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REVIEW

Functionally biased signalling properties of 7TM receptors – opportunities for drug development for the ghrelin receptor

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The ghrelin receptor is a 7 transmembrane (7TM) receptor involved in a variety of physiological functions including growth hormone secretion, increased food intake and fat accumulation as well as modulation of reward and cognitive functions. Because of its important role in metabolism and energy expenditure, the ghrelin receptor has become an important therapeutic target for drug design and the development of anti-obesity compounds. However, none of the compounds developed so far have been approved for commercial use. Interestingly, the ghrelin receptor is able to signal through several different signalling pathways including $G\alpha_q$, $G\alpha_{i/o}$, $G\alpha_{12/13}$ and arrestin recruitment. These multiple signalling pathways allow for functionally biased signalling, where one signalling pathway may be favoured over another either by selective ligands or through mutations in the receptor. In the present review, we have described how ligands and mutations in the 7TM receptor may bias the receptors to favour either one G-protein over another or to promote G-protein independent signalling pathways rather than G–protein-dependent pathways. For the ghrelin receptor, both agonist and inverse agonists have been demonstrated to signal more strongly through the $G\alpha_q$-coupled pathway than the $G\alpha_{12/13}$-coupled pathway. Similarly a ligand that promotes $G\alpha_q$ coupling over $G\alpha_i$ coupling has been described and it has been suggested that several different active conformations of the receptor may exist dependent on the properties of the agonist. Importantly, ligands with such biased signalling properties may allow the development of drugs that selectively modulate only the therapeutically relevant physiological functions, thereby decreasing the risk of side effects.

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Abbreviations

7TM, 7 transmembrane segment; AgRP, agouti-related peptide; AMPK, AMP-activated PK; AP2, adaptor-related protein complex 2; AT_{1A} receptor, angiotensin 1A receptor; AVP, arginine-vasopressin; CREB, cAMP response element binding protein; ECL, extracellular loop; FAS, fatty acid synthase; GH, growth hormone; GHS, growth hormone secretagogues; GRK, GPCR kinase; IP, inositol phosphate; IP_3, inositol tris-1,4,5-phosphate; Isn, isonipecotic acid; MC4, melanocortin 4; NPY, neuropeptide Y; SRE, serum response element; UCP2, uncoupling protein 2; VTA, ventral tegmental area; WT, wild type

Introduction to ghrelin receptor physiology

The ghrelin receptor, previously known as the growth hormone secretagogue (GHS) receptor 1a (for receptor nomenclature see Alexander *et al.*, 2011), is the receptor for the anabolic hormone ghrelin. This receptor is involved in GH secretion, appetite regulation, fat accumulation and energy expenditure. In addition it can also modulate behaviour and mood (van der Lely *et al.*, 2004; Dickson *et al.*, 2011; Figure 1).

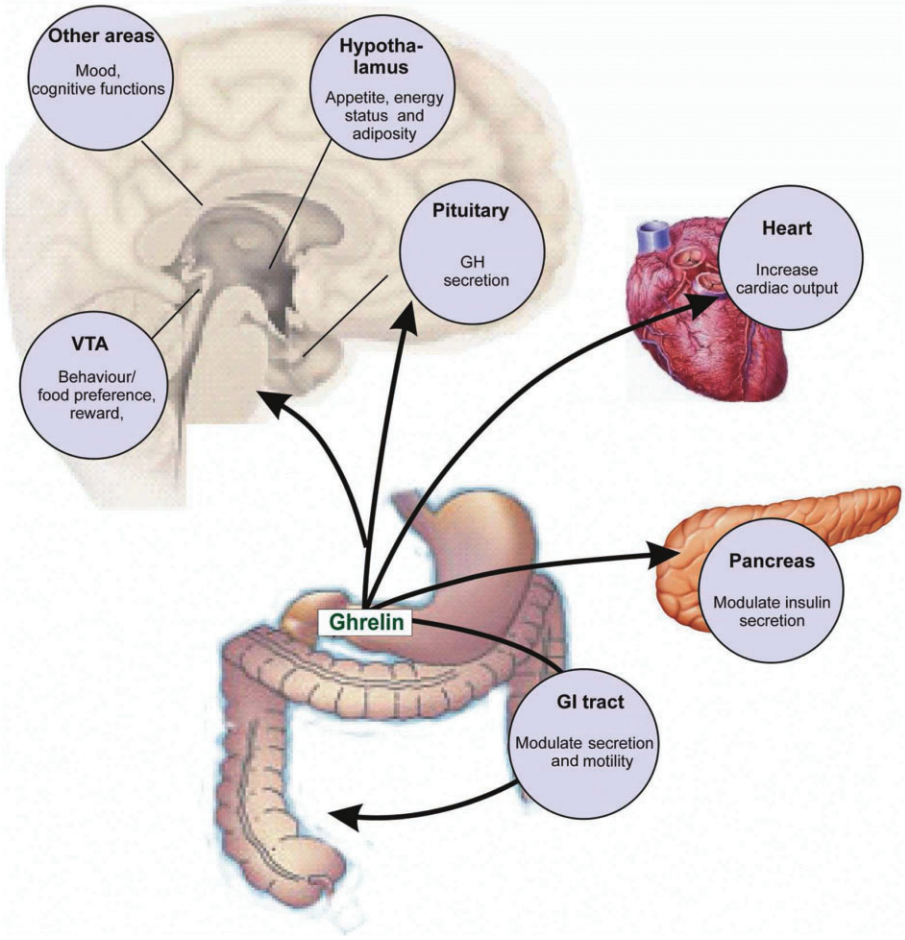

Figure 1
Cartoon of the most well-known ghrelin-mediated physiological functions.

GH

The ghrelin receptor was initially cloned as a receptor important for GH secretion. In the mid-1970s, it was found that enkephalin-like peptides had the ability to release GH from pituitary cells (Bowers, 2001), acting through a receptor different from the previously discovered GH-releasing hormone receptor, but did not affect the release of other pituitary hormones (Bowers *et al.*, 1990). The pharmaceutical industry was encouraged by this observation and established large drug discovery programmes in order to develop GH-releasing substances (Smith *et al.*, 1997). Highly potent peptide and non-peptide compounds, collectively named GHS, were developed and were important for the cloning of the receptor for these synthetic compounds (Howard *et al.*, 1996). Some of the GHS compounds were tested for their ability to induce GH secretion in a large clinical trial. However, the results were not good enough to support further clinical development with GH secretion as primary focus (Murphy *et al.*, 2001; Bach *et al.*, 2004).

Appetite and adipose tissue regulation

The ghrelin receptor is mostly associated with its expression in the neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons of the arcuate nucleus of the hypothalamus, from where it exerts its orexigenic effect (Howard *et al.*, 1996; Kojima *et al.*, 1999). Acute administration of ghrelin induces a rapid increase in food intake, while continuous ghrelin administration augments both food intake and weight gain, primarily by increasing the mass of adipose tissue (Tschop *et al.*, 2000). Plasma levels of ghrelin increase before a meal and subsequently decrease after energy intake in human subjects, suggesting ghrelin is a meal initiator or meal preparator (Cummings *et al.*, 2001). Additionally, the plasma levels of ghrelin are regulated in response to the general energy balance, as obese individuals have decreased plasma ghrelin levels compared with lean individuals (Tschop *et al.*, 2001). The orexigenic effect of ghrelin has been shown to be dependent on various other neuropeptides and signalling molecules, in addition to NPY and AgRP, acting in the hypothalamic nuclei, such as cannabinoids, melanocortin and orexin.

