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Novel and conventional receptors for ghrelin, desacyl-ghrelin and pharmacologically related compounds

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Abbreviations: ACTH, adrenocorticotropic hormone; CD 36, Cluster of Differentiation 36, also known as FAT (fatty acid translocase); CRF, corticotrophin releasing factor; CRFR2, CRF receptor 2; GH, growth hormone; GHS, growth hormone secretagogue; GHSR, growth hormone secretagogue receptor; GOAT, ghrelin O-acyl transferase; GLP-1, glucagon like peptide 1; GRLR, ghrelin receptor-like receptors; icv, intracerebroventricular; IRS, insulin receptor substrate; MSP, modified substance P; NMU, neuromedin U; NO, nitric oxide; NOS, nitric oxide synthase; SOD, superoxide dismutase; UAG, unacylated ghrelin, also referred to as desacyl-ghrelin.

Abstract - The only molecularly identified ghrelin receptor is the growth hormone secretagogue receptor, GHSR1a. Its natural ligand, ghrelin, is an acylated peptide whose unacylated counterpart (UAG) is almost inactive at GHSR1a. A truncated, non-functional, receptor, GHSR1b, derives from the same gene. We have critically evaluated evidence for effects of ghrelin receptor ligands that are not consistent with actions at GHSR1a. Effects of ghrelin are observed in cells or tissues where the expression of GHSR1a is not detectable, or after the *Ghsr* gene has been inactivated. In several, effects of ghrelin are mimicked by UAG and ghrelin binding is competitively reduced by UAG. Effects in the absence of GHSR1a and sites at which ghrelin and UAG have similar potency suggest the presence of novel non-specific ghrelin receptors (ghrelin receptor-like receptors). A third class of receptor, the UAG receptors, at which UAG, but not ghrelin, is an agonist has been proposed. None of the novel receptors, with the exception of the glycoprotein CD36, that accounts for ghrelin action at a limited number of sites, have been identified. GHSR1a and GHSR1b combine with other G-protein coupled receptors to form heterodimers, whose pharmacologies differ from their components. Thus it is feasible some ghrelin receptor-like receptors and some UAG receptors are heterodimers. Effects mediated through ghrelin receptor-like or UAG receptors include adipocyte lipid accumulation, myoblast differentiation, osteoblast proliferation, insulin release, cardioprotection, coronary artery constriction, vascular endothelial cell proliferation and tumor cell proliferation. The molecular identification and pharmacological characterisation of novel ghrelin receptors are thus important objectives.

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I. The story of ghrelin

Two threads of history led to ghrelin, the first being the discovery of novel compounds that cause growth hormone release, and the second being the hypothesis that there exists a peripheral source of an orexigenic hormone.

In the early 1980s, synthetic peptides that were potent as stimulants of growth hormone release from pituitary cells, but which did not act at the growth hormone releasing hormone receptor, were discovered (Bowers et al., 1984). These were termed growth hormone secretagogues, and substantial effort was made to discover the growth hormone secretagogue receptor, GHSR. This took until 1996, when a G protein coupled receptor (GPCR) that responded to growth hormone secretagogues was discovered and cloned (Howard et al., 1996). This receptor protein had two natural forms, a functional 7 membrane spanning receptor, GHSR1a, and a truncated, non-functional receptor, GHSR1b. A natural ligand for the receptor was not found until 1999 (Kojima et al., 1999). Surprisingly, this ligand, ghrelin, was extracted from the stomach. Ghrelin, like the earlier discovered artificial growth hormone secretagogues, increased food intake (Nakazato et al., 2001). Much earlier studies had predicted the existence of a signal originating from the stomach or another peripheral source that promotes feeding (Cannon and Washburn, 1912; Carlson, 1913), but the identification of a peripheral orexigenic hormone had eluded researchers. In the few years after its discovery, it was firmly established that ghrelin had physiological roles in controlling growth hormone release and appetite (Kojima and Kangawa, 2005; van der Lely et al., 2004).

Ghrelin is a 28 amino acid peptide that has an 8 carbon fatty acid side chain on the serine at position 3, whose addition is catalysed at the precursor stage by the enzyme ghrelin O-acyl transferase, GOAT (Gutierrez et al., 2008). Although by definition ghrelin has an acyl side chain (Kojima et al., 1999), it is sometimes referred to in publications as acyl-ghrelin. The 28 amino acid peptide without the side chain is referred to in the literature as desacyl-ghrelin (commonly meaning ghrelin from which the side chain has been cleaved) or unacylated ghrelin (UAG). We have used the abbreviation UAG to refer to this peptide.

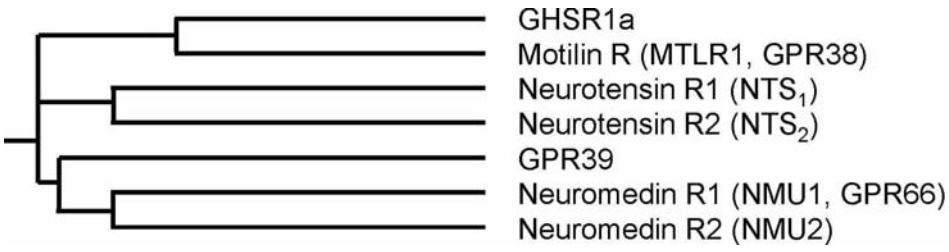
Experimental studies, recently reviewed in *Pharmacological Reviews* (Chen et al., 2009), soon revealed many other effects of ghrelin, effects of other products of the ghrelin gene (ghrelin, UAG, obestatin and des-Gln14-ghrelin), and effects of small molecule ghrelin receptor ligands, some of which have been tested for their therapeutic potential (Table 1). It also emerged that not all effects of ghrelin, ghrelin mimetics and related compounds could be explained by actions at the unmodified GHSR1a, and some were independent of GHSR1a (Muccioli et al., 2007). Several previous

reviews provide evidence for the existence of novel ghrelin and desacyl-ghrelin receptors (Muccioli et al., 2007; Seim et al., 2011; Soares and Leite-Moreira, 2008).

II. Properties of GHSR1a

A. The receptor and agonists

The molecularly identified ghrelin receptor, GHSR1a, is a typical GPCR. It is a family A receptor and a member of the ghrelin receptor group, which includes receptors for motilin (~52% homology with GHSR1a), neurotensin 1 and 2 (~35% homology), neuromedin receptors 1 and 2 (~30% homology) and GPR39, with ~30% homology (Holst et al., 2004).



(Fig 1 here)

The original discovery paper showed that ghrelin at nanomolar concentrations activated GHSR1a in pituicytes and in transfected CHO cells, but that GHSR1a did not respond to UAG (Kojima et al., 1999). Later studies revealed a weak but full agonism and low potency displacement of binding by UAG at GHSR1a (Bednarek et al., 2000; Callaghan et al., 2014; Gauna et al., 2007b; Matsumoto et al., 2001). A difference in potency of over 1000-fold was reported in two studies: UAG had an EC_{50} of between 1.6 and 2.4 µM, compared to an EC_{50} of 2-2.6 nM for ghrelin at GHSR1a (Callaghan et al., 2014; Gauna et al., 2007b). In another study, the difference in potency was greater: 1-2 nM for ghrelin and >100 µM for UAG (Matsumoto et al., 2001). Radioligand binding experiments confirm a greater than 1000 fold difference in binding efficiency: UAG displaced labelled ghrelin from GHSR1a with an IC_{50} of 10-13 µM, whereas the IC_{50} for ghrelin was 7-10 nM (Bednarek et al., 2000; Gauna et al., 2007b). Ghrelin administered to humans stimulates appetite and increases circulating GH, ACTH, cortisol, prolactin and glucose (Garin et al., 2013). In contrast, growth hormone release, in human, is not affected by UAG (Broglio et al., 2004).

