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# Biased signaling: A viable strategy to drug ghrelin receptors for the treatment of obesity

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ABSTRACT

Obesity is a global burden and a chronic ailment with damaging overall health effects. Ghrelin, an octanoylated 28 amino acid peptide hormone, is secreted from the oxyntic mucosa of the stomach. Ghrelin acts on regions of the hypothalamus to regulate feeding behavior and glucose homeostasis through its G protein-coupled receptor. Recently, several central pathways modulating the metabolic actions of ghrelin have been reported. While these signaling pathways can be inhibited or activated by antagonists or agonists, they can also be discriminatingly activated in a "biased" response to impart different degrees of activation in distinct pathways downstream of the receptor. Here, we review recent ghrelin biased signaling findings as well as characteristics of ghrelin hormone and its receptors pertinent for biased signaling. We then evaluate the feasibility for ghrelin receptor biased signaling as a strategy for the development of effective pharmacotherapy in obesity treatment.

## 1. Introduction

Ghrelin hormone, identified for stimulating growth-hormone secretion has also been recognized as an appetite-stimulating peptide, stimulating food reward pathways in the brain and influencing food preferences, especially high fat/sugar diets [1], indicating ghrelin's role in determining hedonic eating behavior [2]. Easy access to palatable food is one potential contributing factor to the prevalence of obesity, a chronic ailment with damaging overall health effects [3]. The only effective treatment in achieving long-term weight loss over and above lifestyle and diet therapy is bariatric surgery [4]. This surgical procedure involves excising ghrelin hormone producing regions of the stomach. Follow-up studies show behavioral changes, *i.e.* patient's preference for food alters after undergoing bariatric surgery, such that their choice for a high-fat diet becomes far less than it was before surgery. Although the underlying molecular mechanisms for improvement in weight loss and the changes in behaviors are not clear, ghrelin has been associated with both the improvement in weight loss and aversion for a high-fat-diet. Thus, since its discovery in 1999, ghrelin has been studied for the development of a novel drug for treating obesity [5]. However, to date there are no drugs that target the ghrelin receptor that are effective for inducing weight loss.

Here, we review characteristics of ghrelin structure, its synthesis and ghrelin receptor biased signaling and assess whether the quest for signaling bias is a viable strategy for the development of pharmacological ghrelin agonists that might facilitate a targeted and more effective approach to weight loss in obesity treatment.

## 2. Ghrelin structures

The human ghrelin gene consists of 5 exons, and it encodes the 117-amino acid, preproghrelin. Exon 1 and 2 encode the functional processed ghrelin 28-amino acid peptide. Subsequent post-translational processing of ghrelin includes the addition of an octanoyl fatty acid moiety to serine at the 3rd position of the mature 28-amino acid peptide hormone (Fig. 1). This unusual modification among peptide hormones is mediated by the enzyme Ghrelin *O*-acetyltransferase.

The Serine-3 residue is conserved across species (Fig. 2) (GenBank accession number: Human NP_057446; Chimp XP_016795941; Dog NP_001003052.1; Cow XP_005223078; Mouse NP_001366058; Rat NP_067701; Frog NP_001267573). The lack of acetylation leads to desacyl ghrelin which renders ghrelin unable to bind to its receptor, growth hormone secretagogue receptor type 1a (GHSR1a). The ratio of plasma acylated ghrelin/total ghrelin is 1:5 [6] and the receptor for desacyl ghrelin is yet to be identified. Interestingly, a mass spectrometry analysis of human plasma acyl ghrelin suggests that all ghrelin in

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circulation is acylated and that des-acyl perhaps is an artefact owing to sample handling [7]. However, there is evidence showing that des-acyl ghrelin has some biological activity [8,9]. Furthermore, chemical synthesis of ghrelin derivatives revealed that although octanoylated ghrelin is the most active form of ghrelin, modification of ghrelin's serine-3 residue by other fatty acids, for example, palmitoyl group, or by unsaturated fatty acid 3-octanoyl (C8:1) or branch fatty acid 4-methylpentanoyl also preserve ghrelin-stimulated activity. However, a reduced ghrelin activity is reported using a calcium mobility assay with shorter-chain fatty acids acylated synthetic-ghrelin at serine-3 [1,10].

Acetylation of ghrelin is essential for most of the physiological effects of ghrelin, including the activation of orexigenic hypothalamic neurons. The exact function of serine-3 acyl-modification is unclear. However, a recent structural study suggests that the modification may play a role in stabilizing the ghrelin in a specific receptor-bound confirmation [11] thus conferring an active conformation on the ghrelin receptor by binding to the hydrophobic environment of the ligand binding pocket. This is akin to how lipid G protein-coupled receptors (GPCRs) accommodate hydrophobic moieties of their lipophilic ligands [12]. In addition to the mature 28-amino acid long ghrelin, a multi-species sequence comparative analysis shows there also exists an endogenous truncated version: "minighrelin". Minighrelin has been identified in human and mice, and treatment with exogenous minighrelin induces food intake in mice in a manner similar to the full-length ghrelin [13]. Further, recently discovered peptide hormone, the liver-expressed antimicrobial peptide 2, synthesized in the liver and small intestine has been reported as an endogenous antagonist of the ghrelin receptor regulated by metabolic status [14,15]. Similarly, the melanocortin receptor accessory protein 2, expressed in the paraventricular nucleus in the hypothalamus has been shown to interact with the ghrelin receptor, enhance ghrelin-induced orexigenic signaling [16], and regulate both biased signaling as well as constitutive activity of the ghrelin receptor [17].

## 3. Ghrelin secretion system: GPCRs and 7TM receptors

Ghrelin is synthesized primarily by the enteroendocrine "P/D1" cells in human and "X/A-like" cells in rat of the oxyntic glands of the gastric fundus of the stomach [18,19]. Since this initial observation, several subsequent reports described the tissue distribution profile for ghrelin secreting cells and ghrelin action [20]. However, the underlying molecular mechanisms and pathways necessary for ghrelin biosynthesis and secretion are not fully known yet. These cells are diffusely dispersed within the complex of gastric mucosa, therefore until recently direct investigations of native living "X/A-like" cells was not possible. However, this restriction has been mitigated by generating transgenic mice in whom "X/A-like" cells are labelled with a fluorescent protein [21,22]. Ghrelin synthesis in the central nervous system has also been reported; however, lack of experimental reproducibility and the apparent ghrelin low concentration have called into question whether brain-derived ghrelin has physiological relevance [23,24].