The intracellular signalling leading to activation of the orexigenic pathways by ghrelin has been studied in the hypothalamus; primarily in the ventromedial hypothalamus and the arcuate nucleus. Part of this process involves activation of sirtuin 1, which deacetylates p53 and forkhead box protein O1 (FoxO1) and thereby makes these two transcription

factors available for regulating gene transcription. This leads to modulation of the activity of AMP-activated PK (AMPK; Velasquez *et al.*, 2011). The phosphorylation of AMPK leads to the inactivation of acetyl-CoA carboxylase (Munday *et al.*, 1988), preventing malonyl-CoA formation, the substrate of fatty acid synthase (FAS). In addition, phosphoAMPK decreases mRNA expression levels of FAS, resulting in a decreased level of free fatty acids in the brain (Zhou *et al.*, 2001; Lopez *et al.*, 2008). The decreased malonyl-CoA levels then lead to increased activity of carnitine palmitoyl transferase 1 and changes in the mitochondrial respiration and production of ROS, a process that is dependent on the activation of uncoupling protein 2 (UCP2) (Andrews *et al.*, 2008; Lopez *et al.*, 2008). UCP2 has been shown to be highly important for not only ghrelin-induced mitochondrial proliferation but also activation of NPY/AgRP neurons and ghrelin-induced food intake (Andrews *et al.*, 2008). Ghrelin also increases the activation of the transcription factors cAMP response element binding protein (CREB), FoxO1 and brain-specific homobox transcription factor that are important for the transcription of NPY and AgRP genes (Sakkou *et al.*, 2007; Lage *et al.*, 2010). In addition, results from a recent study indicate that the mammalian target of rapamycin and its substrate S6K are also involved in the orexigenic effect of ghrelin (Martins *et al.*, 2012; Stevanovic *et al.*, 2012).

Extra-hypothalamic regions are involved in the hedonic aspects of ghrelin-induced appetite regulation. For example, activation of the ghrelin receptor in dopaminergic neurons within the ventral tegmental area (VTA) increases their firing rates and food intake. This effect was reversed by infusion of a ghrelin receptor antagonist into the VTA, which reduced ghrelin-induced feeding behaviour (Abizaid *et al.*, 2006).

Other functions

Extra-hypothalamic expression of the ghrelin receptor has been shown to be important for a number of other functions. For example administration of ghrelin into the VTA augmented alcohol intake by increasing the alcohol reward signal, whereas a ghrelin receptor antagonist attenuated alcohol intake in mice (Jerlhag *et al.*, 2009). Additionally the ghrelin receptor appears to be important for other reward related behaviours such as sucrose preference, and the responses to cocaine and amphetamine (Jerlhag *et al.*, 2012; Figure 1).

As mRNA for the ghrelin receptor is present in many areas of the brain, several groups have investigated the importance of the ghrelin/ghrelin receptor-axis in cognitive function. In early experiments it was shown that ghrelin improves memory retention and anxiety behaviour (Carlini *et al.*, 2002) and synthetic ghrelin receptor agonists have been shown to enhance learning and exploratory behaviour (Atcha *et al.*, 2009). It was also shown that ghrelin promotes dendritic spine formation and contributes to long-term potentiation, consistent with its involvement in memory retention (Diano *et al.*, 2006).

In addition to the ubiquitious expression of ghrelin receptors in the brain, ghrelin receptors are also present in peripheral tissues, but at much lower expression levels (Guan *et al.*, 1997; Gnanapavan *et al.*, 2002). Their pattern of expression in the heart has led to the speculation that the ghrelin receptor system influences cardiovascular function, both in the presence and absence of growth hormone (Papotti *et al.*, 2000). For example, it was demonstrated that ghrelin has a beneficial effect on blood pressure, can prevent ischaemia, heart failure and even angiogenesis in vascular endothelial tissue. However, these effects on the CVS may, to a large extent, result from central ghrelin-mediated regulation of the sympathetic nervous system (Nagaya *et al.*, 2001; Chang *et al.*, 2004; Li *et al.*, 2007; Sato *et al.*, 2011; Yang *et al.*, 2012).

In the pancreas, ghrelin receptors have been shown to be expressed both on the endocrine α and β cells as well as potentially on a novel cell type, the ε cells. Conflicting data have been presented with respect to the functional effect of ghrelin on the pancreas (Broglio *et al.*, 2003a; Briggs and Andrews, 2011). However, in most studies it was demonstrated that acute administration of ghrelin induces a transient decrease in spontaneous insulin secretion and that antagonists are able block this effect (Broglio *et al.*, 2003b; Esler *et al.*, 2007). Interestingly, non-acylated ghrelin has been shown to counteract the ghrelin-induced modulation of insulin and glucose levels without affecting the neuroendocrine actions of ghrelin (Broglio *et al.*, 2004).

Given the wide range of effects of the ghrelin receptor system on physiology, a key challenge for ghrelin receptor drug discovery is to develop drugs that selectively modulate the desired aspects of ghrelin receptor function – for example, hypothalamic feeding behaviour. In potential treatments for obesity or cachexia, the effects of ghrelin receptor ligands on various metabolic parameters may be beneficial. However, the risks of side effects related to pituitary growth hormone secretion, mood and memory, and peripheral cardiovascular events, must be minimized. Modulation of the ghrelin/ghrelin receptor axis by exogenous ligands is clearly a delicate process that requires control of the full array of physiological responses induced by ghrelin. Therefore, we should pursue opportunities for functionally biased ligands towards the relevant signalling pathways, if we want to create a new generation of superior drugs for disease treatment.

7TM receptor signalling and activation

7TM receptor signalling

The ghrelin receptor belongs to the large family of 7 transmembrane segment receptors (7TM receptors), which are localized in the plasma membrane and transduce extracellular signals to intracellular responses. In addition to peptide hormones such as ghrelin, these extracellular stimuli also include large proteins, lipids, small organic molecules, ions and photons. Importantly a large percentage of drugs on the market targets the 7TM receptor superfamily, including some anti-histamines and β-blockers (Rask-Andersen *et al.*, 2011). To differentiate the extracellular stimuli from this large array of 7TM receptors, cells have developed a sophisticated intracellular signalling network, governed by G–protein-dependent and G–protein-independent mechanisms. The G-protein is a heterotrimer composed of Gα and Gβγ subunits, which when inactive is assembled and bound to GDP. Upon receptor activation the GDP is exchanged with GTP, thereby allowing the Gα and Gβγ subunits to dissociate and initiate their respective signalling cascades. There are four