Thus the potency of ghrelin at GHSR1a depends on the integrity of the octonyl side chain at serine 3, although other hydrophobic side chains can be substituted with retention of activity (Bednarek et al., 2000). The minimum octonylated sequence for effective stimulation of the receptor is the N-terminal 4 amino acids of ghrelin, Gly-Ser-Ser(*n*-octanoyl)-Phe (Bednarek et al.,

2000). The EC_{50} of this acylated tetrapeptide was about half that of full length ghrelin and it produced 90% of the maximum response to ghrelin.

It is notable that that circulating levels of ghrelin and UAG are in the range 0.1 to 0.5 nmol/L (Crujeiras et al., 2010; Patterson et al., 2005; St-Pierre et al., 2007; Tschöp et al., 2001), although higher levels (3-4 nmol/L) are measured by assays that detect bound peptide (Lambert et al., 2011; Patterson et al., 2005). Thus UAG in the circulation is at a considerably lower concentration than that necessary to activate GHSR1a.

The hexapeptide growth hormone secretagogues, such as hexarelin, which were the first synthetic GHSR1a agonists (Bowers et al., 1984) have poor bioavailability and short *in vivo* half lives. However chemical screens soon identified potent non-peptide, small molecule agonists (Table 1), including the Merck compounds, L163255, L163191 (MK677) and L692429 (Barakat et al., 1998; Smith, 2005). Development of agonists accelerated once the receptor was cloned, particularly after identification of its natural ligand, and soon numerous potent small molecule agonists, and some peptides with improved *in vivo* stability, became available (Chollet et al., 2012; Chollet et al., 2009). New compounds included NN703 from NovoNordsk (Hansen et al., 1999), capromorelin and related pyrazolinone-piperidine dipeptides from Pfizer (Carpino et al., 2002), GSK892491 (Witherington et al., 2008), anamorelin (Paul et al., 2006) and ulimorelin, a macrocyclic distinctly different from other synthetic agonists (Hoveyda et al., 2011) (Table 1).

Cortistatin is also a natural ligand of GHSR1a. This peptide has close structural homology to somatostatin, both peptides sharing the ability to bind and activate all five somatostatin receptor subtypes (Tringali et al., 2012). Because cortistatin and somatostatin display a number of different biological actions, specific cortistatin receptors, able to bind selectively cortistatin but not somatostatin, have been postulated (Dello Russo et al., 2009). Cortistatin, but not somatostatin (or somatostatin fragments) displaced ghrelin from its GHSR1a binding sites in the pituitary (Deghenghi et al., 2001; Muccioli et al., 2001). The role or roles of cortistatin at GHSR1a are not yet resolved (Córdoba-Chacón et al., 2011; Prodam et al., 2008).

Second messengers

GHSR1a is promiscuous in its coupling to second messengers and downstream effectors (Camiña, 2006; Sivertsen et al., 2013). It was first recognised to couple to phospholipase C, inositol phosphate production and Ca^{2+} mobilisation in somatotrophs and when heterologously transfected into host cells (Adams et al., 1995; McKee et al., 1997). This appears to be its primary G-protein coupling, through $G\alpha_q$ (Alexander et al., 2011; Holst et al., 2003). Evidence of activation of $G\alpha_s$, and thus activation of adenylate cyclase and PKA, has been reported for neurons in the arcuate nucleus (Kohno et al., 2003). In these cells, the increase in cytoplasmic Ca^{2+} caused by

ghrelin was prevented by the PKA inhibitor, H89 and by blocking N-type Ca^{2+} channels. Thus ghrelin is deduced to activate $G\alpha_s$, and, via PKA, phophorylate and open N-type Ca^{2+} channels. Adenylate cyclase and PKA are also downstream from the novel ghrelin receptor in chondrocytes (Caminos et al., 2005). $G\alpha_{i/o}$ 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). In pancreatic islets, interaction of GHSR1a with the somatostatin receptor, SST5, results in coupling through $G\alpha_{i/o}$ (Park et al., 2012). Activation of $G\alpha_{12/13}$ in response to ghrelin is observed in transfected cells (Liu et al., 2007).

Agonist binding to GHSR1a also causes β-arrestin recruitment and internalisation, independent of G-protein signalling (Damian et al., 2012; Mokrosiński et al., 2012). There is in addition constitutive (ligand independent) internalisation of the receptor that is prevented by inverse agonists (Holst et al., 2004).

B. Constitutive activity of GHSR1a

GHSR1a exhibits a high level of constitutive activity, generally estimated to be 50% of the maximum activity induced by ghrelin. Studies of GHSR1a reconstituted in lipid discs indicate that this activity is intrinsic to the receptor, rather than from its activity in a cell environment. In lipid discs, in the absence of ligand, GHSR1a induced inositol triphosphate (IP_3) accumulation and GTPγS binding, are attenuated by an inverse agonist and increased by an agonist (Damian et al., 2012). Thus the PLC/IP_3 pathway is constitutively active. Moreover, in intact cells where the receptor is constitutively active, tagged ghrelin is internalised but the internalisation ceases when constitutive activity is blocked with an inverse agonist (Holst et al., 2004). However, ERK phosphorylation and $G\alpha_i$ coupling require agonist-mediated receptor activation (Sivertsen et al., 2013).

The physiological relevance of constitutive activity for growth hormone release is demonstrated by a naturally occurring mutation (Ala204Glu) that is associated with familial short stature (Pantel et al., 2006). The heights of these individuals are about 3 standard deviations below unaffected individuals from the same family. When the mutated receptor was investigated in transfected cells, it was found to have lost constitutive activity, but not responsiveness to ghrelin (Liu et al., 2007; Pantel et al., 2006). However, affected family members were sometimes overweight or obese, indicating that loss of constitutive activity throughout life does not cause weight loss, consistent with ghrelin and GHSR knockout models (Wortley et al., 2004; Zigman et al., 2005). Nevertheless, there is evidence that constitutive activity contributes to appetite when normal GHSR1a is present through life. Intracerebroventricular application (by minipump) of an inverse agonist (a modified substance P, MSP, see Table 1) decreased food intake, reduced weight and lowered hypothalamic

NPY expression in rats (Petersen et al., 2009). After 6 days, weight was about 5% less than in the controls.

C. Interaction sites for agonists and inverse agonists at GHSR1a

Site-directed mutagenesis and modelling have determined that the key sites for agonist binding differ from the sites that determine constitutive (ligand-independent) activity, although the sites are close to each other (Mear et al., 2013; Mokrosiński et al., 2012; Sivertsen et al., 2013). A number of modulatory sites have also been identified (Fig 2). The discovery that a single mutation (Ala204Glu) in the 2nd extracellular loop (ECL2) of the human GHSR1a causes idiopathic short stature syndrome (Pantel et al., 2006) provided clear evidence that constitutive and ligand dependent activation could be separated. When the properties of the native and mutated receptor were compared, it was found that both responded equally to agonists, but that constitutive activity was substantially diminished for the mutant (Liu et al., 2007; Pantel et al., 2006).

A critical agonist interaction site is the glutamic acid residue in the extracellular part of the 3rd transmembrane domain (TM3). Mutation of this residue, GluIII:09, using the numbering system of Schwartz (1994), to Gln rendered the receptor unresponsive to ghrelin and a range of GHSR1a agonists, but did not change ligand independent activity (Feighner et al., 1998; Holst et al., 2009). Detailed comparison of mutational effects on the agonism of ghrelin, GHRP-6, MK667, SM130686 (Table 1) and L692429 have identified residues in TM3 (including GluIII:09) and TM6 as essential to ghrelin potency, and indicate that the most important sites for ghrelin agonist action occur in a restricted region within the centre of the binding pocket, spanning TM3 and TM6 (Fig 2). For the synthetic, non-peptide agonists, sites in the ghrelin binding pocket (GluIII:09, PheVI:16, ArgVI:20) are also important for their agonist potency, but influential interaction sites also occur in the external parts of other helices, including TM2, TM 4, TM5 and TM7 (Holst et al., 2009; Holst et al., 2006). Some of these sites are important for non-peptide agonists, such as AspII:20 whose mutation reduces MK677 potency over 1000 fold (Feighner et al., 1998; Holst et al., 2009), while having less influence on the potency of ghrelin. There are also interactions with the extracellular loops that may influence efficacy (Holst et al., 2009; Holst et al., 2006).

