement in Coot65 and Phenix66, respectively.

NANOBIOT-G-protein dissociation assay. To measure the activity of inverse agonist PF-05190457 (Tocris, Cat. No. 6350), we carried out IP1 accumulation assays for ghrelin receptor independent of agonist. The agonist ghrelin and antagonist compound 21 were also performed as the control. The cDNA of ghrelin receptor subcloned into pCDNA3.1 (+)–expression vector with a HA signal sequence followed by a Flag tag at the N-terminus. Point mutations in our study were generated by using Q5 site-Directed Mutagenesis kit (NEB). All the constructs were verified by sequencing. The cell culture and transfection methods were expressed in HEK293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Cells were harvested 48 h post transfection.

Nanoscale biofilm formation and characterization. The biofilm formation and characterization were performed using NanoBiT-G-protein dissociation assay56 in which the expression of luciferase coupled with a fluorescent-cryptate-labeled anti-IP1 monoclonal antibody dissolved in Lysis Buffer were added to each well of a six-well plate at a concentration of 0.3 million/ml (2 mL per well). Baseline coelenterazine 400a (Maokangbio) solution diluted 0.01% BSA and 5 mM HEPES was added to the wells. After 1 h incubation at room temperature, IP1 was quantified using Synergy H1 microplate reader (BioTek) with excitation at 520 nm and emission at 595 nm. The accumulation of IP1 was calculated according to a standard dose–response curve in GraphPad Prism 8 (GraphPad Software). Data were represented as the mean ± SEM from three independent experiments and all experiments were repeated at least three times.
change signals over vehicle treatment were used to show G-protein dissociation response.

**Enzyme-linked immunosorbent assay (ELISA).** Cell surface expression of the receptor subunits was detected by ELISA. Plasmids corresponding to WT and mutant ghrelin receptors were transfected as described above. After transfection, cells were re-seeded onto cell adherent reagent (Appylyn) treated 96-well plates at a density of 3 x 10^4 cells per well. Twenty-four hours later, cells were washed with PBS and fixed with 100% formaldehyde for 10 min followed by three times washing with PBS. Following fixation, cells were blocked with blocking buffer (1% BSA in PBS) for 1 h at RT. Afterward, plates were incubated with a 1:10,000 dilution of anti-FLAG M2 HRP-conjugated monoclonal antibody (SigmaAldrich) in blocking buffer for another 1 h at RT. After careful washing, 80 µL/well dilluent SuperSignal Elisa Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) was added, and the luminescence was measured using a luminescence microplate reader (Tecan).

**Synthesis.** All solvents and chemicals used for compound 21 synthesis were reagent grade and supplied by commercial sources. Silica gel thin-layer chromatography was performed on pre-coated plates GF-254 (Qingdao HaiYang, China). The compound purity and characterization were established by a combination of liquid chromatography-mass spectroscopy (LCMS) and nuclear magnetic resonance (NMR) analytical techniques. The LC/MS using Waters Acuity_Arc-2489-Qda (Column: Xbridge C18; Column size: 3.5 µm 2.1 × 50 mm, mobile phase: A: Water, B: Acetonitrile; gradient 8%: 40% over 60 min). The reaction mixture was washed with saturated sodium bicarbonate and EtOAc. The residue was dried under vacuum.

3.4 mmol) and TEA (0.9 g, 8.7 mmol) in DCM (12.5 mL) was stirred overnight at room temperature. The reaction mixture was washed with saturated sodium bicarbonate and EtOAc. The residue was dried under vacuum. 80 µL/well dilluent SuperSignal Elisa Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) was added, and the luminescence was measured using a luminescence microplate reader (Tecan).

3×10^4 cells per well. Twenty-four hours later, cells were washed with PBS and seeded onto cell adherent reagent (Applygen) treated 96-well plates at a density of 10^5 cells per well. 10% formaldehyde for 10 min followed by three times washing with PBS. The reaction mixture was extracted by the saturated sodium bicarbonate and EtOAc. The residue was dried under vacuum. The reaction mixture was concentrated in a vacuum to give the title compound.

Statistics and reproducibility: All functional study data were analyzed using Prism 8 (GraphPad) and presented as means ± SEM. From at least n = 3 biologically independent experiments performed in triplicate. Concentration–response curves were evaluated with a standard dose–response curve. Statistical differences were determined by two-sided, one-way ANOVA with Dunnett’s multiple comparisons test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The structural data generated in this study have been deposited in the Protein Data Bank (http://www.pdb.org/) under accession number 7TR3 for the PF-05190457–ghrelin receptor, 7W22 and Electron Microscopy Data Bank (EMDB) accession number EMDB-32268 for the ghrelin–ghrelin receptor-Go complex. All the other data generated in this study are provided in the Supplementary information and source data files. Source data are provided with this paper.