highly studied Gα-mediated signalling pathways: (i) $G\alpha_s$ activates adenylate cyclase, leading to cAMP accumulation and cAMP-mediated responses whereas (ii) $G\alpha_{i/o}$ inhibits adenylate cyclase and thereby the cAMP accumulation; (iii) $G\alpha_{q/11}$ activates PLC leading to inositol tris-1,4,5-phosphate [IP_3; in the rest of this review article we will refer to the inositol phosphates (IP_3, IP_2, IP) as IP, IP is the most predominant form of the IPs measured in experiments of $G\alpha_q$ and subsequently PLC activation] and DAG production; and (iv) $G\alpha_{12/13}$ activates Rho guanine exchange factors, resulting in activation of Rho associated kinases, and cytoskeletal rearrangement. The Gβγ subunits have also been shown to regulate signalling pathways such as calcium release (Stehno-Bittel *et al.*, 1995), PLB, adenylate cyclase, but also MAPK, nucleic histone deacetylase 5 and adipocyte enhancer-binding protein 1 among others (Tang and Gilman, 1991; Crespo *et al.*, 1994; Zhang *et al.*, 1996; Park *et al.*, 1999; Spiegelberg and Hamm, 2005).

The most commonly described G–protein-independent signalling pathway involves phosphorylation of the 7TM receptor by GPCR kinases (GRKs) followed by β-arrestin adaptor recruitment. β-Arrestins are able to activate various kinases such as Src, Akt and MAPK (e.g. ERK 1/2 and p38; Violin and Lefkowitz, 2007). β-Arrestin recruitment is also one of the possible pathways for internalization of most 7TM receptors, initiating processes of intracellular trafficking, which determine whether receptors are recycled or degraded during chronic agonist exposure (Zhang *et al.*, 1998; Hanyaloglu and von Zastrow, 2008).

Ghrelin receptor mediated signalling

The ghrelin receptor was originally discovered to induce calcium release during an investigation into its growth hormone-releasing properties (Howard *et al.*, 1996). When its endogenous ligand, ghrelin, was eventually detected (Kojima *et al.*, 1999) more thorough studies of its signal transduction properties demonstrated a ghrelin receptor-dependent elevation of the PLC endproduct IP. Ghrelin has also been reported to activate other downstream signalling pathways, such as CREB-mediated transcription, in a dose-dependent manner – presumably through $G\alpha_q$, as CREB can be activated by calcium calmodulin kinase (Dash *et al.*, 1991; Kojima *et al.*, 1999; Holst *et al.*, 2003; 2004). In addition to the $G\alpha_q$-coupled signalling the ghrelin receptor couples to $G\alpha_{12/13}$ and thereby activates RhoA kinase. The combined actions of $G\alpha_q$ and $G\alpha_{12/13}$ are responsible for the majority of the ghrelin-induced activation of serum response element (SRE), whereas $G\alpha_i$ coupling is not relevant for this pathway (Sivertsen *et al.*, 2011), as the signal is unaffected by pertussis toxin. However $G\alpha_{i/o}$ coupling has been demonstrated in GTPγS assays in model systems (Bennett *et al.*, 2009) as well as in isolated lipid discs (Damian *et al.*, 2012; Mary *et al.*, 2012). Furthermore, activation of the ghrelin receptor leads to the recruitment of the clathrin adaptor-related protein complex 2 (AP2), or β-arrestins, in a manner that is independent of G-protein coupling (Damian *et al.*, 2012; Mokrosinski *et al.*, 2012). Stimulation of the ghrelin receptor also induces ERK1/2 phosphorylation in a dose-dependent manner. This process has been shown to be dependent on PKC stimulation and phosphatidylcholine accumulation in a G–protein-dependent manner. In contrast β-arrestin does not play a role in this signalling, because a dominant negative mutant of β-arrestin failed to decrease ERK1/2 phosphorylation (Mousseaux *et al.*, 2006; Chu *et al.*, 2007). This wide range of signalling possibilities has allowed the development of ligands with functionally biased signalling properties (Figure 2).

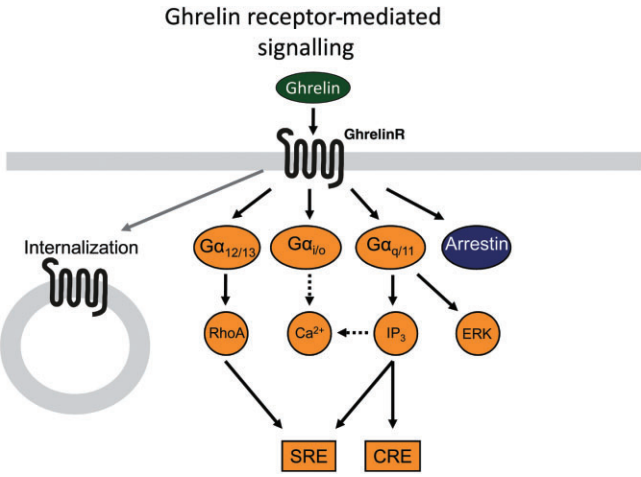

Figure 2

Multiple signalling pathways of the ghrelin receptor. Dotted arrows represent not fully verified signalling pathways, and black full arrows indicate pathways that have been described or suggested for the ghrelin receptor. The ghrelin receptor is able to signal through three different G-proteins, for example $G\alpha_q$, $G\alpha_{i/o}$, $G\alpha_{12/13}$ in addition to G–protein-independent arrestin coupling and internalization. $G\alpha_q$, that activates PLC and leads to increased IP and DAG formation can induce an increase in Ca^{2+} signalling. However, the pharmacological profiling of different ghrelin receptor agonists indicates that the Ca^{2+} signalling and IP accumulation originate from separate signalling pathways (Holst *et al.*, 2005), which explains the dotted line. In addition, $G\alpha_q$ coupling may also lead to CRE-mediated transcription activity and probably contributes to the SRE-mediated transcriptional activity. Finally, ligand-mediated $G\alpha_q$ coupling may also stimulate ERK1/2 phosphorylation. $G\alpha_{12/13}$ activates RhoA and ROCK resulting in SRE transcription. $G\alpha_{i/o}$, generally inhibits adenylate cylase to decrease cAMP accumulation; however, this has not been shown for the ghrelin receptor and it is possible that it can couple to $G\alpha_{i/o}$ and induce Ca^{2+} release. Ligand activation of the ghrelin receptor induces recruitment of β-arrestin, which might lead to receptor internalization. Both the constitutive and ligand activation of the receptor induce internalization of the receptor. β-Arrestins might lead to ERK phosphorylation but this is still uncertain.

Unusually, ligand binding is not required for significant ghrelin receptor activation. In the absence of any ligand present, the receptor signals with almost 50% constitutive activity as measured in IP accumulation assays. This property has been demonstrated for several different signalling pathways including SRE- and CREB-mediated transcriptional activity (Holst *et al.*, 2004). Recruitment of arrestin has also been shown to occur in a ligand-independent manner in heterologous expression systems, whereas in isolated lipid discs the arrestin recruitment is ligand dependent (Mary *et al.*, 2012; Mokrosinski *et al.*, 2012). Internalization of the ghrelin receptor can occur in a ligand-independent manner, but is dependent on receptor constitutive activity and domains within the C-terminal tail (Holliday *et al.*, 2007).