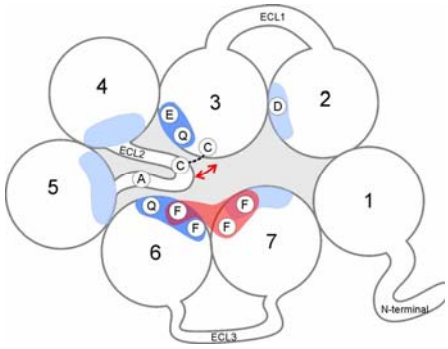


Fig. 2 here

Substitutions revealed a critical role for constitutive activity of a cluster of aromatic amino acids in TM6 and 7 (Fig 2). These were PheVI:16, plus the residues PheVII:06 and PheVII:09 on

the inner faces of the external pocket of GHSR1a, distinct from the essential interaction sites for agonists (Holst et al., 2004). The close spatial proximity of these amino acids is hypothesised to place GHSR1a in its active conformation in the absence of agonist (Holst et al., 2004; Mokrosiński et al., 2012). The level of constitutive activity can be modified up or down by manipulating the size and hydrophobicity of the residue in position VI:16. It has also been suggested that the aromatic residue VI:16 may work as a tethered agonist located strategically at the interface between TM3, TM6 and TM7, locking the extracellular ends of these TM domains in a conformation that drives high constitutive activity of GHSR1a (Schwartz et al., 2006). A conserved aromatic link crucial for high basal signaling of GHSR1a is formed by the TrpVI:13 and PheV:13 residues (Holst et al., 2004). Specific residues in the vicinity of this cluster have been proposed to orchestrate finely tuned microswitches critical for setting the activation level in absence of ligand (Holst et al., 2004; Valentin-Hansen et al., 2012). Three deeply located residues, Trp276 (TrpVI:13), ValIII:16, and IleIII:19, also significantly impact on GHSR1a constitutive activity (Gozé et al., 2010). Gozé et al. (2010) introduced the mutation TrpVI:13Ala and mutated the two surrounding amino acid residues in adjacent TM3, ValIII:16, and IleIII:19. The TrpVI:13A1a mutation reduced the ligand-independent activity but, surprisingly, the double mutation, ValIII:16Leu plus IleIII:19Met, increased GHSR1a ligand independent activity 2 fold, presumably by re-aligning TM3, 5, 6 and 7.

The molecular basis for reliance of ligand-independent activity on the ECL2 site (Ala204) identified by Pantel (2006) has been recently investigated (Mokrosiński et al., 2012). The study revealed that restricting the flexibility of ECL2, either by mutation or by ligand binding, reduces ligand independent signaling (Mokrosiński et al., 2012). ECL2 links TM4 and TM5, and has a disulphide link to TM3 (Fig 2). It is proposed that flexing of ECL2, in particular the propensity of the part of ECL2 that links TM3 and TM5 (ECL2b) to form an α–helix, is strongly influenced by Ala204, the mutation favouring the α–helix and restriction of the free movement of TM3 relative to TM5 (Mokrosiński et al., 2012).

Other studies showed that ligand properties can be switched from agonist to inverse agonist or from inverse agonist to agonist by a single amino acid or space generating substitution in the ligand peptide sequence depending on the residue mutated (Els et al., 2012; Holst et al., 2007). These studies identified the C-terminal heptapeptide (fQwFwLL) of MSP as the sequence necessary for inverse agonism, the D-Phe residue being essential (Holst et al., 2006).

III. Identification of GHSR1a and 1b expression by RT-PCR

GHSR1a in the human, pig and rat is encoded by two exons and GHSR1b is encoded by a single exon (Howard et al., 1996; McKee et al., 1997). A major issue with RT-PCR identification of the GHSR1a receptor transcript is that some studies have used primers that cannot distinguish

between the 1a and 1b isoforms or genomic DNA because the forward and reverse primer are both targeted to the first exon, for example Murata (2002) reported expression of GHSR1a mRNA in liver samples and liver cell lines using primers designed against exon 1 and not spanning the intron, whereas several other groups have not been able to detect GHSR1a mRNA in the same liver cell line or human liver samples, using primers appropriate for distinguishing 1a and 1b (Gnanapavan et al., 2002; Thielemans et al., 2007; Ueberberg et al., 2009). Others have used primers that should be able to detect the 1a isoform over the 1b isoform as the primers select against regions in exon 2 (Beiras-Fernandez et al., 2010; Iglesias et al., 2004). However, as the primers do not span an intron, there is potential that the primers were detecting genomic DNA.

Two studies quantitatively profiled expression of GHSR1a and 1b in human tissues using intron spanning primers (Gnanapavan et al., 2002; Ueberberg et al., 2009). The Gnanapavan studies used tissue obtained from surgery whereas Ueberberg used Human Total RNA Master Panel II from BD Biosciences. Both studies found the highest levels of GHSR1a mRNA expression in pituitary, consistent with its role in regulating GH function. The only other organs in which GHSR1a expression has been consistently and reliably detected by these and other studies are brain, spinal cord, pancreas, adrenal glands and heart. GHSR1b expression occurs in many more tissues and organs. Notable amongst organs where GHSR1b but not GHSR1a was expressed are adipose tissue, breast, liver, skeletal muscle and prostate (Gnanapavan et al., 2002; Ueberberg et al., 2009). As detailed below, in each of these tissues that lack GHSR1a, ghrelin has effects on cell functions.

IV. Heteromeric receptors

Several functional receptors, consisting of GHSR1a or GHSR1b in combination with another GPCRs have been identified (Jiang et al., 2006; Kern et al., 2012; Park et al., 2012; Rediger et al., 2011; Schellekens et al., 2013; Takahashi et al., 2006). Heterodimerisation with GHSR can change G-protein coupling (Park et al., 2012), can change agonist potency (Schellekens et al., 2013) and can create a receptor with pharmacology different to its component GPCRs (Takahashi et al., 2006). Experiments with pancreatic islet cells demonstrate change in G-protein coupling. In normal pancreas, GHSR1a and the somatostatin receptor, SST5, are expressed together, and ghrelin inhibits insulin secretion. In contrast, ghrelin stimulated insulin secretion from pancreas-derived cells that did not express SST5, but when these cells where transfected with *Sst5*, insulin secretion was inhibited by ghrelin (Park et al., 2012). The authors present evidence that the excitation is through $G\alpha_q$. and that inhibition is via the GHSR1a/SST5 heterodimer, coupled through $G\alpha_{i/o}$ (Park et al., 2012). Taking clues from lung cancers, in which expression of the peptide neuromedin U (NMU) and of the GHSR1b and neurotensin 1 receptors are increased, the GHSR1b/ neurotensin 1 receptor

combination was investigated. Quite surprisingly, this heterodimer functions as a NMU receptor (Takahashi et al., 2006).

The dopamine D2 receptor (DRD2)/ GHSR1a heterodimer occurs in hypothalamic neurons (Kern et al., 2012). In wild type mice, but not in *Ghsr* null mice, the dopamine agonist, cabergoline, inhibited feeding, indicating that the combination with GHSR1a was necessary for DRD2 activation. The selective GHSR1a antagonist, JMV2959 (Table 1), blocked the anorectic effect of cabergoline, suggesting that by binding to GHSR1a, JMV2959 changes the conformation and responsiveness of the coupled DRD2 receptor. Dopamine D1 receptors also form heterodimers with GHSR1a (Jiang et al., 2006; Schellekens et al., 2013)

A heterodimer between GHSR1a and the melanocortin3 receptor, MC3R, has been identified. Melanocortin signalling is enhanced 2-fold at this heterodimer, and the enhancement is reduced if a constitutively inactive GHSR1a mutant (A204E; see Section above: **Interaction sites for agonists and inverse agonists at GHSR1a**) forms the heterodimer, or if substance P, which is hypothesised to act as a GHSR1a inverse agonist, is added (Rediger et al., 2011).