At a systemic level, for example, it is known that consistent with a role for ghrelin in enhancing appetite, plasma levels of acyl ghrelin rise during fasting, pre-prandially to anticipated meal time and return to baseline levels within the first hour after a meal. In this respect, ghrelin is almost unique among gut hormones except for Insulin-like peptide 5 which we have recently identified as a product of colonic L-cells, and demonstrated that levels were increased in calorie-restricted mice and reduced after feeding [25]. Ghrelin secreting cells in the oxyntic glands of the gastric fundus in the stomach are closed type cells that have no continuity with the lumen; thus, the stimulus triggering ghrelin secretion or inhibition could come from the basolateral side, from the lumen, or both [19].

GPCRs have been suggested to play essential roles in the control of ghrelin secretion. Traditionally, ligands for GPCRs have been characterized as agonists, which stabilize or promote conformational changes in the receptor that result in the activation of heterotrimeric G proteins and generation of second messengers, or antagonists that inhibit activation. However, over the past two decades, studies have found that ligands can bring about a unique "active" receptor conformation which stimulate only a specific subset of a given receptor's functional repertoire. In this regard, ligands could exhibit "bias" for particular G proteins. Indeed, exploration of G protein family members and seven-transmembrane spanning receptors expressed in ghrelin secreting cells have revealed several such receptors that could potentially be targeted for the development of biased agonists and antagonists that modulate ghrelin secretion. Studies in mice show that these GPCRs and transmembrane spanning receptors signal ghrelin secretion or inhibition. Interestingly, the ligand for these receptors includes nutrients (*e.g.* short-chain fatty acids), neurotransmitters (*e.g.* dopamine), hormone peptides (*e.g.* glucose-dependent gastric inhibitory polypeptide) and metabolites (*e.g.* lactate) (Table 1). Furthermore, secretion experiments show that these ligands stimulate ghrelin release in native ghrelin secreting cells, indicating a direct stimulation effect. Also, a study by Engelstoft MS identified transcripts for a number of orphan receptors in ghrelin expressing cells [21], thus the potential for discovering many more ligands that modulate plasma ghrelin levels.

## 4. Ghrelin receptors

Ghrelin signals through its receptor, a GPCR coupled protein that is expressed primarily in the hypothalamus and pituitary. It is becoming apparent that ghrelin's signaling in the central nervous system has effects on eating. For example, intravenous injections of ghrelin into human volunteers elevated their calorie consumption by 28% (an additional 1284 KJ (306 kcal in a single meal) [26].

Two types of ghrelin receptors are known: Growth Hormone Secretagogue Receptor (GHSR)-1a and GHSR1b. The human GHSR1a gene encodes seven transmembrane segments and consists of 366 amino acid residues. The GHSR1a receptor, structurally, belongs to a member of the beta-branch in the class A GPCRs. Among the seven transmembrane domains, the 2nd and 3rd are considered the ghrelin activation domains with the amino-terminus located on the extracellular side and the carboxy-terminus on the intracellular side [1,27]. Recent results of mutagenesis studies suggest that the binding pocket of GHSR1a is split into two cavities, and one-half is more critical for ghrelin binding [12]. In contrast, the function of GHSR1b has been enigmatic. It is a product of alternative splicing resulting in a truncated version of GHSR1a, lacking transmembrane domains 6 and 7. Thus, ghrelin is unable to bind


**Fig. 1.** Schematic representation of the octanoylated ghrelin.
Ghrelin, acylated (green) on the third serine (blue) residue.

GHSR1b and therefore unable to mediate the signal. However, recent *in vitro* studies focusing on the changes that arise due to manipulation of the relative expression of GHSR1a and GHSR1b in the plasma membrane show that GHSR1b may modulate GHSR1a activity [28]. Furthermore, the data suggests that GHSR1b also determines GHSR1a capacity to dimerize and form complexes with other GPCRs and to modulate ghrelin-induced signaling [28].

## 5. Ghrelin receptor and heterodimerizations

The idea that ligand binding to an allosteric site can induce receptor conformational changes and that protein interactions could have a similar effect on the receptor's conformation has been recognized for some time. However, additional layers of complexity in ghrelin signaling comes from at least two aspects- 1) GHSR1a has an unusually high constitutive signaling activity 2) GHSR1a can form heterodimers with other GPCRs (Fig. 3, Table 2). GPCR heteromers, where one protein is likely to induce changes in the active conformation of its interacting partner, could lead to novel pharmacological properties of the receptor [29,30]. Similarly, it is also thought that GHSR1a has the potential to form heterodimers with several GPCRs and has the capacity to modulate feeding. Indeed, studies show GHSR1a dimerization partners occur under normal biological conditions with significant evidence of activity resulting from a dimerized complex [31]. Furthermore, GHSR1a appears to have signaling activities even in the absence of its natural ligand, ghrelin, and potentially mediates physiological effects. This GHSR1a activity in the absence of ghrelin is thought to result from ghrelin receptor dimerization (Table 2) [32].

## 6. Ghrelin, orexigenic agent

The empty stomach "hunger pangs" inform the brain to engage in appetitive behavior. The role of ghrelin in this respect is clear, but as to how exactly this information is communicated to the brain remains contentious. Several data show that gut-CNS communication is through afferent fibres of the vagus nerve that project from the gut to the nucleus of the solitary tract in the brain. Thus, vagal afferent sensory signaling is necessary for ghrelin stimulated effects [33]. In support of this hypothesis, for example, a recent functional analysis using RNA-interference to knockdown GHSR revealed that GHSR1a of gut-innervating vagal afferent neurons is necessary for ghrelin signaling in rat. Also, GHSR expression in the nodose ganglion is reduced with targeted RNA-interference [34].