The recent demonstration of ligand-independent AP2 recruitment to the ghrelin receptor may therefore contribute to these basal receptor internalization properties (Damian *et al.*, 2012). Interestingly, however, some signalling pathways, such as ERK phosphorylation and $G\alpha_i$ coupling, do require receptor activation by exogenous agonists (Holst *et al.*, 2004; Damian *et al.*, 2012). Recent studies have shown that the constitutive activity of the ghrelin receptor is an intrinsic property of the receptor; when the receptor is embedded in lipid discs it can induce IP accumulation and GTPγS binding and these signals can be reduced by addition of the inverse agonist or increased by the agonist (Damian *et al.*, 2012). Thus, the scope for functionally selective ghrelin receptor ligands may also include those that might differentially modulate constitutively active receptor signalling rather than ghrelin-stimulated responses, known as 'protean' agonism (Ganguli *et al.*, 1998; Gbahou *et al.*, 2003).

The ghrelin receptor has been shown to dimerize both with other 7TM receptors as heterodimers and with itself as a homodimer. The interface responsible for the dimerization has not been studied for this receptor family. However, it has been demonstrated that the dopamine D_1 receptor (Jiang *et al.*, 2006), dopamine D_2 receptor (Kern *et al.*, 2012), melanocortin MC_3 receptor (Rediger *et al.*, 2012) and the 5-HT_{2C} receptor (Schellekens *et al.*, 2013) are co-expressed with the ghrelin receptor under physiological conditions. Importantly it has been shown that co-expression of the ghrelin receptor with either the MC_3, the D_1 or the $5HT_{2C}$ receptor leads to decreased ghrelin-mediated signalling in heterologous expression systems. In addition α-melanocyte stimulating hormone (α-MSH) signalling is enhanced by co-expression of the ghrelin receptor with the MC_3 receptor (Rediger *et al.*, 2012).

Ligand development for the ghrelin receptor was initially focused on agonists to increase growth hormone secretion. Several high-potency efficacious agonists – based on either peptide or non-peptide scaffolds – were studied in clinical trials, when the appetite promoting effects of the ghrelin system was revealed and ligands to block the function of ghrelin subsequently became the major focus. The substance P analogue ([D-Arg^1, D-Phe^5, D-$Trp^{7,9}$, Leu^{11}]-substance P), was the first antagonist and inverse agonist to be identified for the ghrelin receptor. Since then, several other ligands for the ghrelin receptor have been discovered. Based on a conserved motif wFwLL (where wFwLL denotes one letter abbreviations of amino acids, using the small letters to denote the D-form and capital letters to denote the L-form.) of the substance P analogue, a series of inverse agonists has been developed (Holst *et al.*, 2003). The penta-peptide wFwLL in itself binds to the ghrelin receptor with submicromolar affinity. However, it has the potential to act both as an agonist with a high potency and as an inverse agonist with a slightly lower potency. Modification by the N-terminal elongation of this motif with an alanine (AwFwLL) produced a more effective partial agonist whereas addition of lysine (KwFwLL) produced an inverse agonist (Holst *et al.*, 2007). Thus, even subtle changes in the structure of this motif can give rise to large variations ranging from negative to positive efficacy. This family of peptides interacts with the ghrelin receptor through a different subset of receptor interaction points compared with the endogenous ligand ghrelin, as shown by mutational analysis and computational docking simulations (Holst *et al.*, 2006). More recently, in an attempt to develop a treatment for obesity, small-molecule antagonists have been discovered for the ghrelin receptor. However, the molecular pharmacological properties of these compounds have yet to be fully characterized (Xin *et al.*, 2006; Rudolph *et al.*, 2007; Palus *et al.*, 2011).

Using mutation studies in combination with computational chemistry, the interaction of ligands with the ghrelin receptor has also been studied for several agonist compounds and for the previously mentioned peptide-based inverse agonists. The most important ligand interaction site described in the ghrelin receptor is a glutamic acid in the extracellular part of TM III. This is pivotal for the binding and function of both ghrelin and almost all ghrelin receptor ligands. Furthermore, mutations of aromatic and positively charged residues in TM VI affect the potency of ghrelin significantly (Feighner *et al.*, 1998; Holst *et al.*, 2006; 2009). Apart from these residues, no other substitutions in the binding pocket affect ghrelin-induced activation, indicating that ghrelin only makes a few key interactions within the centre of the binding pocket of the receptor in addition to potential interactions in the extracellular loops (ECLs; Holst *et al.*, 2006; 2009). However, the constitutive activity of the ghrelin receptor is diminished by substitutions in several other receptor domains, including substitutions of aromatic and charged residues in the extracellular part of TM III, TM VI and VII (Holst *et al.*, 2004; Goze *et al.*, 2010). These studies demonstrate the importance of a hydrophobic cluster connecting the extracellular parts of TM VI and VII for the activation of the receptor. Finally, inverse agonists generally require interactions deep in the transmembrane-binding pocket compared with the interactions made by agonists, which occur more superficially towards the extracellular ends of the TM domains (Holst *et al.*, 2007; 2009).

Biased 7TM receptor signalling

Traditionally, the same agonist–receptor complex has been thought to signal with the same intrinsic efficacies to all downstream signalling pathways. This classical paradigm is sufficient to account for variations in agonist potency and maximum response, which depend on the extent of signal amplification and receptor reserve. However, multiple lines of evidence challenge this classical view, suggesting that that some ligands may activate 7TM receptors differently through pathway-dependent intrinsic efficacies (Kenakin and Miller, 2010; Kahsai *et al.*, 2011; Kenakin, 2012). From a cellular signalling perspective, this phenomenon predicts ligands with the capacity to bias the functional response and favour a selection of some signalling pathways over others of the receptor's signalling repertoire. This allows the receptor to activate only a subset of specific effectors and thereby fine tune cellular responses and associated physiological responses. In addition to the ligand-induced signalling bias, structural changes in the receptor by amino acid substitutions may also allow the receptor to selectively favour some signal transduction pathways over others. This concept, known as 'ligand-directed trafficking', 'functional selectivity' or 'biased agonism', indicates that receptors are capable of generating

a more complex response than classical pharmacological agonism or antagonism would predict.

Quantifying the bias component of a ligand by application of an operational model may facilitate prediction of the biased signalling properties in all systems without actually quantifying biased signalling in all systems (Ehlert, 2008; Kenakin, 2009). In the model defined by Ehlert, the relative intrinsic response of a biased agonist compared with the standard agonist can be estimated based on data from simple binding and dose–response curves, for example affinity and efficacy. This method has been applied successfully to the muscarinic M_2 receptor system where agonists with variable efficacy through $G\alpha_{i/o}$, $G\alpha_s$ and $G\alpha_{15}$ signalling have been calculated and observed (Griffin *et al.*, 2007).