The $5HT2_C$ receptor occurs in several isoforms that are produced by RNA editing, which modifies the second intracellular loop and alters signalling properties. The unedited form, $5HT2_C$R-INI has the highest constitutive activity. This isoform dimerises with GHSR1a to form a receptor that responds to both 5HT and ghrelin (Schellekens et al., 2013). The heterodimer was less responsive to ghrelin and the ghrelin receptor agonist, MK677 (Table 1) than homomeric GHSR1a. Responsiveness was restored by a $5HT2_C$ antagonist. GHSR1a/D1 and GHSR1a/MC3 heteromeric receptors also exhibited lowered responses to GHSR1a agonists (Schellekens et al., 2013). An inverse agonist of the GHSR1a receptor increased responsiveness of the GHSR1a/$5HT2_C$R-INI heterodimer to $5HT2_C$ agonism.

When GHSR1b was co-expressed with GHSR1a in HEK293 cells, the signal transduction capacity of GHSR1a was attenuated, suggesting that GHSR1b may interact with GHSR1a (Chan and Cheng, 2004). Heterodimerisation was demonstrated in lipid rafts incorporating GHSR1a and GHSR1b (Mary et al., 2013). In these rafts, GHSR1b exerted a dominant negative inhibition of GHSR1a signaling.

V. Evidence for novel receptors: ghrelin receptor-like receptors and unacylated ghrelin receptors

We have grouped the evidence for the existence of ghrelin receptor-like receptors by the cell types and organs where the evidence has been found. The most convincing evidence is from cells or tissues in which there is no detectable expression of GHSR1a, or from experiments in which responses were observed after knockout of *Ghsr*. Some of the evidence comes from experiments in

which ghrelin and UAG have parallel effects at similar concentrations, remembering that ghrelin is over 1000 times more potent than UAG at GHSR1a. The most convincing of these experiments are those in which competition for binding has been demonstrated, suggesting that the same receptor, a ghrelin receptor-like receptor, is responsible for effects of ghrelin and UAG in these tissues.

Some ligands that stimulate GHSR1a also act at sites where ghrelin is not an agonist. The sites include the UAG receptors. A UAG receptor is a receptor for UAG and related compounds at which ghrelin is a much poorer agonist or not an agonist at all. In some cases, mimicry between ghrelin and other ligands in a particular tissue could be because both GHSR1a and UAG receptors are present.

A. Adipose tissue

Overview: Published data indicate that proliferation, differentiation, lipid accumulation and glycerol handling by pre-adipocytes and adipocytes are mediated by receptors that do not discriminate between ghrelin and UAG and by UAG-selective receptors. On the other hand, inflammation of adipose tissue involves GHSR1a expressed by macrophages and possibly also adipocytes, whereas UAG agonists are anti-inflammatory. GHSR1a may also have a role in thermogenesis in brown fat (Lin et al., 2011). It is feasible that agonists of UAG receptors could assist in maintaining fat stores in lean subjects and in reducing peaks in circulating free fatty acids, for example after meals (Benso et al., 2012; Kos et al., 2009) as well as reducing inflammation in fat stores, such as occurs in metabolic disease (Delhanty et al., 2013).

Gnanapavan (2002) has reported that GHSR1a mRNA is not expressed in human adipose tissue although low levels of GHSR1b mRNA were present. On the other hand, in a study using intron-spanning primers, GHSR1a expression was found in rat adipose tissue (Choi et al., 2003). While GHSR1a expression in white and brown adipose tissues is below the detectable level in young mice, GHSR1a expression was readily detectable in visceral white fat and interscapular brown fat of old mice (Lin et al., 2011). This may be contributed to by GHSR1a expression in inflammatory cells in fat from old mice (Ma et al., 2013). It would be desirable to use *in situ* hybridisation histochemistry to determine the precise cellular sites of expression of *Ghsr1a* in adipose tissues of old animals.

1. Lipid accumulation:

Both ghrelin and UAG stimulate lipoprotein lipase levels and cause lipid accumulation in rat and human visceral adipocytes and rat bone marrow adipocytes (Kos et al., 2009; Rodríguez et al., 2009; Thompson et al., 2004). Ghrelin and UAG had similar potencies, with thresholds for increasing lipoprotein lipase levels of about 1-10 nM in human adipocytes (Kos et al., 2009). Labeled ghrelin binding in rat epididymal adipose tissue was displaced by ghrelin, UAG, MK0677 and hexarelin. All 4 agents inhibited β adrenergic agonist mediated lipolysis (Muccioli et al.,

2004). Investigation of the second messenger pathway showed that both ghrelin and UAG blocked isoproterenol induced cAMP accumulation, via activation of PI3 kinase γ/AKT and phospodiesterase 3B (Baragli et al., 2011). PI3 kinase γ, also known as a class IB PI3 kinase, is reported to be strictly GPCR activated (Andrews et al., 2007). Ghrelin and UAG were also similarly potent in bone marrow, but their actions were not reduced by the GHSR1a antagonist, L163255 (Table 1) (Thompson et al., 2004). In human adipocytes, this effect was blocked by the Y1 receptor blocker, BIBP3226, suggesting that ghrelin and UAG may act indirectly, by releasing a peptide of the pancreatic peptide, peptide YY, neuropeptide Y family (Kos et al., 2009). In human volunteers, a 16 h infusion of UAG (1 μg/kg/h) reduced plasma free fatty acid levels (Benso et al., 2012).

On the other hand, UAG, but not ghrelin, at concentrations of 10-100 nM, suppressed glycerol secretion and reduced the expression of glycerol-releasing hormone sensitive lipase, an effect not prevented by the Y1 antagonist, BIBP3226 (Kos et al., 2009). Similarly, release of glycerol and non-esterified fatty acids was reduced by UAG (10-100 pM), but not ghrelin, in differentiated adipose 3T3-L1 cells (Miegueu et al., 2011). The stimulation of fatty acid uptake in an adipose cell line (3T3-L1) was inhibited by the antagonist, (D-Lys3)-GHRP-6, a lipid raft disruptor, PI3 kinase and phospholipase C blockade (Miegueu et al., 2011). As indicated below, in the section on bone, (D-Lys3)-GHRP-6 appears to be an antagonist of ghrelin receptor-like receptors that respond to both ghrelin and UAG, as well as being a GHSR1a antagonist.

2. Adipose tissue inflammation:

Adipose tissues tend to become inflamed with age, an effect that is exacerbated by a high fat diet. Macrophages that infiltrate the fat express GHSR1a; the adipose inflammation caused by high fat diets is attenuated in *Ghsr* null mice, and numbers of pro-inflammatory macrophages in intra-abdominal fat and liver steatosis are both reduced (Ma et al., 2013). Inflammation in white fat caused by high fat diets in mice is reduced by UAG and the UAG mimetic, AZP531 (Table 1) (Delhanty et al., 2013). Thus GHSR1a and an UAG receptor may regulate opposing inflammatory reactions in fat deposits.

B. Skeletal muscle/ myoblasts

Overview: Ghrelin and UAG, through ghrelin receptor-like receptors, reduce skeletal muscle atrophy and enhance muscle repair. Identifying selective GRLR agonists is a priority, because such agonists may have therapeutic utility in treating muscle wasting and injury.

Studies in skeletal muscle and skeletal muscle derived myoblasts that do not express *Ghsr*, and in *Ghsr* knockout animals, indicate that ghrelin and UAG have parallel metabolic and tissue protective effects that cannot be through GHSR1a (Reano et al., 2014).