However, there also exists an alternative view that purports that gut vagal afferents are not necessary for the ghrelin mediated eating-stimulatory effect [35]. Equally elegant work by Egerod KL in which comprehensive profiling of GPCRs in vagal afferent sensory neurons was carried out using Nav1.8 Cre-DTA mice revealed that GHSR expression was below the detection limit [36]. This supports the notion that hypothalamic neurons are sufficient for ghrelin's orexigenic effect [36]. The apparent controversy and confusion about the relative importance of whether stomach-derived ghrelin through the circulation, or afferent vagal neurons, or both, and what is the involvement of the enteric nervous system in ghrelin mediated stomach-brain signaling remains unresolved.

Ghrelin-producing neurons are present in the hypothalamus and project to brain regions, including the arcuate nucleus [18]. Within the brain, the strongest expression of the ghrelin receptor transcript is detected in several hypothalamic nuclei, including the arcuate nucleus [37]. Moreover, two classes of peptide-producing neurons in the arcuate nucleus of the hypothalamus are known for the regulation of food intake: anorexigenic neurons that express pro-opiomelanocortin (POMC) and the orexigenic neurons that express neuropeptide Y (NPY) and agouti-related peptide (AgRP) [38,39]. Ghrelin receptor mRNA is expressed by the majority (~94%) of NPY expressing neurons in the arcuate nucleus [38], an observation consistent with a recent study using single-cell RNA sequencing [39]; whereas, the expression in POMC neurons is limited to ~9% [38]. Increased ghrelin level due to fasting appears to significantly enhance NPY/AgRP neuronal activities as determined by monitoring fluorescent protein-tagged NPY (NPY-GFP) expressing neurons in mice [40], and to increase ghrelin receptor signaling components downstream of ghrelin receptor in AgRP neurons [41]. Ghrelin induced activities in NPY-GFP labelled neurons is reversed upon refeeding; however, the changes in POMC neuronal activities were insignificant [40] and the ghrelin receptor expression was almost absent [41]. Nonetheless, POMC neuron activity is modulated by synaptic input from AgRP neurons leading to the inhibition of POMC neuronal activities [42,43].

The predominant point of view is that ghrelin initiates feeding because of its direct effect on enhancing the electrical activity of AgRP neurons [44], and expression levels of the ghrelin receptor GHSR1a are upregulated in AgRP neurons after food deprivation [41]. Ghrelin-induced food intake is blunted in NPY/AgRP null mice [45,46] or when these neurons were inhibited chemically [47]. Interestingly, in the absence of NPY/AgRP neurons, ghrelin-dependent feeding behavior becomes sensitive to nutrient type (palatability), *i.e.* these transgenics fail to respond to ghrelin-stimulated food intake when put on the standard chow diet. However, ghrelin's orexigenic effect was restored when the animals were fed a high fat/high sucrose diet [48]. Taken together, POMC neurons are critical nodes in the control of body weight [39], and AgRP neurons, which are directly activated by ghrelin, play an essential role for normal feeding behavior and could be a potential target for stimulating appetite and glycemic control.

## 7. Ghrelin, motivation agent

The idea that caloric restriction (internal need state) likely interacts with environmental cues (such as the scent of food) at several levels of neuronal processing has been identified as critical to discern the underlying molecular mechanisms for specific motivated behaviors. Thus,


**Fig. 2.** Amino acids sequence alignment for the mature ghrelin peptides.

**Table 1**
Summary of GPCRs expressed in ghrelin secreting cells and their effect on ghrelin secretion in mice.

| Receptor Full name | Abbreviation | Ligand | G protein binding | Main findings and conclusion | Ref |
|---|---|---|---|---|---|
| Glucose-dependent insulinotropic polypeptide receptor | GIPR | Gastric inhibitory polypeptide (GIP) | Gs | In primary gastric culture, GIP stimulation increases ghrelin secretion<br>In MGN3-1 cell culture, GIP treatment did not show changes in ghrelin levels | [21]<br>[22] |
| Taste receptor type 2 | TAS2R | Bitter taste-receptor agonists | Ggustducin, Gtransducin, Gi | Immunohistochemistry show colocalization of octanylated ghrelin with gustatory G proteins, α-gustducin and α-transducin<br>Administration of bitter taste-receptor agonists increase plasma ghrelin level and food intake. Those effects were blunted in α-gustducin knockout mice | [101] |
| Oxytocin receptor | OXTR | Oxytocin | Gq/11 | In primary gastric culture, oxytocin did not stimulate ghrelin secretion<br>In MGN3-1 cell culture, oxytocin stimulation increases ghrelin secretion | [21]<br>[102] |
| Dopamine receptors | DRD1a and DRD2 | Dopamine | Gi/o, Gs | In primary gastric culture, Dopamine did not stimulate ghrelin secretion<br>In MGN3-1 cell culture, the DRD1a agonist (fenoldopam) stimulated ghrelin secretion, whereas the DRD2 agonist (bromocriptine) did not. Dopamine stimulation also increases ghrelin secretion in MGN3-1 cells | [21]<br>[102] |
| Secretin receptor | SCTR | Secretin | Gs | In primary gastric culture, secretin stimulation increases ghrelin secretion<br>In MGN3-1 cell culture, secretin stimulation increased ghrelin secretion | [21] |
| β1-adrenergic receptor | ADRB1 | Epinephrine, norepinephrine and isoproterenol | Gs | In primary gastric culture, isoproterenol stimulated ghrelin secretion<br>In MGN3-1 cell culture, epinephrine, norepinephrine and isoproterenol stimulation increased ghrelin secretion | [21]<br>[22] |
| Prostaglandin E receptor 4 | EP4 | Prostaglandin E2 (PGE2) | Gs | In primary gastric culture, EP4 and ghrelin colocalized, but PGE2 treatment did not stimulate ghrelin secretion<br>In MGN3-1 cell culture, PGE2 stimulation increased ghrelin secretion. | [22] |
| Somatostatin receptor | SSTRs (1,2,3) | Somatostatin | Gi | In primary gastric culture, somatostatin inhibited ghrelin secretion<br>In MGN3-1 cell culture, somatostatin inhibited ghrelin secretion | [21]<br>[22] |
| Melanocortin 4 receptor | MC4R | MC4 | Gs | In primary gastric culture, MC4 stimulation increases ghrelin secretion<br>In MGN3-1 cell culture, MC4 stimulation increases ghrelin secretion | [21]<br>[22] |
| Calcitonin gene-related peptide receptor | CGRPR or CALCRL | Calcitonin gene-related peptide (CGRP) | Gs | In primary gastric culture, CGRP stimulation increases ghrelin secretion<br>In MGN3-1 cell culture, no stimulation in ghrelin secretion was observed | [21]<br>[22] |
| G protein-coupled receptor 41 | GPR41 or FFAR3 | Short-chain fatty acids | Gi | In primary gastric culture, short-chain fatty acids inhibit ghrelin secretion | [21] |
| G protein-coupled receptor 43 | GPR43 or FFAR2 | Short-chain fatty acids | Gi/o, Gq/11 | In primary gastric culture, short-chain fatty acids inhibit ghrelin secretion<br>In MGN3-1 cell culture, short-chain fatty acids stimulation showed no effect in ghrelin secretion | [21]<br>[22] |
| G protein-coupled receptor 81 | GPR81 | Lactate | Gi | In primary gastric culture, lactate stimulation inhibits ghrelin secretion<br>In MGN3-1 cell culture, lactate stimulation inhibits ghrelin secretion | [21]<br>[22] |
| G protein-coupled receptor 120 | GPR120 or FFAR4 | Long-chain fatty acids | Gi, Gq | In primary gastric culture, long-chain fatty acids inhibit ghrelin secretion<br>GPR120 is expressed on the membrane of ghrelin-expressing GFP (Ghr-GFP) cells. Long-chain fatty acids inhibit ghrelin secretion in Ghr-GFP cells.<br>In MGN3-1 cell culture, long-chain fatty acids inhibit ghrelin secretion | [21]<br>[20]<br>[22] |
| G protein-coupled receptor 142 | GPR142 | Tryptophan | Gi, Gq | In MGN3-1 cell culture, tryptophan stimulation increases ghrelin secretion | [22] |
| Calcium sensing receptor | CaSR | Aromatic amino acids/calcium ions | Gi, Gq | In primary gastric culture, the calcimimetic compound R-568 can both inhibit and stimulate ghrelin secretion dependent upon the concentration of calcium | [21] |
| Vasopressin V1b receptor | V1bR | Vasopressin | Gq | In primary gastric culture, vasopressin stimulated ghrelin secretion | [21] |