G-protein coupling versus G–protein-independent signalling

The most thoroughly investigated biased ligands are those that selectively differentiate between G-protein and G–protein-independent mediated signalling (Berg *et al.*, 1998). The clinical and pharmaceutical perspective for this kind of ligand is that it may allow for activation of only the therapeutic or clinically relevant pathways, not activating pathways associated with adverse effects. An example of such a biased ligand was discovered for the niacin receptor, GPR109A, where novel nicotinic acid derivatives only induce the anti-lipolytic response mediated through inhibition of cAMP accumulation. This is in contrast to nicotinic acid itself, which also activated an unwanted flushing response mediated through the G–protein-independent pathway of ERK1/2 activation (McKenney *et al.*, 1994; Richman *et al.*, 2007). This kind of success story has encouraged research within the field of functionally biased ligands targeting the 7TM GPCRs.

Examples of receptors

Several different 7TM receptors have the ability to couple to more than one signalling pathway and within the last decade an increasing number of receptor ligands have been developed or characterized for biased signalling properties. (Kenakin, 1995a; Berg *et al.*, 1998)

For the angiotensin II (AT_{1A}) receptor, the ligand TRV120027 is an excellent example of how biased ligands can provide advantages over classical unbiased drugs. TRV120027 stimulates ERK1/2 phosphorylation in a β–arrestin-dependent manner while antagonizing G-protein coupling such as $G\alpha_q$-mediated IP production or GTPγS recruitment (Wei *et al.*, 2003). This signalling profile allows the ligand to inhibit angiotensin II-mediated vasoconstriction while increasing cardiomyocyte contractility via β-arrestin coupling (Violin *et al.*, 2010; Boerrigter *et al.*, 2011). *In vivo*, TRV120027 reduces mean arterial pressure in a manner similar to that of unbiased AT_{1A} receptor antagonists. However, unlike the unbiased antagonists, which tend to decrease cardiac performance, TRV120027 increases cardiac performance and preserves cardiac stroke volume. Thus, this ligand alleviates the symptoms of acute heart failure, and has now reached phase 2 clinical trials. The striking differences *in vivo* between unbiased and arrestin-biased ligands illustrate the potential of biased ligands for optimized targeting of therapeutic effects while minimizing adverse events.

For the chemokine CCR5 receptor synthetic ligands have been developed in an attempt to prevent HIV entry. Importantly, to decrease the risk of side effects, these ligands have been selected for biased signalling properties that favour internalization and do not affect G-protein coupling (O'Hayre *et al.*, 2010; Steen *et al.*, 2013).

Mutation-induced signalling bias

In a large number of other class A 7TM receptors, functionally biased ligands have also been found to distinguish between G-protein coupled and arrestin-mediated signalling (Stallaert *et al.*, 2011). Interestingly, such bias can also be generated by mutations located in various areas of the 7TM receptor, which selectively shift the balance for activating one pathway rather than another. Substitutions of residues located in the ligand binding pocket can in some cases be responsible for biasing the signalling towards either the G-protein or G–protein-independent pathways. For example, in the muscarinic M_2 cholinoceptor, mutations in both the orthosteric and the allosteric pockets have been identified that selectively affect coupling to the ERK1/2 pathway. Substitution of a tyrosine in the orthosteric site selectively abolished signalling to the ERK1/2 pathway while retaining the ability to activate G-proteins and mediate intracellular calcium mobilization. In contrast, mutation of a second tyrosine in the allosteric site selectively enhanced signalling through the ERK1/2 pathway (Gregory *et al.*, 2010).

More common are substitutions in the intracellular part of the receptor that selectively uncouple either G-protein or β-arrestin coupling. For example, mutations in the conserved TM III DRY motif of the AT_{1A} receptor result in the loss of G-protein coupling, measured by GTPγS binding or IP production. However, this receptor mutant retains the ability to induce ERK1/2 phosphorylation via β-arrestin recruitment (Wei *et al.*, 2003). Another AT_{1A} mutation with a substitution in the second intracellular loop displayed impaired IP accumulation compared with wild type (WT) receptors, but still retained WT-like receptor internalization properties (Gaborik *et al.*, 2003). Several other mutations in intracellular loop 2 preserved receptor activation of Src and ERK1/2, but could not induce $G\alpha_q$-mediated activation of PLC or $G\alpha_i$-mediated inhibition of forskolin-induced cAMP accumulation (Seta *et al.*, 2002). These studies suggest that mutations of the intracellular surface of the AT_{1A} receptor are important for discrimination between the intracellular signalling proteins, e.g. β-arrestins versus G proteins. Thus some types of signalling bias, normally invoked by substitutions in the intracellular domains, induce selective manipulation of the interaction sites between an active receptor conformation and its G-protein or arrestin effectors. However other biasing mutations, which can be located in almost any region of the 7TM receptor, clearly indicate the possibility of pathway-specific receptor conformations.

Biased coupling to one G-protein versus another

The majority of the biased ligands described in the literature distinguish between G-protein coupled and G–protein-independent signalling (Violin and Lefkowitz, 2007). However, in some cases signal transduction of one G-protein

is favoured over another G-protein (Berg *et al.*, 1998). Both mutations and ligands can bias receptors to distinguish between the different G-proteins as described later.

The melanocortin MC_4 receptor represents an example of a receptor where ligands have been shown to favour one G-protein coupling over another. Activation of the MC_4 receptor stimulates both cAMP accumulation and Ca^{2+}-release, which involve both $G\alpha_s$ and $G\alpha_i$ proteins (Buch *et al.*, 2009). These G-protein coupled pathways can be separated by different agonists. For example, the non-peptide ligand tetrahydroisoquinoline (Van der Ploeg *et al.*, 2002) displays a 100-fold higher potency for $G\alpha_s$ coupling, as measured by cAMP accumulation, compared to Ca^{2+} release. In contrast, other non-peptide agonists such as NBI 58704 show the opposite signalling bias with an 11-fold impaired potency for cAMP accumulation compared with Ca^{2+}-release (Nickolls *et al.*, 2005). Even the well-described MC_4 receptor antagonist AgRP displays biased signalling properties as it is able to *activate* $G\alpha_{i/o}$ coupling by stimulating the MC_4 receptor (Buch *et al.*, 2009).

The thyrotropin (TSH) receptor represents an example of a receptor where substitutions of residues in the transmembrane region alter G-protein signalling preferences. In the constitutively active TSH receptor, subtle substitutions of aromatic and hydrophobic residues in TM V and VI resulted in bias of the functional response with respect to $G\alpha_s$ coupled cAMP accumulation compared with $G\alpha_q$ coupled IP accumulation. Substitutions in TM VI resulted in strongly decreased $G\alpha_s$ coupled constitutive efficacy as measured by cAMP, whereas the constitutive $G\alpha_q$ coupled IP accumulation was only slightly decreased. The same phenomenon was observed for ligand-induced activation, as the same substitutions showed almost WT-like ligand-induced stimulation of cAMP accumulation, but strongly decreased efficacy for IP accumulation. This suggests that specific residues in these helices are involved in G–protein-specific coupling (Kleinau *et al.*, 2011).