Both UAG and ghrelin promote differentiation and fusion of C2C12 skeletal muscle cells through what appears to be the same receptor (Filigheddu et al., 2007) and both forms of ghrelin inhibit skeletal muscle atrophy. Ghrelin and UAG were approximately equipotent, with EC_{50}s of about 1 nM, in stimulating differentiation of myoblasts in culture (Filigheddu et al., 2007). Neither motilin nor the ghrelin C-terminal fragment, ghrelin (9-28), were effective. No GHSR1a expression could be detected in undifferentiated or differentiated myoblasts, using intron spanning primers (Filigheddu et al., 2007). The IC_{50} values for the displacement of labeled ghrelin from myoblasts were 1.20 ± 0.09 nM for ghrelin and 1.32 ± 0.08 nM for UAG. In another study it was found that both ghrelin and UAG (100 µg/kg, twice daily) reduced fasting-induced skeletal muscle wasting in *Ghsr* knockout mice (Porporato et al., 2013). Both the acylated and unacylated forms of ghrelin reduced dexamethasone-induced skeletal muscle atrophy. In mice, muscle cell damage, including cell apoptosis, caused by doxorubicin, was abrogated by both ghrelin and UAG, given 12 hours later as a single injection, then by repeated doses, twice daily, over 4 days (Yu et al., 2014). In another study, UAG reduced muscle protein loss in rats with burn injury to the skin (Sheriff et al., 2012).

In addition to its positive effects on muscle cell differentiation and its inhibition of muscle atrophy, ghrelin stimulates glucose uptake by myoblasts. In the mouse myoblast cell line, C2C12, which does not express *Ghsr* (Filigheddu et al., 2007; Gershon and Vale, 2014), ghrelin stimulated glucose uptake with an EC_{50} of about 10 nM (Gershon and Vale, 2014). Surprisingly, the effect of ghrelin was inhibited by the corticotrophin releasing factor receptor2 (CRFR2) antagonist anti-sauvagine (Gershon and Vale, 2014). Both ghrelin and UAG, effective at 1 nM, increased CRFR2 expression in the muscle cells.

Skeletal muscle is repaired following ischemic damage by recruitment of resident stem cells (satellite cells) that form myoblasts that are necessary for muscle regeneration. It has been found that UAG, but not ghrelin, facilitated the division of satellite cells and their involvement in the repair process (Togliatto et al., 2013). The enhanced repair was associated with the increased expression of superoxide dismutase-2 (SOD-2) in satellite cells.

C. Bone

Overview: Ghrelin and UAG stimulate osteoblast activity and compounds that act on the GRLR, common to ghrelin and UAG, in bone may be effective in combating age-associated bone loss.

GHSR1b mRNA, but not GHSR1a, occurs in human bone and osteoblasts, and both ghrelin and UAG stimulate osteoblast proliferation (Delhanty et al., 2006). Peak responses occurred at 1 and 10 nM for ghrelin and UAG, respectively. Proliferation was reduced by inhibition of ERK or PI3 kinase (Delhanty et al., 2006). In another study, which showed that *Ghsr* expression was

undetectable in bone cells and cell lines from rat and mouse, ghrelin was also shown to increase mitogenic activity in osteoblasts (Costa et al., 2011). Ghrelin also increased the bone-resorbing activity of rat osteoclasts (Costa et al., 2011). The proliferative effect of ghrelin on rat osteoblast cell lines was prevented by the antagonist (D-Lys3)-GHRP-6 (Kim et al., 2005), but this antagonist does not appear to be specific for GHSR1a, as these cell lines do not express *Ghsr*. Ghrelin and UAG (1 nM) both reduced reactive oxygen species production and protected cells of the murine osteoblast cell line, MC3T3-E1, from peroxidase-induced oxidative injury (Dieci et al., 2014). There was no effect of the potent GHSR1a agonist, macimorelin (EP1572; (van der Lely et al., 2004)) and the effect was also not antagonised by (D-Lys3)-GHRP-6.

D. Chondrocytes

Human and mouse chondrocytes respond to ghrelin, but do not express GHSR1a (Caminos et al., 2005). Binding studies revealed high affinity (Kd = 3.8 nM) and lower affinity binding (Kd = 130 μM) sites (Caminos et al., 2005). Ghrelin increased cAMP with a threshold effect at about 1 nM. Ghrelin decreased chondrocyte metabolic activity and decreased basal and insulin stimulated fatty acid uptake. Whether chondrocytes respond to UAG was not tested.

E. Pancreatic islets

Overview: Published evidence points to expression of GHSR1a, GRLR and UAG receptors in the pancreas. GHSR1a is expressed by pancreatic β cells, where ghrelin inhibits insulin secretion (see section IV, Heteromeric receptors) , and ghrelin, also through its GHSR1a -dependent action leading to increased release of insulin-like growth factor, is diabetogenic (Vestergaard et al., 2008). However, through GRLR and UAG receptors in the islets, ghrelin and UAG increase glucose tolerance and insulin release. They also increase β cell survival and proliferation. Compounds selective for non-GHSR1a receptors in the islets may have the potential to be anti-diabetogenic.

It is well established that ghrelin, acting through GHSR1a, inhibits insulin secretion (Broglio et al., 2001; Dezaki et al., 2007; Tong et al., 2010). *Ghsr* null mice demonstrate a lower fasting blood glucose and increased insulin sensitivity relative to wild type littermates when fed a high fat diet (Lin et al., 2011; Qi et al., 2011). However, ghrelin has other effects that cannot be explained by actions at GHSR1a.

Both ghrelin and UAG increase cell survival and inhibit apoptosis of pancreatic β-cells induced by serum starvation or inflammatory mediators, with similar potencies (Granata et al., 2007). Investigation of the pancreatic β-cell line, HIT-T15, and human pancreatic β-cells, points to the presence of receptor that bind ghrelin, UAG, UAG fragments and modified UAG fragments, notably cyclised ghrelin6-13 (AZP531; Table 1). The sequence of AZP531 is distinct from the sequence necessary for activation of GHSR1a, which is ghrelin1-4 (Bednarek et al., 2000).

Moreover, neither GHSR1a mRNA nor protein was detected in HIT-T15 cells (Granata et al., 2007). Labeled ghrelin bound to the cells and was displaced by ghrelin (IC_{50} = 3.8 ± 0.6 nM), UAG (IC_{50} = 2.3 ± 0.4 nM) and hexarelin (IC_{50} = 20.8 ± 2.3 nM). Scatchard analysis indicated the presence of a single binding site with similar affinity for ghrelin and UAG. Ghrelin and UAG both caused proliferation of HIT-T15 and human pancreatic β-cells (Granata et al., 2007; Granata et al., 2012). NF449, a Gαs protein-coupled receptor antagonist completely blocked ghrelin and UAG-induced cell proliferation, whereas pretreatment with pertussis toxin was ineffective. Consistent with Gαs coupling, ghrelin and UAG both caused accumulation of cAMP and activation of PI3kinase/AKT and ERK1/2 (Granata et al., 2012).

AZP531, which has no effect at GHSR1a, administered by a subcutaneous osmotic minipump prevented glucose intolerance and insulin resistance that was induced by 2 or 4 weeks of high fat diet in mice (Delhanty et al., 2013). AZP531 and UAG also reduced the body weight gain and fat mass increase caused by the high fat diet, without altering energy intake. Part of the insulin resistance induced by high fat may be hepatic, as AZP531 prevented the HFD-induced suppression of hepatic insulin receptor substrate 1 (*Irs1*) gene expression (Delhanty et al., 2013). AZP531, given intravenously, increases blood pressure in the anaesthetised rat, whereas ghrelin has a hypotensive effect (Callaghan et al., 2014).

UAG, in contrast to ghrelin, stimulates insulin release. Exogenous UAG (3-30 nmol kg^{-1}) enhanced insulin secretion in i.v. glucose challenged sedated rats, and ghrelin (30 nmol kg^{-1}) reversed the effect of UAG (Gauna et al., 2007a). Infusion of UAG in human volunteers (1.0 mg/kg/h for16 h), compared to saline infusion, improved glucose clearance and enhanced insulin release in response to meals taken during the infusion (Benso et al., 2012). Free fatty acid levels were also decreased, suggesting increased uptake or decreased lipolysis. Consistent with this observation, again in human volunteers, infusion of ghrelin inhibited insulin release, an effect that was antagonised by UAG (1.0 μg/kg i.v. bolus) (Broglio et al., 2004). Another study found that co-administration of ghrelin and UAG as a single i.v. bolus injection causes a significant decrease in insulin concentration in non-diabetic subjects suffering from morbid obesity (Kiewiet et al., 2009). However another group employing 4 μg/kg UAG in human volunteers did not observe any counterbalancing between the effect of ghrelin on blood glucose by UAG (Tong et al 2014). The glucose concentration did not change in the first hour after combined administration, suggesting an improvement in insulin sensitivity.