for example, both prior caloric deficit and proximity to tasty foods stimulate the appetite. The astonishing differences in these two causes of appetite have, at times, led to the view that they are mutually exclusive: hedonic *versus* homeostatic eating. However, in addition to the differences, they also share commonalities and interrelatedness. In this respect, ghrelin has been implicated in both [49]. For example, in patients with Prader-Willi syndrome, food condition stimulus enhanced nonspecific desire of Pavlovian-instrumental transfer to a greater extent than in control subjects. Circulating ghrelin levels in these patients is high and they are hyperphagic. This observation has led to the hypothesis that perhaps these patients are unusually vulnerable to overeating because of heightened "wanting" of food [50,51]. Indeed, a recent human neuroimaging study using fMRI of the brain activity following intravenous ghrelin injection suggests that the action of ghrelin is food-odour specific and heightens food odour conditioning [52], thus making us more vulnerable to tasty food smells and excess calorie intake. In this experiment, the areas controlled by ghrelin were very diverse, including the ventral tegmental area, ventromedial prefrontal cortex (vmPFC) and ventral striatum (vSTR). The exact mechanism underlying the selective effects of ghrelin on food stimulus is not known, and could not be addressed using the system due to the limitation in the resolution of fMRI [52].

Intriguingly, despite several supportive evidences for ghrelin's role as a signal for initiating appetitive and maintaining consummatory behaviors, ghrelin, GHSR1a and ghrelin-secreting cells are dispensable for feeding. In studies in which the gene for ghrelin or GHSR1a are deleted or in animals in which the ghrelin-secreting cells are ablated, no detectable difference on feeding was identified. These findings have led to the propositions that perhaps the primary function of ghrelin is not in controlling feeding, but it might be to resist hypoglycemia [49], a suggestion consistent with the findings showing that disruption of ghrelin causes extensive hypoglycemia in starved mice [53].

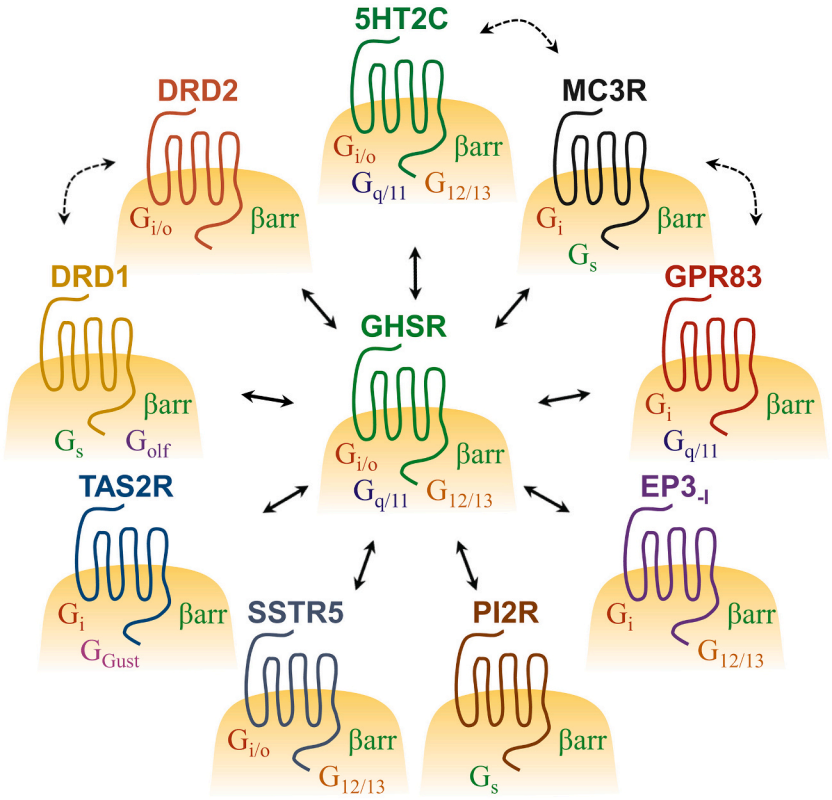


**Fig. 3.** Signaling pathways of GPCRs reported to form heterodimers with the ghrelin receptor.
Ghrelin receptors have been shown to form heterodimers with multiple GPCRs. This scheme represent the different signaling pathways activated by those receptors. Straight arrows indicate interactions between GHSR and multiple GPCRs to for heterodimers. Moreover, some of the GPCRs have been reported to constitute heteromers among themselves (dotted arrows).