Received: 27 July 2021; Accepted: 29 December 2021; Published online: 13 January 2022

References
1. Berg, K. A., Harvey, J. A., Spampinato, U. & Clarke, W. P. Physiological relevance of constitutive activity of 5-HT2A and 5-HT2C receptors. Trends Pharmacol. Sci. 26, 625–630 (2005).
2. Meye, F. J., van Zessen, R., Smidt, M. P., Adan, R. A. & Ramakers, G. M. Morphine withdrawal enhances constitutive µ-opioid receptor activity in the ventral tegmental area. J. Neurosci. 32, 16120–16128 (2012).
3. Van der Stelt, S. et al. Peripheral hyperalgesia in a murine model of hyperalgesia reduces obesity by reversing leptin resistance. Cell. Metab. 16, 167–179 (2012).
4. Damiano, M. et al. High constitutive activity is an intrinsic feature of ghrelin receptor protein: a study with a functional monomeric GHS-R1a receptor reconstituted in lipid discs. J. Biol. Chem. 287, 3630–3641 (2012).
5. Smit, M. J. et al. Pharmacogenomic and structural analysis of constitutive ghrelin peptide–coupled receptor activity. Annu. Rev. Pharmacol. Toxicol. 47, 53–87 (2007).
6. Bond, R. A. & Ijzerman, A. P. Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. Trends Pharmacol. Sci. 27, 92–96 (2006).
7. Howard, A. D. et al. A receptor in pituitary and hypothalamus that functions as a growth hormone release factor. Proc. Natl. Acad. Sci. U.S.A. 95, 10755–10757 (1998).
31. Holst, B. et al. Common structural basis for constitutive activity of the ghrelin receptor family. J. Biol. Chem. 279, 53806–53817 (2004).
32. Yan, W. et al. Structure of the human gonadotropin-releasing hormone receptor GnRHR reveals an unusual ligand binding mode. Nat. Commun. 11, 5287 (2020).
33. Ping, Y. Q. et al. Structures of the glucocorticoid-bound adhesion receptor GPR97-(g) complex. Nature 589, 620–626 (2021).
34. Djam, J. et al. Cryo-EM structure of an activated VIP1 receptor-G protein complex revealed by a NanoBt tethering strategy. Nat. Commun. 11, 4121 (2020).
35. Wang, S. et al. Structure of the D2 dopamine receptor bound to the atypical antipsychotic drug risperidone. Nature 555, 269–273 (2018).
36. Nagiri, C. et al. Crystal structure of human endothelin ET(B) receptor in complex with peptide inverse agonist IRL2500. Commun. Biol. 2, 236 (2019).
37. Shao, Z. et al. High-resolution crystal structure of the human CBI cannabinoid receptor. Nature 540, 602–606 (2016).
38. Kung, D. W. et al. Identification of spirocyclic piperidine-azetidine inverse agonists of the ghrelin receptor. Bioorg. Med. Chem. Lett. 22, 4281–4287 (2012).
39. Wang, Y. et al. Molecular recognition of an acyl-peptide hormone and activation of ghrelin receptor. Nat. Commun. 12, 5064 (2021).
40. Ring, A. M. et al. Adrenaline-activated structure of β2-adrenoceptor stabilized by an engineered nanobody. Nature 502, 575–579 (2013).
41. Xiao, P. et al. Ligand recognition and allosteric regulation of DRD1-Gs signaling complexes. Cell 184, 943–956.e198 (2021).
42. Ohgusu, H. et al. Ghrelin O-acyltransferase (GOAT) has a preference for n-hexanoyl-CoA over n-octanoyl-CoA as an acyl donor. Biochem. Biophys. Res. Commun. 386, 153–158 (2009).
43. Gutierrez, J. A. et al. Ghrelin octanoylation mediated by an orphan lipid transferase. Proc. Natl Acad. Sci. USA 105, 6320–6325 (2008).
44. Krumm, B. E., White, J. F., Shah, P. & Grisham, R. Structural prerequisites for G-protein activation by the neurotensin receptor. Nat. Commun. 6, 7895 (2015).
45. Zhang, H. et al. Structural basis for chemokine recognition and receptor activation of chemokine receptor CCR5. Nat. Commun. 12, 4151 (2021).
46. Kim, K. et al. Structure of a hallucinogen-activated Gq-coupled 5-HT(2A) serotonin receptor. Cell 182, 1574–1588.e1519 (2020).
47. Peng, Y. et al. 5-HT(2C) receptor structure reveals the structural basis of GPCR polypharmacology. Cell 172, 719–730.e714 (2018).
48. Hanson, M. A. et al. Crystal structure of a lipid G protein-coupled receptor. Science 335, 851–855 (2012).
49. Hua, T. et al. Crystal structures of agonist-bound human cannabinoid receptor CB1(1). Nature 547, 468–471 (2017).
50. Lin, X. et al. Structural basis of ligand recognition and self-activation of orphan GPR52. Nature 579, 152–157 (2020).
51. Deluigi, M. et al. Complexes of the receptor 1 with small-molecule ligands reveal structural determinants of full, partial, and inverse agonism. Sci. Adv. 7, eabe5044 (2021).
52. Vortmeier, G. et al. Integrating solid-state NMR and computational modeling to investigate the structure and dynamics of membrane-associated ghrelin. PLoS ONE 10, e0122444 (2015).
53. Ferré, G. et al. Structure and dynamics of G protein-coupled receptor-bound ghrelin reveal the critical role of the octanoyl chain. Proc. Natl Acad. Sci. USA 116, 17525–17530 (2019).
54. Inoue, A. et al. Illuminating G-protein-coupling selectivity of GPCRs. Cell 177, 1933–1947.e1925 (2019).
55. Cherezov, V. Lipidic cubic phase technologies for membrane protein structural studies. Curr. Opin. Struct. Biol. 21, 559–566 (2011).
56. Caffrey, M. & Cherezov, V. Crystallizing membrane proteins using lipidic mesophases. Nat. Protoc. 4, 706–731 (2009).
57. Li, X. et al. Crystal structure of the human cannabinoid receptor CB2. Cell 176, 459–467.e413 (2019).
58. Kabsch, W. XDS. Acta Crystallogr D Biol. Crystallogr 66, 125–132 (2010).
59. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr D Biol. Crystallogr 66, 213–221 (2010).
60. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Crystallogr D Biol. Crystallogr 66, 486–501 (2010).
61. Schüttelkopf, A. W. & van Aalten, D. M. PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. Acta Crystallogr D Biol. Crystallogr 60, 1355–1363 (2004).
62. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. Nat. Methods 14, 331–332 (2017).
63. Zhang, K. Geet: real-time CTF determination and correction. J. Struct. Biol. 193, 1–12 (2016).
64. Scheres, S. H. RELION: implementation of a Bayesian approach to cryo-EM structure determination. J. Struct. Biol. 180, 519–530 (2012).
65. Heymann, J. B. Guidelines for using BioSft for high resolution reconstruction and validation of biomolecular structures from electron micrographs. Protein Sci. 27, 159–171 (2018).
66. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol. Crystallogr 60, 2126–2132 (2004).
67. Goddard, T. D. et al. UCSF ChimeraX: meeting modern challenges in visualization and analysis. Protein Sci. 27, 14–25 (2018).
68. Potters, E. F. E. et al. UCSF ChimeraX: structure visualization for researchers, educators, and developers. Protein Sci. 30, 70–82 (2021).
69. Kato, H. E. et al. Conformational transitions of a neurotransmitter receptor 1-G(II) complex. Nature 572, 80–85 (2019).