F. Heart, cardioprotection and ionotropic effects

1. Cardioprotection:

Overview: Ghrelin protects the heart against isoproterenol-induced injury (Chang et al., 2004b), ischemia-reperfusion injury (Chang et al., 2004a; Zhang et al., 2009) and reduces damage due to myocardial infarct (Locatelli et al., 1999; Soeki et al., 2008). Evidence that is reviewed below indicates that there are several receptor types in the heart, including GRLR for ghrelin and UAG, UAG receptors and GHSR1a. Cardioprotection appears to be mediated, primarily, through non-selective receptors for ghrelin and UAG (GRLRs) on cardiomyocytes. Receptors on endothelial cells (Section H below) may also contribute to cardiac protection and repair.

When compared directly, ghrelin and UAG have similar potencies in protecting the myocardium against drug-induced injury, inhibition of cardiomyocyte apoptosis and inhibition of cardiac endothelial cell death (Baldanzi et al., 2002; Pei et al., 2014).

The use of cardiomyocyte cell lines has partially clarified the mode of action of ghrelin and UAG in cardioprotection. The cancer therapeutic, doxorubicin, exhibits cardiotoxicity and causes death of rat-derived H9c2 cardiomyocytes, an effect that is inhibited by both ghrelin and UAG (Baldanzi et al., 2002). Although H9c2 cardiomyocytes do not express *Ghsr1a*, ghrelin and UAG recognize a common high affinity binding site (Kd = 4 nM) on these cells. Two other agonists, MK677 and hexarelin, recognized the common ghrelin and UAG binding sites. The affinity of ghrelin binding sites on H9c2 cardiomyocytes was about 10-fold lower than the affinity at GHSR1a in pituitary and hypothalamic membranes (Baldanzi et al., 2002). Ghrelin also protects H9c2 cells from H_2O_2 induced apoptosis (Zhang et al., 2011). Binding experiments on ventricular membranes from guinea-pigs found that ghrelin, UAG and hexarelin recognized a common high-affinity (Kd 0.51± 0.06 nM) binding site (Bedendi et al., 2003). Ghrelin binding to rat cardiomyocytes revealed a single binding site, with a Kd of 0.25 nM (Chang et al., 2004a).

In vivo studies confirm that UAG is cardioprotective (Pei et al., 2014). Mice in which cardiac function was compromised by doxorubicin were given UAG (100 µg/kg, i.p., twice daily for 4 days). Decreases in ventricular fractional shortening, increased fibrosis and apoptosis caused by doxorubicin were reversed by UAG. Cardiac protection by hexarelin, *in vivo*, after ischemia and reperfusion was observed in hypophysectomised rats, and is thus not an indirect effect of GH release by ghrelin receptor agonists (Locatelli et al., 1999).

In vivo effects (D-Lys3)-GHRP-6 (Pei et al., 2014) differ from those observed *in vitro* (Baldanzi et al., 2002); the prevention of doxorubicin-induced myocardial fibrosis and apoptosis by UAG was not antagonised by (D-Lys3)-GHRP-6 applied *in vivo* (Pei et al., 2014), whereas this compound reversed UAG effects on cardiomyocytes *in vitro* (Baldanzi et al., 2002). Another study

that provides contrasting data on cardioprotection is that of Frascarelli (2003). It was reported that neither the potent GHSR1a agonist, MK667 (Table 1) nor UAG was cardioprotective, whereas ghrelin and hexarelin were cardioprotective in perfused rat hearts subjected to ischemia/reperfusion injury.

Other experiments have identified cardiac binding sites that do not recognise ghrelin. One site in rat cardiac membranes was identified as CD36, a glycoprotein that is expressed in adipose tissue, platelets, monocytes/ macrophages, dendritic cells, and microvascular endothelium. Hexarelin binding to CD36 was displaced by hexarelin-related peptides, including the antagonist (D-Lys3)-GHRP-6, but not by ghrelin (Demers et al., 2004). The functional effects of hexarelin binding to CD36 of cardiomyocytes have not been reported.

A recent binding study (Lear et al., 2010) using a murine cardiomyocyte line, HL-1, and isolated mouse and rat cardiomyocytes, has identified two binding sites for UAG, with Kds of 0.65 and 47 nM, that were not effectively displaced by ghrelin, ghrelin 9-28 or motilin. Displacement of labelled UAG with cold UAG yielded IC_{50} values of 4 and 400 nM (Lear et al., 2010). In the HL-1 cells, UAG increased medium-chain fatty acid uptake, whereas ghrelin did not modify fatty acid uptake.

2. Inotropic effects:

Studies on the inotropic effects of ghrelin and UAG have showed that they both reduced guinea pig papillary muscle mechanical tension in a dose dependent manner, their action depended on NO synthesis as well as on endocardial endothelium integrity (Bedendi et al., 2003). The negative inotropic effect of ghrelin on papillary muscle has been studied in rat where it was also found to be an action independent of GHSR1a (Soares et al., 2006). In contrast to the endothelium mediated, GHSR1a independent, negative inotropic effects of ghrelin and UAG, ghrelin also has positive inotropic effects on isolated rat ventricular myocytes (Sun et al., 2010). This effect of ghrelin is GHSR1a dependent, involves augmented L type Ca^{2+} channel currents and was blocked by (D-Lys3)-GHRP-6.

G. Heart, Coronary artery constriction

Ghrelin, 0.1 and 1 nM, increased arterial resistance in the isolated perfused rat heart and 1 nM constricted isolated coronary artery segments (Pemberton et al., 2004). These effects were blocked by the L-type Ca^{2+} channel antagonist, diltiazem, and by the PKC inhibitor bisindolylmaleimide. Both GHRP6 and hexarelin constricted rat coronary arteries (Xu et al., 2003). In anaesthetised pigs, direct infusion of ghrelin also caused coronary artery constriction (Grossini et al., 2007). The effect was prevented by the antagonist of β-adrenoceptors, butoxamine. It was suggested that ghrelin blocks a tonic β_2-adrenergic receptor-mediated vasodilatation. Radiolabelled ghrelin binds to

human coronary arteries with a Kd of 0.2 nM and binding is competed off with hexarelin (Katugampola et al., 2001). Binding was reported to be on the smooth muscle (Katugampola et al., 2001).

In mice, hexarelin caused coronary vasoconstriction that was lacking in CD36 knockout animals (Bodart et al., 2002). Moreover, coronary arteries of spontaneous hypertensive rats, which are CD36 deficient, were substantially less responsive to hexarelin, compared to normotensive rats. In a GHSR1a reporter mouse, no evidence of GHSR1a expression was found in coronary arteries (Callaghan et al., 2012).

H. Endothelial cells: nitric oxide (NO) production, proliferation and angiogenesis

Overview: Protective effects of ghrelin and UAG against endothelial cell damage caused by serum starvation, oxidative stress or hyperglycaemia through as yet unidentified receptors have been reported. There is some evidence that the actions of UAG are through activation of endothelial NOS (eNOS). This effect could possibly be targeted to assist tissue revascularisation or to attenuate endothelial cell damage in diabetes.