Biased signalling of the ghrelin receptor

The ghrelin receptor is coupled to at least three different G-proteins, $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$, and in addition it is also able to recruit β-arrestin and AP2 upon activation. Most of these pathways may be activated ligand-independently, whereas ERK phosphorylation is only activated by agonists (Holst *et al.*, 2004). The wide spectrum of signalling pathways available for the ghrelin receptor provide many possibilities for ligand- and mutation-induced selectivity of the functional response.

A classical biased agonist, wFw-isonipecotic acid (Isn), has been discovered for the ghrelin receptor, which favours signalling through one G-protein rather than another (Sivertsen *et al.*, 2011). This development was built on the knowledge of the wFw peptide binding modalities and the transition from inverse to positive agonism that this peptide series displays at the ghrelin receptor (described earlier). Thus, the biased agonist contains the core binding wFw motif, and is linked to a synthetic amino acid Isn at the C-terminus. Mutational studies and computational docking studies revealed that the interaction pattern of wFw-Isn is different from that of all the other peptide and non-peptide ghrelin receptor ligands described. Importantly, the activity of this agonist is not dependent on the highly conserved negatively charged glutamic acid in TM III that serves as the receptor anchoring point for all the other ligands described for the ghrelin receptor. This ligand induces IP accumulation and ERK1/2 phosphorylation, but it is unable to activate the $G\alpha_{12/13}$ coupled SRE-induced transcriptional activity. These data and calculations suggest that wFw-Isn is a biased ligand that favours pathways such as $G\alpha_q$ and ERK1/2 phosphorylation over $G\alpha_{12/13}$ coupling. In support of this notion, wFw-Isn does not activate RhoA in a pituitary cell line naturally expressing the ghrelin receptor, unlike ghrelin itself (Sivertsen *et al.*, 2011; Figures 3B and 4B). Interestingly, wFw-Isn administered i.c.v. to rats was unable to stimulate food intake, which is one of the most solid and reproducible effects of ghrelin receptor stimulation. These data suggest that based on the elucidation of the ligand receptor interaction it is possible to rationally develop biased agonists.

In addition the hexapeptide ligand, KwFwLL, has been identified, unusually, as a biased *inverse* agonist for the ghrelin receptor. It is a potent inverse agonist, when measuring IP accumulation, with an EC_{50} value of 32 nM. However, when measuring SRE transcriptional activity it is a very low-potent inverse agonist that only induces a weak decrease in the signalling at 1 μM (Holst *et al.*, 2007). This observation is most likely due to biased inverse agonist signalling favouring inhibition of the $G\alpha_q$ coupled pathway compared to that coupled to $G\alpha_{12/13}$ (Figure 3C).

The examples mentioned earlier of biased ligands for the ghrelin receptor were characterized *in vitro*, predominantly in heterologous expression systems. However, recently the ghrelin receptor was reconstituted in lipid discs, a phospholipid bilayer lined by amphipathic helical membrane scaffold proteins to form a disc like structure (Bayburt *et al.*, 2002). In this reconstitution method, the receptor is expressed in bacteria, extracted via inclusion body formation, which requires solubilization before purification, but yields functional receptors (Mary *et al.*, 2012). Receptor activation was measured by monitoring conformational changes via fluorescent probes in the intracellular and in the extracellular domains. Under these conditions the ghrelin receptor was able to couple to $G\alpha_i$ in addition to the previously mentioned signalling pathways. In this system, a biased agonist, JMV3018, was demonstrated to couple to $G\alpha_q$ to the same extent as ghrelin, but was unable to couple to $G\alpha_i$ or recruit β-arrestin (Mary *et al.*, 2012; Figure 4B).

The phenomenon of signalling bias in the ghrelin receptor system has also been demonstrated by the introduction of mutations in the receptor. For example, we observed that substitutions of an alanine residue in one of the ECLs (position 204) resulted in clear differences in relative receptor signalling through IP production, SRE-induced transcriptional activity or β-arrestin mobilization. Substitution of the alanine with either a charged residue or an aromatic residue only decreased the basal constitutive $G\alpha_q$-mediated signalling, measured as IP accumulation. However, these mutations had a greater effect on both SRE-mediated transcription activity and arrestin recruitment, where decreased ghrelin-induced efficacy was also observed. In particular arrestin recruitment was almost eliminated (Mokrosinski *et al.*, 2012). The potencies of ghrelin-induced SRE-induced transcription activity and IP accumulation were only affected when the alanine was substituted with a small negatively charged

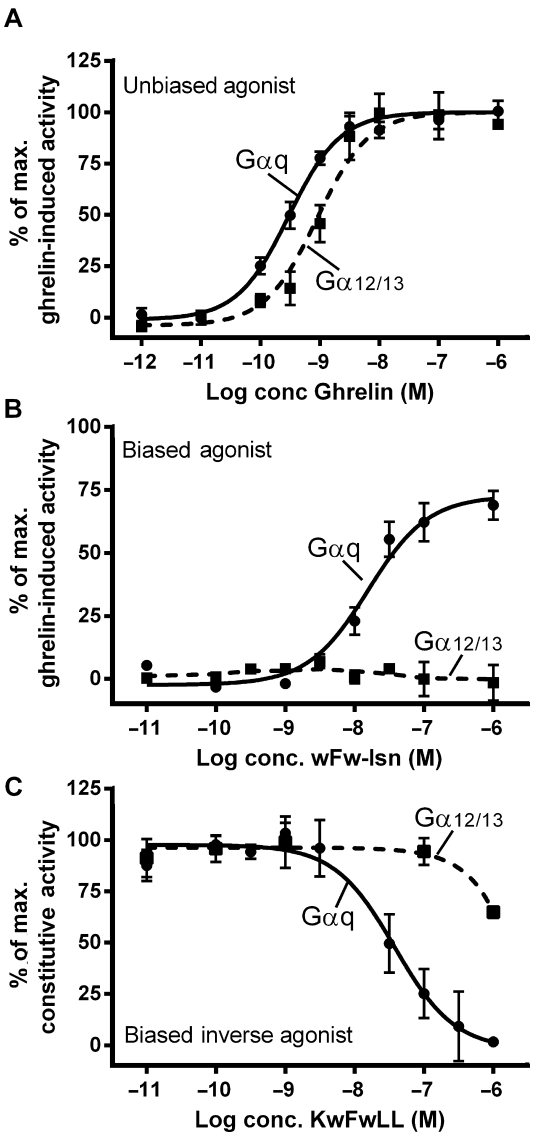

Figure 3

Dose–response curves of an example of an unbiased agonist, a biased agonist and a biased inverse agonist. The dose–response curve was performed in HEK293 cells transiently transfected with the ghrelin receptor where the response of the ligands is measured by the accumulation of IP ($G\alpha_q$ coupled) and by activation of SRE-mediated transcriptional control ($G\alpha_{12/13}$ mediated; Holst *et al.*, 2006). Dose–response curves for (A) the un-biased ligand, ghrelin, (B) the biased agonist, wFw-lsn and (C) the biased inverse agonist are shown.

aspartic acid. However, the potency of ghrelin in the β-arrestin mobilization assay was not affected by any substitution. The molecular background for this substitution-induced signalling bias is probably due to changes in the secondary structure in ECL2 resulting in an extended constrained α-helix that then affects the intracellular coupling repertoire of the receptor. The findings of this study also indicate that the less constrained ECL2 is important for the constitutive activity of the ghrelin receptor (Mokrosinski *et al.*, 2012).