## 8. Biased agonism at the ghrelin receptor

The ghrelin receptor GHSR1a signals through a variety of G protein-dependent and -independent pathways. Once activated, those pathways initiate numerous intracellular signaling cascades to mediate physiological effects including appetite, food intake [54], fat accumulation [55], and maintenance of energy homeostasis [56], making GHSR1a an attractive target for the development of anti-obesity treatments. However, the ghrelin receptor is also involved in inducing addiction [57,58], growth hormone secretion [54], and gastric emptying [59]. Thus, ligands targeting that receptor to activate or block a specific physiological effect may also induce undesirable side effects, which limit their clinical use. Biased agonism has been proposed as an approach to tackle this issue. This concept, also called "functional selectivity" or "biased signaling", refers to the ability of a ligand to selectively modulate a limited fraction of signaling pathways among all those controlled by a receptor [60,61]. Hence, biased ligands may allow a more targeted modulation of the desired physiological functions mediated by the activated receptors while avoiding those associated with side effects [62]. Also, studies have shown that introducing mutations to the seven transmembrane core could also bias the receptor to favor one signaling pathway over another. Thus, understanding the mechanisms of biased signaling at the GHSR1a, using either specific ligands or mutations in the receptor, may lead to the development of more selective drugs to treat obesity with minimal adverse effects.

## 9. GHSR biased ligands

In the pharmacological concept of biased signaling, each agonist is thought to stabilize the receptor in a specific active conformation among several others that the receptor could adopt. This variety in signaling conformations would also indicate that the agonist-induced activation of the receptor is not uniform but rather biased toward specific signaling pathways over others. In other words, the conformational information generated by different ligands is transmitted downstream to specific signaling proteins to finally mediate distinct physiological responses.

This concept was verified for the GHSR1a. More precisely, data generated using purified ghrelin receptors in lipid discs showed that GHSR1a was able to adopt distinct conformations following its stimulation by ligands that differ in their efficacies in activating $G\alpha_q$ subunits or recruiting β-arrestin proteins [63]. A similar set of experiments also showed that unlike the conformation stabilized by ghrelin, the endogenous ligand of GHSR1a, the partial agonist JMV3018 stabilizes GHSR1a in a distinct conformation that was unable to activate $G\alpha_{i/o}$ or recruit β-arrestin in lipid dishes [64]. Together, these findings indicate that GHSR1a can adopt distinct conformational features when activated by different ligands.

The GHSR1a ghrelin receptor belongs to a family of G protein-coupled receptors that signals through $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{13}$ as well as β-arrestin scaffold proteins [65]. Interestingly, it was recently shown that some of those signaling pathways were involved in mediating distinct ghrelin physiological responses [65–69]. Therefore, biasing GHSR1a signaling with specific ligands could be adopted as a strategy for the development of anti-obesity drugs that could stimulate signaling pathways involved in reducing food intake and fat accumulation, while at the same time, avoiding other pathways that modulate mood, gastric motility, and the development of seizures.

A recent study characterized the physiological importance of activating $G\alpha_{q/11}$ or β-arrestin signaling pathways on food intake and gastric emptying using biased GHSR ligands for those pathways [66]. In this study, the appetite-modulatory effect of ligands was assessed by monitoring the hypothalamic effects to avoid influences from gastric emptying, for which a delay may translate into nausea and lead to reduced food intake [67]. Results showed that appetite regulation is mainly mediated by $G\alpha_{q/11}$ activation, while other downstream pathways engaged by GHSR, such as $G\alpha_{i/o}$, $G\alpha_{13}$, or β-arrestin might be responsible for promoting the gastric emptying effect. The role of $G\alpha_q$ and $G\alpha_{11}$ in the ghrelin-induced food intake was confirmed in mice