Protective effects were first reported in porcine aortic endothelial cells stressed by serum starvation (Baldanzi et al., 2002). Apoptosis was reduced by about 80% with either ghrelin or UAG (1 μM). It was later reported that UAG, but not ghrelin, attenuated circulating endothelial progenitor cell damage in diabetic patients (Togliatto et al., 2010). In these experiments, patients were given UAG (3 μg/kg/h) or ghrelin (1 μg/kg/h) as an intravenous infusion for 12 h, and blood samples were collected to isolate endothelial progenitor cells and circulating angiogenic cells. UAG infusion doubled the number of viable cells, whereas ghrelin (at 1/3 the dose) had no effect. The cells were capable of *de novo* vessel formation, which was enhanced by UAG. Similar observations were made on cells from obese mice. Fluorescent-tagged UAG bound to endothelial progenitor cells and circulating angiogenic cells. It was displaced by unlabeled UAG, but not by ghrelin. In experiments on mouse cells, from wild-type and eNOS knockouts, it was shown that the actions of UAG were dependent on rescue of eNOS activity (Togliatto et al., 2010). Several other studies have also shown that ghrelin activates eNOS, including in endothelial cells from human umbilical vein (Xu et al., 2008) and bovine, human (Iantorno et al., 2007), rat (Shimizu et al., 2003) and mouse (Xu et al., 2008) aortic endothelial cells. However, effects of UAG were not tested in these studies. In contrast, Shimada et al (2014) have confirmed that UAG protects human vascular endothelial cells, but comparison with ghrelin was not made. The activation of eNOS is caused through Akt and AMP kinase mediated phosphorylation (Xu et al., 2008). The effects in bovine aortic endothelial cells were inhibited by knockdown of *Ghsr1a* by treatment with siRNA (Iantorno et al., 2007), suggesting that GHSR1a is involved in this species.

There is evidence that actions on the capillary endothelium within human pancreatic islets contributes to islet protective effects of both ghrelin and UAG (Favaro et al., 2012). Treatment with 3 ghrelin gene products, ghrelin, UAG and obestatin, reduced NO and inflammatory cytokine release from the endothelium. Competitive binding data suggested that obestatin, but not ghrelin or UAG, exerted its effects through binding to the GLP-1 receptor. Ghrelin and UAG increased survival and significantly inhibited glucose-induced apoptosis of the endothelial cells, through increased cAMP and activation of PI3K/Akt, ERK1/2 phosphorylation.

I. Liver

The literature is consistent in failing to detect GHSR1a in liver. GHSR1b, but not 1a, expression was detected in human liver (Gnanapavan et al., 2002; Ueberberg et al., 2009) and GHSR1a could not be detected in the liver of mouse (Lim et al., 2013) or rat (Moreno et al., 2010). GHSR1a expression was low to undetectable in pig hepatocytes, whereas the authors found high levels of expression in the pancreas (Gauna et al., 2005). Western blot analysis failed to reveal GHSR1a protein in the mouse liver (McGirr et al., 2011), although the sensitivity is such that small amounts of protein would probably remain undetected.

Despite the absence of GHSR1a, ghrelin (100 nM) stimulates glucose production in isolated porcine liver cells whereas they do not respond to the potent GHSR1a agonist, hexarelin (Gauna et al., 2005). Similarly, hexarelin did not alter glucose levels in human (Broglio et al., 2004). Effects on liver are also observed in *Ghsr* null mice, in which UAG changes the hepatic expression of genes involved in cell death, growth and proliferation (Delhanty et al., 2010).

Hepatic insulin sensitivity is decreased in obesity, an effect that is associated with decreased expression of insulin receptor substrate 1 (IRS-1). In mice, a high fat diet decreased *Irs1* gene expression, which was prevented by infusing the mice with UAG or the UAG receptor agonist, AZP531 (Delhanty et al., 2013). Consistent with effects on insulin/ glucose pathways in liver, in *Ghsr* knockout mice, UAG increased the hepatic expression of *Lcn2* and *Nox4*, genes that are linked to insulin sensitivity (Delhanty et al., 2010).

J. Tumor cells

Overview: Receptors for ghrelin, UAG and related ligands are present in several tumor types, and in cancer cell lines derived from tumors (Chopin et al., 2012). This includes cancers of breast (Cassoni et al., 2004; Jeffery et al., 2005), the gastroenteropancreatic system (Leontiou et al., 2007; Nikolopoulos et al., 2010), liver (Murata et al., 2002), pituitary (Korbonits et al., 2001) and prostate (Jeffery et al., 2002; Lanfranco et al., 2008) in all of which ghrelin influences cancer cell proliferation. However, both proliferative and anti-proliferative effects are observed and no useful therapeutic agents based on this pharmacology have emerged.

Cassoni (2001) used displacement of (^{125}I)Tyr-Ala-hexarelin to characterise ghrelin receptor-like receptors in breast tumour tissue and breast cancer cell lines. Ghrelin and UAG displaced hexarelin binding with IC_{50}s of 190 and 800 nM. By contrast, UAG is over 1000 times less potent than ghrelin at GHSR1a (see above). Unrelated peptides did not displace hexarelin from the tumor cells. Binding to these ghrelin receptor-like receptors was found in well-differentiated carcinomas of the breast and in several breast cancer-derived cell lines, including the commonly used MCF7 and MDA-MB-231 lines. Two laboratories have shown that neither line exhibits detectable GHSR1a gene expression (Cassoni et al., 2001; Jeffery et al., 2005), whereas GHSR1b gene expression is readily detected (Gahete et al., 2011; Jeffery et al., 2005). It is possible that the ghrelin receptor-like receptors of breast cancers are heterodimers of the non-functional GHSR1b with another GPCR (see earlier section: Heteromeric receptors).

Proliferation of the estrogen-dependent breast cancer cell line, MCF7, is *inhibited* by ghrelin (Cassoni et al., 2001). It is notable that ghrelin does not have an effect in the absence of estrogen (Jeffery et al., 2005). Consistent with an inhibitory role of ghrelin in breast cancers, ghrelin expression is significantly correlated to low histological grade, estrogen receptor positivity, small tumor size and low proliferation of human breast tumors (Grönberg et al., 2008). In contrast, ghrelin at concentrations of 10 and 100 nM significantly *increased* proliferation of the estrogen-independent breast cancer cell line, MDA-MB-231 (Jeffery et al., 2005). Another product of the ghrelin gene, in1-ghrelin (in which intron 1 is translated) also increases proliferation (Gahete et al., 2011). Because most breast cancers express the ghrelin precursor gene and the acylating enzyme, GOAT (Gahete et al., 2011; Grönberg et al., 2011; Grönberg et al., 2008), it is a reasonable hypothesis that ghrelin or other ghrelin gene products modulate breast cancer growth, the literature indicating that growth is reduced in estrogen-dependent breast cancers and increased in aggressive estrogen-independent breast cancers.

In prostate carcinomas, there is absence of both GHSR1a and GHSR1b expression, although GHSR1b expression occurs in 50% of benign prostate hyperplasias (Cassoni et al., 2004). Ghrelin had a dose dependent proliferative effect on the prostate cell line, LNCaP, with a threshold of about 0.1 nM, and a peak response with 10 nM ghrelin (Yeh et al., 2005). Both ghrelin and UAG, at 10-100 pM, increased proliferation of the PC-3 cell line (Cassoni et al., 2004). However, neither GHSR1a nor GHSR1b expression was found in the LNCaP or PC-3 cell lines (Cassoni et al., 2004). In the human prostate cell lines, DU-145 and PC-3, and cells from prostate carcinomas, both ghrelin and UAG displaced labeled (Tyr 4) ghrelin (Cassoni et al., 2004). Unlike the other prostate cancer cell lines, DU-145 does express GHSR1a (Cassoni et al., 2004) and agonists of GHSR1a inhibit proliferation (Pellegrino et al., 2013). The displacement of label with UAG suggests that this cell line may express a ghrelin receptor-like receptor.

K. Appetite

Overview: Consistence evidence indicates that the orexigenic effects of ghrelin released from gastric ghrelin stores are mediated through GHSR1a. However, a number of studies indicate that actions at both GRLR and UAG receptors, most likely in the hypothalamus, can influence appetite. Whether non-GHSR1a have physiological roles in control of food intake has not been determined.