7TM receptor conformational changes during biased signalling

Biased signalling properties may, from a molecular perspective, refer to the fact that several different distinct 'active' as well as 'inactive' conformations of the receptor exist. These 'active' conformations may interact differently with the intracellular signalling partners and generate different signalling patterns (Kenakin, 1995b). It has recently been shown that multiple active conformations are stabilized during ligand binding in the β_2-adrenoceptor (Kahsai *et al.*, 2011).

The active conformation of 7TM receptors

Transition to the active conformation of 7TM receptors, stabilized by ligands of highly variable chemical nature, is believed to involve similar global changes. These conformational changes between the active and the inactive conformation have been intensively studied over the last two decades by use of various biochemical and biophysical methods, and more recently, complementary X-ray crystallographic data have been obtained. Using spin-labelling studies in rhodopsin, Farrens *et al.* (1996) showed that the intracellular parts of the TM segments moved relative to each other upon activation and that constraining the intracellular part of the transmembrane segments by covalent bonds impaired signal transduction. The most dramatic changes were an outward movement and rotation of the TM VI relative to TM III (Farrens *et al.*, 1996). These data were confirmed both by environment-sensitive probes in the β_2-adrenoceptor, by NMR studies and by introduction of zinc sites (Sheikh *et al.*, 1996; Gether and Kobilka, 1998; Ye *et al.*, 2010). More recently crystal structures of the β_2-adrenoceptor bound to either an agonist or an inverse agonist, as well as the receptor in complex with the G-protein have been resolved (Rasmussen *et al.*, 2007; 2011). The difference between these structures is primarily that the intracellular part of TM VI is rotated and moved away from the helix bundle in the structure of the agonist-bound receptor compared with that of the inverse agonist (Schwartz and Sakmar, 2011). This allows for an interaction with the C-terminal part of $G\alpha_s$ protein that initiates the intracellular activation cascade (Rasmussen *et al.*, 2011). The crystal structure of the A_{2A} adenosine receptor shows a similar outwards rotation of the intracellular part of TM VI upon binding to its endogenous ligand, adenosine (Jaakola *et al.*, 2008; Lebon *et al.*, 2011). This mechanism of activation is therefore believed to be a general characteristic of 7TM receptors of the rhodopsin family. The conformation of the extracellular part of the receptor has only been shown to differ to a very small degree between the active and the inactive state (Bokoch *et al.*, 2010). This change involves weakening of a salt bridge between ECL II and III in the active conformation (Bokoch *et al.*, 2010) and in the extracellular located transmembrane part the most significant changes involved minor movements of TM V (Rasmussen *et al.*, 2011).

Several active conformations

Interestingly, biophysical data suggest that full agonists and partial agonists targeting the most well studied receptor, the β_2-adrenoceptor, apparently bind to and activate the receptor through different mechanisms and with different kinetics –

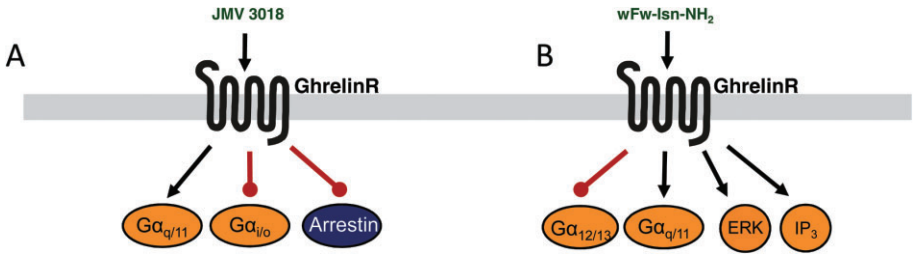

Figure 4

The effects of two different biased agonists. (A) The biased agonist JMV3018 is able to stimulate $G\alpha_q$ recruitment in purified ghrelin receptors in lipid discs, but no recruitment of $G\alpha_q$ or arrestin was observed for this compound. (B) The biased agonist wFw-Isn is able to couple to $G\alpha_q$ and the downstream signalling pathways IP and ERK phophorylation, but it is unable to activate the $G\alpha_{12/13}$ coupled SRE pathway when compared with ghrelin.

irrespective of the fact that they have very similar chemical structures (Swaminath *et al.*, 2005). For example fluorescent probes in the intracellular part of the receptor revealed different conformations induced by the full agonists, adrenaline and isoprenaline, and the partial agonist noradrenaline (Swaminath *et al.*, 2005; Reiner *et al.*, 2010).

Distinct receptor conformations could also lead to differential recruitment of intracellular regulatory proteins, such as G-proteins, β-arrestins and GRKs, biasing the receptor towards a given pattern of response (Breit *et al.*, 2011; Ulloa-Aguirre *et al.*, 2011). This is supported by data showing that a β–arrestin-biased ligand of the β_2-adrenoceptor, carvedilol, selectively mediates a specific phosphorylation pattern, thus leading to stimulation of ERK (Nobles *et al.*, 2011). The specific phosphorylation pattern has been shown to be due to selective recruitment of GRK6, suggesting a specific active conformation stabilized by the biased agonist compared with the un-biased agonists. The distinct active conformation induced by β–arrestin-biased agonists has also been studied by site-specific 19F-NMR label studies of the β_2 adrenoceptor in complex with ligands. These data suggest that the biased agonist stabilizes a conformation in the intracellular part of the receptor, slightly different from un-biased ligands. Specifically for the β_2-adrenoceptor, β–arrestin-biased ligands that do not, or only to a small extent, activate G–protein-dependent signalling, induced a characteristic pattern of TM VII movements. This effect on TM VII was different from the changes observed for unbiased ligands that induced major changes in TM VI and VII (Bokoch *et al.*, 2010; Liu *et al.*, 2012). A similar observation has been made in a prototypical peptide activated 7TM receptor, the V_2 arginine-vasopressin, in which the receptor conformations stabilized, either by an un-biased agonist or by two different agonists biased either towards β-arrestin or $G\alpha_s$ coupling, were studied. In this receptor system, ligand-induced conformational changes were studied by fluorescent probes incorporated strategically into the purified V_2 receptor. $G\alpha_s$ biased agonists induced changes in the conformation between intracellular loop 3 and TMVI, whereas the β-arrestin biased agonist required movements in the interphase between TMVII and helix VIII (Rahmeh *et al.*, 2012).