**Table 2**
Effect of heterodimerization on GHSR1a and GPCRs functions [101–109].

| Receptor full name | Receptor Pair for GHSR | System | Main findings and conclusion | Ref |
|---|---|---|---|---|
| Serotonin 2 receptor | 5HT2C | *In vitro* | Data sugget that heterodimerization may attenuate ghrelin-stimulated signaling. | [31] |
| G-protein coupled receptor 83 | GPR83 | *In vivo* *In vitro* | Gpr83 colocalized with GHSR1a and AgRP in the arcuate nucleus of the mouse hypothalamus. Feeding behavior was attenuated in response to changes in Gpr83 expression, suggesting a direct link with ghrelin receptor activity in the brain. Heterodimerization of Gpr83 with GHSR1a reduced ghrelin receptor activity. | [103] |
| Melanocortin 3 Receptor | MC3R | *In vivo* *In vitro* | MC3R colocalized with GHSR in the arcuate nucleus of the mouse hypothalamus. Both constitutive and ligand-induced activities of GHSR1a were impaired by receptor heterodimerization. | [104] |
| E-type prostanoid receptor | EP3-I | *In vitro* | Heteromerization was detected in HEK293 transfected cells. In these cells, the total expression level and constitutive activity of GHSR1 were reduced. In addition, the constitutive internalization of GHSR1 was increased in the presence of EP3-I. The physiological relevance of these observations is still not known. | [105] |
| Prostacyclin receptor | PI2R | *In vivo* | *In situ* hybridization show expression of prostacyclin PGI2 receptor in the ghrelin-producing cell of the rat stomach. Prostacyclin decreased circulating ghrelin that was induced by acute inflammation. | [106] |
| Somatostatin receptor | SSTR5 | *In vitro* | Data suggest that ghrelin suppression of glucose-stimulated insulin secretion is a consequence of the heterodimer formation. Heterodimer formation depends on the ratio of ghrelin to SST. | [107] |
| Taste receptor | TAS2R | *In vivo* | Ghrelin secreting cells co-express α-gustducin (bitter taste chemosensor) and α-transducin (bitter and sweet taste chemosensor), which are essential components for TAS2R functioning. Treatment with TAS2R agonists increases plasma ghrelin level in WT but not α-gustducin knockout mice. Those changes in plasma ghrelin levels might be due to TAS2R dimerization with GHSR1a. | [101] |
| Dopamine receptor D1 | DRD1 | *In vivo* *In vitro* *In vivo* | DRD1 coexpressed with GHSR in mouse cortex, hippocampus, substantia nigra, midbrain, and ventral tegmental areas. GHSR activation by ghrelin amplifies DRD1 signaling by a mechanism associated with heterodimer formation. Data suggest that heterodimerization may attenuate GHSR1a mediated signaling. | [108] [31] |
| Dopamine receptor D2 | DRD2 | *In vivo* *In vitro* *and* *In vivo* | DRD2 colocalized with GHSR1a in mouse striatum, hippocampus, and hypothalamus. The heterodimer formation modifies DRD2 signal transduction. DRD2 activation by cabergoline in the heterodimer induces anorexigenic effect upon interactions with GHSR1a. Anorexia induced by cabergoline was blocked by a selective GHSR1a antagonist. | [109] |

models lacking both subunits in AgRP neurons of the hypothalamus [66], the main neuronal population targeted by ghrelin [70]. Based on these findings, it can be postulated that a ligand capable of directing the GHSR stimuli toward pathways downstream of $G\alpha_{i/o}$, $G\alpha_{13}$, or β-arrestin while inducing inverse, or at least less agonistic activity on the $G\alpha_{q/11}$ signaling could be considered as an effective treatment for obesity with reduced side effects such as constipation and nausea (Fig. 4).

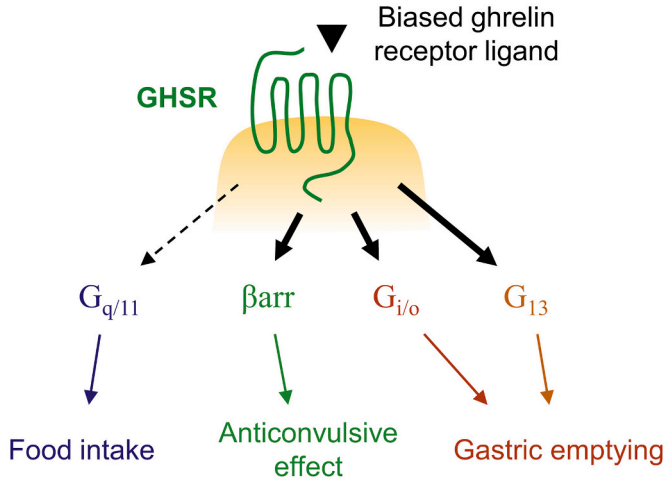


**Fig. 4.** Schematic representation of an optimal biased ghrelin receptor ligand for obesity treatment.
Progress in the development of biased agonists for the ghrelin receptor has allocated desired and undesired effects to specific signaling pathways. A biased ligand that would favor activation of the $G\alpha_{i/o}$, $G\alpha_{13}$ and β-arrestin pathways while simultaneously inducing less agonistic activity on the $G\alpha_{q/11}$ signaling could be an effective treatment for obesity that is expected to produce anorexigenic and anticonvulsive effects while avoiding undesired effects such as constipation and nausea.

However, it is worth mentioning that in contrast with the previous theory, some GHSR1a ligands displaying partial agonist efficacy toward $G\alpha_q$ such as JMV2959 [65] have showed effectiveness in decreasing food intake [68]. This behavior could be attributed to the ability of those ligands to delay gastric emptying associated with nausea as described above, or their capacity to stabilize the receptor in specific conformations that allow activation of other downstream signaling pathways responsible for reducing food intake.

Beside mechanisms involved in food intake and gastric emptying, signaling pathways responsible for mediating the anticonvulsive effects of some GHSR ligands were also investigated *in vivo* using multiple seizure or epilepsy models [71–74]. For instance, recent data showed that mice treated with YIL781 suffered from longer and more severe seizures compared to saline-treated controls, whereas treating mice with JMV1843 induced fewer and less severe seizures. While both GHSR agonists activated $G\alpha_q$ and $G\alpha_{12}$ pathways [65,66,75], only JMV1843 recruited β-arrestin proteins to the receptor [65,66]. Together, these results indicate that $G\alpha_q$ and $G\alpha_{12}$ signaling pathways are not involved in mediating the anticonvulsant action of JMV18430 and suggest a possible involvement of the β-arrestin signaling in inducing the anticonvulsive effect (Fig. 4).

Furthermore, several studies performed in animal models also investigated the effect of multiple GHSR ligands on GH secretion, which has been reported to have direct lipolytic effects on the adipose tissue [76,77]. Therefore, GHSR-mediated activation of GH secretion could be beneficial for weight loss. Interestingly, results also showed a complete dissociation between the effect of the tested GHSR ligands on GH release and their effects on food intake and body weight control. Specifically, ligands such as GSK1614343 and BIM-28163 behaved *in vivo* as antagonists for ghrelin-induced GH secretion but as agonists in stimulating food intake [78–80]. In the same line, a specific inhibitor of the intracellular enzyme SIRT1 showed an ability to block the ghrelin-induced food intake without affecting GH secretion [81]. Together, these results demonstrate a clear inability of GHSR ligands to induce simultaneously GH secretion and food intake, which suggests that GHSR-induced modulation of both effects is controlled by distinct intracellular signaling pathways. A similar dissociation between both physiological effects of the ghrelin system was also observed using small molecule GHSR ligands such as JMV2951 and JMV2894. While both ligands stimulated GH secretion when administrated alone, only JMV2951 showed an ability to induce food intake stimulation [68]. In addition, it should be noted that different ligands acting as GHSR antagonists in the *in vitro* calcium mobilization assay were able to induce distinct effects on GH release *in vivo*. Thus, for instance, while GSK1614343 and BIM28163 acted as antagonists for ghrelin-induced GH release [79,80], another antagonist for calcium release such as JMV2959 [82] was unable to inhibit GH secretion [68]. Therefore, screening for ligands that behave as antagonists in calcium release assays might not help in predicting their effect on GH secretion.