Acknowledgements
This work was supported by Natural Science Foundation of China grant 31972916 to Z.S.; Ministry of Science and Technology of China grant 2019YFA0508800 (Z.S. and Y.Z.); Science and Technology department of Sichuan Province 2020YJ0208 (Z.S.); National Natural Science Foundation of China 81922071 (Y.Z.); Zhejiang Province Science Fund for Distinguished Young Scholars LR19H31001 (Y.Z.); Key R & D Projects of Zhejiang Province 2021C03039 (Y.Z.); the National Natural Science Foundation of China (32100959 to C.M.); Y.Z. is also supported by MOE Frontier Science Center for Brain Science & Brain-Machine Integration, Zhejiang University. We thank Dr. Liang Ma for his help to synthesize compound 21 used in this study. We also thank staffs of the Center of Cryo-Electron Microscopy, Zhejiang University, BL14U beamline at National Center for Protein Sciences Shanghai (NCPSB) and BL14XU beamline of Spring-8. The diffraction data collection was performed at the BL41XU of Spring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI) (proposal number 2019B2705).

Author contributions
J.Q. and Y.C. designed the expression constructs, purified the ghrelin receptor complexes, and prepared the samples for data collection toward the structures. J.Q., C.M., and D.D.K. performed the cryo-EM experiments and prepared the cryo-EM grids, collected cryo-EM images, and performed map calculations. Q.M. built and refined the cryo-EM structure. Y.C. and Z.X. developed the ghrelin receptor construct, purification, and crystallization. K.H. and Z.X. collected diffraction data. Z.X. solved and refined the crystal structures with help of J.Z., Q.J., S.Y.J., H.Z., and C.W. designed the cellular assays and analyzed results. Y.M., Y.Z., and Z.S. planned and coordinated the project, W.Y., Y.Z., and Z.S. supervised the overall project, and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.