Given that several 7TM receptors can exist as dimers or oligomers (Pin *et al.*, 2005; Milligan, 2008), it seems feasible that signalling would be modulated in these states compared with signalling of a monomeric receptor. Dimerization both as homo-dimerization and as heterodimerization has been demonstrated to modulate signalling bias. For example, when co-expressing the cannabinoid with the dopamine D_2 receptor a switch in CB_1-mediated G_s coupling was observed, even in the absence of a D_2 agonist (Jarrahian *et al.*, 2004), supposedly because the D_2 receptor stabilizes the CB_1 receptor in a conformation more favourable for Gs coupling (Ellis *et al.*, 2006; Hudson *et al.*, 2010). A recent publication showed that homodimerization may affect the signalling bias in the family B 7TM receptor glucagon like peptide-1 (GLP-1) receptor; intracellular Ca^{2+} release was completely abolished when dimerization was disrupted, whereas ERK and cAMP signalling was unchanged (Harikumar *et al.*, 2012). The association of a 7TM receptor with other membrane proteins could therefore affect signalling bias of 7TM receptors.

Active and inactive conformations of the ghrelin receptor

The ghrelin receptor has also been studied in terms of conformational changes during activation and inactivation. The observation that inverse agonists interact differently with the receptor compared with agonists and thereby may stabilize a different conformation, has been observed in mutational studies. The KwFwLL, an inverse agonist, and the structurally similar AwFwLL, an agonist, interact in a different way with the receptor. They are both dependent on key residues in TM II, III, IV, V and VII; however, mutations in TM V strongly decreased the potency of AwFwLL, whereas the inhibitory potency of KwFwLL was unchanged. This indicates that the two structurally-related molecules interact differently with the receptor to generate different pharmacological effects by stabilizing the inactive and active receptor conformations respectively (Holst *et al.*, 2007). In agreement with the crystal structure data, the active state requires conformational changes in TM V and agonism was dependent on residues in TM V. In contrast, the inverse agonist did not share these interaction sites. Biophysical data from fluorescence spectroscopy studies using reconstituted monomeric ghrelin receptors in lipid discs support the occurrence of distinct active and inactive ghrelin receptor conformations differentially stabilized by agonists and inverse agonists. Thus, the ghrelin receptor occupied by the inverse agonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P analogue adopts a confirmation

that is different from the conformation stabilized by full agonists, MK-677 and ghrelin. Notably this conformation is also different from that of the unoccupied receptor, which may suggest multiple inactive conformations. The conformation induced by the inverse agonist leads to decreased binding of β-arrestins and G-proteins (Damian *et al.*, 2012). Importantly, the arrestin-biased ligand JMV3018 also induced changes in receptor conformation that differed from those induced by an unbiased agonist. Interestingly, conformational selection was also observed when the ghrelin-receptor complex was exposed to either β-arrestin or $G\alpha_q$. In the presence of β-arrestin, ghrelin induced an active conformation that was different from the conformation stabilized in the presence of $G\alpha_q$, supporting the hypothesis that minor variations in the active conformation can determine the coupling pattern of 7TM receptors.

From the present data, it is postulated that 7TM receptors may adopt many different active and inactive conformations, stabilized by agonists or inverse agonists respectively. Several different active conformations may exist depending on the properties of the agonist. Biased agonists stabilize receptor conformations that couple with different intrinsic efficacies to the different possible signalling pathways (Schellekens *et al.*, 2013). Finally dimerization and intracellular cross-talk between the ghrelin receptor and other 7TM receptors might also contribute to the biased signalling properties, as the ghrelin receptor has been demonstrated to dimerize with MC_3, D_1, D_2 and other receptors (Jiang *et al.*, 2006; Kern *et al.*, 2012; Rediger *et al.*, 2012; Schellekens *et al.*, 2013).

Biased signalling in a physiological setting

Several studies performed in animal models suggest that it is possible to dissociate the physiological effects of the ghrelin receptor system dependent on biased signalling properties of the applied ligand. In particular, the actions of ligands developed in the search for either anti-obesity treatment through inhibition of the ghrelin receptor system, or for anti-cachexia treatment through activation of the system, can be dissociated on the basis of their effects on GH secretion, food intake and body weight.

The small molecule compound GSK161443 represents one example of a physiologically biased agonist. *In vitro*, this compound was shown to act as a potent competitive antagonist in calcium screening assays, although in IP accumulation assays, weak partial agonism was observed. In the initial *in vivo* studies monitoring GH release, GSK161443 behaved as an antagonist. However, this compound turned out to behave as an agonist with regard to food intake and body weight regulation both in acute and chronic studies (Costantini *et al.*, 2011). Importantly, this effect was found to be mediated entirely by the ghrelin receptor as the compound only increased body weight in WT mice and not in ghrelin receptor knockout mice (Sabbatini *et al.*, 2010; Costantini *et al.*, 2011). A similar dissociation between GH secretion and food intake effects was observed for another class of small-molecule ligands – but in the opposite manner (Moulin *et al.*, 2007). These ligands were also described as antagonists in calcium mobilization assays, but they were only able to antagonize the agonist effects induced by ghrelin receptors in food intake studies, not in the GH secretion studies (Moulin *et al.*, 2007). Peptide ligands based on ghrelin itself have also been shown to display a strong degree of selection between the modulation of GH secretion and food intake. The ghrelin analogue BIM-28163 was shown to act as a ghrelinR antagonist in the *in vitro* calcium mobilization assay (Halem *et al.*, 2004). However, in *in vivo* assays, BIM-28163 blocked ghrelin-induced GH secretion but acted as a full agonist on food intake with comparable efficacy to ghrelin (Halem *et al.*, 2004).

These *in vivo* studies show that activation or inhibition of the ghrelin receptor is not a simple one-way road to modulation of all the downstream physiological functions. In particular, it is obvious that modulation of GH secretion and food intake is dependent on distinct subsets of the complex intracellular signal transduction pathways initiated by this receptor. Thus compounds simply screened for antagonism of ghrelin-induced calcium release (via $G\alpha_q$) cannot be predicted to have the same physiological effects *in vivo*, as illustrated in the examples mentioned previously (Halem *et al.*, 2004; Moulin *et al.*, 2007; Sabbatini *et al.*, 2010; Costantini *et al.*, 2011). Even with calcium release, multiple mechanisms may be involved, for example both $G\alpha_q$ coupling and $G\alpha_i$ coupling may also induce an increase in the calcium level through Gβγ-subunit potentiation of PLC (Lee *et al.*, 1993). The dissociation between the pathways modulating GH secretion and food intake has also been shown in *in vivo* studies where a specific inhibitor of the intracellular enzyme SIRT1 selectively blocked the ghrelin-induced food intake, but did not affect GH secretion (Velasquez *et al.*, 2011). In order to predict the physiological effect of a given ligand based on results from *in vitro* studies, it is therefore important to dissect the signalling repertoire of the ghrelin receptor carefully and take into consideration the many possible coupling pathways.

Conclusion

The ghrelin receptor constitutes an interesting therapeutic target because of its important effect on food intake and energy expenditure. However, the drug development process is complicated by the fact that the ghrelin receptor system also modulates neuropsychiatric functions such as mood, reward behaviour and cognition, and GH secretion. The development of functionally biased ghrelin receptor ligands, illustrated in principle by current *in vitro* and *in vivo* studies, demonstrates that ligand modulation of a selected subset of the possible ghrelin receptor signalling pathways is achievable, and a route that could be used to develop drugs with minimal potential side effects.

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Conflict of interest

None.

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