Moreover, it was shown that structural modifications in $_D$-Trp-Phe-$_D$-Trp (wFw)-containing peptides, acting as ligands for the ghrelin receptor, were able to induce changes in ligands' efficacy. For example, extending the ligand structure N-terminally with alanine, a non-polar residue, allowed this new peptide to act as a partial agonist for the ghrelin receptor, whereas, extending the ligand at the same position with lysine, a positively charged residue, resulted in generating an equally potent inverse agonist peptide [83,84]. This notion of balance in the ligand efficacy between agonism and inverse agonism was substantiated by demonstrating that the efficacy of those N-terminally extended wFw-containing peptides could be swapped to the opposite type by mutating residues located in the main ligand-binding pocket of the ghrelin receptor [84]. Data also allowed identification of wFw-Isn-NH2, a biased ligand for the ghrelin receptor that was able to stimulate inositol phosphate accumulation but not the SRE-mediated transcriptional activity, which suggests that this compound mediates signaling through $G\alpha_q$ over the $G\alpha_{12/13}$-related pathway [85]. Taken together, these observations provide proof-of-concept studies into designing novel anti-obesity drugs that could target with more selectivity the GHSR-mediated pathways involved in food intake while avoiding those related to side effects.

## 10. Biased ghrelin receptors

In addition to the ligand-induced conformational changes that could favor one signaling pathway over another, structural changes in the receptor by amino acid substitutions may also allow the receptor to selectively activate a selection of specific signaling pathways. Also, introducing mutations in specific domains of the receptor could help in characterizing the function of each of those areas in transmitting the conformational information to downstream signaling proteins. Thus, it is critical to identify the structural changes in the ghrelin receptor domains that could direct receptor stimulation toward pathways that are therapeutically beneficial while avoiding those associated with undesired effects.

Indeed, the effect of substituting residues from the transmembrane helices III and IV of the ghrelin receptor on inositol phosphate (IP) and serum-responsive element (SRE) activation pathways was characterized [84]. Results showed that mutations such as F119S or I178A were able to change the efficacy of the ghrelin receptor ligand (KwFwLL) from an inverse agonist in respect to IP accumulation when acting on the wild type receptor to a partial agonist on the mutated receptor. In contrast, the S123A mutation swapped the efficacy of the AwFwLL ligand in both IP and SRE signaling pathways from agonism on the wild type receptor into inverse agonism on the mutated receptor. This latter mutation was also able to convert the low potency inverse agonist KwFwLL in the SRE activation assay into a high potency inverse agonist, whereas the F119A mutation induced changes in the ghrelin receptor conformation that allowed KwFwLL to act as a partial agonist [84]. Together, this data demonstrates that introducing amino acid substitutions in the core of the ghrelin receptor could swap the efficacy of ligands from agonism into inverse agonism or the other way around. Results also indicated that making structural changes in the ghrelin receptor by introducing mutations in its transmembrane domains could allow specific ligands to stabilize the receptor into specific conformations that favor the activation of the $G\alpha_q$ or the $G\alpha_{12/13}$-mediated signaling pathway.

Besides introducing mutations that could favor activation of a specific G protein signaling over another, functionally biased ghrelin receptors that could distinguish between G protein and arrestin-mediated signaling have also been described [86,87]. A recent study demonstrated that introducing a single point mutation at the second intracellular loop (ICL2) of the ghrelin receptor was enough to produce GHSR1a mutants with opposite signaling-biased phenotypes [86]. More precisely, substitution of the proline 148 (P148A) residue generated a GHSR1a mutant that had reduced β-arrestin affinity and was biased toward the $G\alpha_{q/11}$ signaling. However, mutating the neighboring leucine 149 (L149G) residue selectively abolished signaling to the $G\alpha_{q/11}$ pathway, generating a complete biased GHSR1a mutant toward the β-arrestin signaling pathway [86,87]. These findings together with spectroscopic [88] and crystal structure data [89] suggest that G protein and β-arrestin cannot simultaneously engage ICL2 residues, which supports a role for conformation-dependent signaling bias at the ghrelin receptor and makes GHSR1a an effective prototype for decoding biased agonism at GPCRs.

Interestingly, it was also shown that substituting the non-polar and neutral residue (alanine) at position 204, located in the second extracellular loop, with the polar and charged amino acid (glutamate) resulted in a complete loss of the constitutive activity of the ghrelin receptor [90]. This ghrelin receptor A204E mutant is a naturally occurring ghrelin receptor variant that was previously reported in patients with a short stature phenotype [91] and found in a young obese patient [92]. A similar loss of a constitutive activity mutation has been described for the melanocortin-4 receptor (MC4R), which was also

shown to be associated with the development of human obesity [93]. The ghrelin receptor A204E mutant was highly expressed at the cell surface and showed similarity to the wild-type receptor in inducing the stimulatory effect of ghrelin on the $G\alpha_q$-mediated IP accumulation and the $G\alpha_{12/13}$-mediated SRE gene gene transcriptional activation pathway [90]. However, the A204E mutant was completely unable to recruit β-arrestin to its vicinity following ghrelin stimulation. This data reveals the critical role of the second extracellular loop in mediating the constitutive activity of the ghrelin receptor and indicate that introducing a charged residue at position 204 could facilitate structural changes in the ghrelin receptor that allow ghrelin to act as a biased agonist that favors the activation of $G\alpha_q$ and $G\alpha_{12/13}$ over the β-arrestin mobilization pathway. The above findings indicate that ligands capable of stabilizing the receptor in a conformation that allows activation of specific G proteins or activation of G protein signaling without inducing β-arrestin recruitment or *vice versa* can provide a blueprint for designing new generations of more selective therapies.

With regard to the above mentioned, it should also be noted that receptors with a broad range of interaction affinities with β-arrestin proteins support the idea that the receptor trafficking information is encoded by different phosphorylation sites positioned either individually or in clusters at the receptor C-terminal tail [94–96]. These sites can spatially coordinate the constitutive and agonist-induced activity of the receptor toward arrestin proteins. Therefore, it would be important to decipher the contribution and interplay of the different receptor phosphorylation sites in mediating β-arrestin interaction, trafficking, and signaling. Mutagenesis experiments demonstrated that phosphorylation sites at the GHSR1a C-terminal tail and the ICL2 core components act in a concerted fashion to regulate GHSR1a/β-arrestin-2 interaction kinetics. While the C-terminal tail was crucial for an initial β-arrestin recruitment, the proximal segment of ICL2 was necessary to maintain a stable and sustained β-arrestin-2 interaction with the ghrelin receptor [86]. Thus, ICL2 and the C-terminal tail determinants orchestrate β-arrestin recruitment and β-arrestin/receptor complex stability, and their interplay could be sufficient to encode a broad repertoire of GHSR1a trafficking fates [86], confirming their key role in regulating a large fraction of the β-arrestin signaling bias. Consequently, identifying the different factors that could affect ICL2 and C-terminal tail functions in regulating β-arrestin interactions and affecting the receptor fate determination should help improving ghrelin receptor expression at the cell surface and facilitate the development of more efficacious therapeutics.

## 11. Challenges in the clinical development of GHSR1a-targeted pharmacotherapy

Ghrelin's important role in controlling appetite is supported by various lines of evidence from studies in rodents and humans. However, there are also studies showing that ghrelin or ghrelin receptors do not affect feeding [49]. This confusion is partly due to technical limitations but also owing to the complexity in ghrelin receptor signaling. Some of these problems are being resolved. For example, the generation of transgenics where ghrelin secreting cells and ghrelin receptor-expressing cells are tagged with fluorescent protein have been a significant development to this field of research, as they facilitate direct investigation of ghrelin receptor biased signaling in native cells, tissues, and whole-organisms [21,22]. Without such tools, biased signaling studies using overexpressed receptors and effectors in cell lines bring into question their relevance to the *in vivo state.* Nonetheless, it is important to note that a simple *in vitro* bias assay is a cost-efficient way of choosing optimal molecules for identifying candidate agonists to advance from a screen to more complex assays. Furthermore, given the crucial insight regarding ghrelin actions, more improved disease models are needed to study ghrelin receptor biased signaling in disease states and its tissue-specific context, where the relative stoichiometry or components and sensitivities of cells may change owing to the pathology. For instance, a recent study showed that obesity induces transcription modification in the neurons of the lateral hypothalamus, a region known to orchestrate feeding [97] as well as for ghrelin mediated hyperphagia [98].

Emerging findings with a detailed mechanism for biased signaling suggest the potential for the rational design of biased ligands [99]. However, even when therapeutic ghrelin receptor biased signaling is identified, other signaling pathways would likely be activated simultaneously along with the desired outcome. This is a likely scenario especially for ghrelin since it acts in the brain where variable co-expression of receptors and interconnections between co-dependent systems and varying endogenous physiological molecules exists. While this has not yet been explicitly demonstrated for the ghrelin system, it has been for bone metabolism studies in mice, where rationally designed biased parathyroid hormone receptor agonist, [D-Trp$^{12}$,Tyr$^{34}$]-bPTH(7–34) resulted in a completely different transcriptomic profile and bone remodeling to the one produced by the natural agonist. And this discrepancy between the natural and the designer bias agonist could not have been foreseen by its *in vitro* bias profile [100]. The chasm to the extrapolations of these studies to human is yet another complexity that will need addressing. However, despite the potential drawback and challenges, the information available to date strongly support the concept of biased signaling as a credible strategy for the development of an effective GHSR1a agonist.

## 12. Concluding remarks and perspectives

Research into the regulation of body weight and food intake has seen a dramatic increase in the last ~30 years in response to the obesity epidemic. Bariatric surgery is the only effective treatment for achieving long-term weight loss. Changes in ghrelin, as well as other gut hormone secretion profiles, are thought to contribute significantly to the efficacy of the surgery. In this respect, the pharmacological modulation of ghrelin mediated effects on appetite and behaviors as a potential pharmacological replacement of bariatric procedures is a strategy that has been sought intensively. However, considering the limited success in the development of effective GHSR1a-specific pharmacotherapy so far, the rationale for thinking that GHSR1a biased signaling as a novel mechanism to achieve functional selectivity and complement existing approaches is plausible and would represent a major advance. For instance, ghrelin receptors can couple to more than one type of GPCR and also bind more than one β-arrestin subtype upon phosphorylation [94].

Therefore, the potential exists to develop and select biased signals that have therapeutic value and discard those that cause an off-target effect. Indeed, among the various ghrelin receptor candidate biased ligands, YIL781 was shown to activate $G\alpha_{q/11}$ and $G\alpha_{12}$ signaling pathways preferentially without influencing the involvement of other G protein coupling and was sufficient to decrease food intake in mice [66]. More knowledge on signaling pathways activated by ghrelin at ghrelin receptors and the pharmacological phenomenon of biased signaling augurs that effective druggable ghrelin will be developed in the future.

## Credit author statement

Karim Nagi and Abdella M Habib conceptualized the idea for the review, wrote and edited the final draft of manuscript.

## CRediT authorship contribution statement

**Karim Nagi:** Conceptualization; Writing - original draft; Writing - review & editing. **Abdella M. Habib:** Conceptualization; Writing - original draft; Writing - review & editing.

GHSR, ghrelin receptor; 5HT2C, serotonin receptor 2C; MC3R, melanocortin-3 receptor; GPR83, G protein-coupled receptor 83; $EP3_{-I}$, E-type prostanoid receptor isoform I; PI2R, prostacyclin receptor;

SSTR5, somatostatin receptor type 5; TAS2R, taste receptor type 2; DRD1, dopamine receptor D1; DRD2, dopamine receptor D2.

## Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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