Comparison of the effects of the GHSR1a ligand, BIM28163, on GH release and appetite also revealed the presence of a novel GRLRs (Halem et al., 2005). BIM-28163 bound to GHSR1a and blocked ghrelin's action at the GHSR1a receptor in transfected cells and it also blocked ghrelin's stimulatory effect on GH secretion (Halem et al., 2005). In contrast, BIM28163 and ghrelin both stimulated food intake and in the dorso-medial hypothalamus and c-Fos, a marker of activated neurons, was induced by both ghrelin and BIM-28163 (Halem et al., 2005). This suggests that some of the receptors through which ghrelin stimulates appetite are different from GHSR1a. Consistent with the effect of ghrelin on appetite being mediated through a receptor other than GHSR1a, the GHSR1a antagonist, GSK1614343, does not block ghrelin-induced feeding (Costantini et al., 2011).

Intracerebroventricular (icv) UAG, but not ghrelin, increases food intake in rats, wild type and *Ghsr* knockout mice, suggesting that there are ghrelin receptor-like receptors in central appetite controlling circuits (Toshinai et al., 2006). In fasted wild type male mice of the ddy strain, icv or ip UAG (~3 nmol) was reported to decrease food intake (Asakawa et al., 2005), however other studies found no effect of icv UAG (7.5 nmol) in fasted male C57Bl6 mice (Neary et al., 2006) or fasted ddy mice (Toshinai et al., 2006). Peripherally applied ghrelin, but not UAG, increases feeding, and *Ghsr* knockout prevents the orexigenic effect of peripherally administered ghrelin (Sun et al., 2004; Toshinai et al., 2006). In humans, food intake was decreased after UAG infusion compared with ghrelin or ghrelin plus UAG infusions, but was not different from saline control (Tong et al 2014).

L. Other evidence suggesting novel receptors

Some other reports that suggest novel receptors are briefly summarised below. In each case these are isolated reports, and in some cases sites and/or mechanisms of effect are unknown.

UAG mimics ghrelin's action in the nucleus tractus solitarius, where injection of either compound lowers blood pressure with almost equal potency (Tsubota et al., 2005). Icv UAG (0.1 and 0.5 nmolar solutions) reduced the temperature of the dorsal body surface and increased tail temperature, suggesting that a blood flow redistribution was elicited by UAG (Inoue et al., 2013). Icv ghrelin mimicked UAG. However, the effect of ghrelin, but not that of UAG was blocked by the antagonist, (D-Lys3)-GHRP-6 (Inoue et al., 2013). Ip injection of UAG (0.03 and 0.1 mg/kg

body weight) caused similar, but less potent effects. Both icv and ip UAG induced cFos expression in neurons of the median pre-optic area, a region known to regulate body temperature.

There are several reports of UAG attenuating effects of ghrelin, for example reducing the appetite stimulatory effects of ghrelin (Inhoff et al., 2008) and reducing its effect on colorectal propulsion (Hirayama et al., 2010) and having opposite effects on insulin secretion (see above). A transgenic mouse overexpressing UAG at levels 10-44 fold greater than in control mice exhibits a small phenotype with a shorter nose to anus length and lower body weights (Ariyasu et al 2005).

It is reported that nanomolar concentrations of UAG, hexarelin, and EP80317 counteract the stimulation of IL-1β and IL-6 synthesis in microglial cells by the Alzheimer disease associated β–amyloid protein (Bulgarelli et al., 2009). Ghrelin was ineffective. Expression GHSR1a was undetectable in the microglial cells.

VI. Conclusions

The only molecularly defined receptor for ghrelin, GHSR1a, is important for many of its actions, but this receptor has very low affinity for UAG, too low for circulating UAG to be a hormone that acts at GHSR1a. However, there is clear evidence of cell and organ responses to low, generally nanomolar, concentrations of UAG, both at sites where ghrelin is also an agonist and at sites where UAG alone has agonist effects. Thus, we postulate that there are two families of ghrelin receptors that are yet to be identified at a molecular level: receptors at which ghrelin and UAG are both potent agonists (ghrelin receptor-like receptors) and receptors at which UAG, but not ghrelin, is a potent agonist (UAG receptors). GRLRs are expressed by adipocytes, myoblasts, osteoblasts, cardiac muscle cells, pancreatic islet cells (which also express GHSR1a), coronary arteries, vascular endothelial cells and cancer cells (breast and prostate). UAG receptors are expressed by hepatocytes and pancreatic beta cells.

It is necessary to determine the molecular natures of the GRLRs that have been reported in many cells types that are essential to human health. Until this is done, there will remain uncertainty about whether the effects at what appear to be non-specific ghrelin receptors or unacylated ghrelin receptors are truly at unique receptors. In the case of the UAG receptors, clinical trials are already underway, using a ghrelin analogue whose molecular site of action is still unknown. No agonist other than UAG has yet been convincingly demonstrated to act at GRLRs, but not ghrelin receptors. Further development of selective ligands will be problematic until the receptors are isolated. It is notable that receptors for ghrelin on pancreatic islet cells are heterodimers of GHSR1a and the somatostatin receptor, SST5. Further exploration of heteromeric GPCRs may uncover other physiologically important GRLRs.

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The authors contributed equally to the literature search, analysis of the literature and to writing the review.

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Footnotes

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Tables

Table 1: Ghrelin receptor ligands

Compound	Properties, characterisation	Relevant pharmacological studies, studies in human	References
Agonists This is a selection of many hundreds of agonists in the patent literature. Except for AZP531 they were developed as GHSR1a agonists. We concentrate on those that have been trialed in human and/or are judged to be useful for biological testing and receptor characterisation.			
Anamorelin (RC1291; ONO7643)	Metabolically stable modified tripeptide. Orally active	Therapy for the treatment of cancer cachexia. Anamorelin increases GH, IGF-1, IGFBP-3, appetite and body weight in human.	(Garcia et al., 2013; Garcia and Polvino, 2009), (Paul et al., 2006), (Garcia and Polvino, 2007)
AZP531	Cyclic ghrelin (and des-acyl ghrelin) 6-13. Not an agonist at GHSR1a	Selective agonist for the UAG receptor. Phase 1 clinical trial, commenced July 2013 to provide data on AZP531 actions on metabolic dysfunction, particularly on hyperglycemia. (Alizé Pharma). Anticipated use in type 2 Diabetes.	(Julien et al., 2012)
Capromorelin (CP424391)	Pyrazolinone-piperidine dipeptide. Centrally penetrant. Orally active.	Being tested for treatment of frailty (Phase 2) and GERD (Phase 1) (RaQualia Pharma Inc) Shown to increase lean body mass Originally produced by Pfizer, now licensed to RaQualia.	(Pan et al., 2001), (White et al., 2009)
GSK894490 (Compound 24)	Non-peptide. Orally active	Readily cross the blood/brain barrier and elicits pro-cognitive effects in recognition and spatial learning and memory tests	(Witherington et al., 2008), (Atcha et al., 2009)
CP464709	Non-peptide	Centrally penetrant agonist with long half life	(Carpino et al., 2002)
GHRP-6	His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2.	The first effective and commonly used GHS. Short *in vivo* half life. Stimulates GH secretion in vivo. EC_{50} 1.5 nM.	(Bowers et al., 1984) (Raun et al., 1998)
Hexarelin	His-2-methyl-D-Trp-Ala-Trp-D-Phe-Lys-NH_2. Methylated analogue of GHRP-6	One of the most commonly experimentally used GHSR1a agonists. Increases GH release and muscle mass. Popular with body builders. EC_{50} 2 nM in pituitary cells.	(Ghigo et al., 1994)
Ibutamoren (MK677, L163191)	Non-peptide, substituted spiropiperidine sulfonamide. Orally active as mesylate	Effective agonists of GHSR1a. MK677, GHRP-6 and L692585 have about 50% greater efficacy than ghrelin and have been termed super agonists. Ibutamoren increases GH, and IGF-I, and IGFBP-3 levels. In postmenopausal women shown to have a positive effect on bone mineral density. Used off-prescription by body builders.	(Codner et al., 2001), (Murphy et al., 2001) (Nass et al., 2008) .

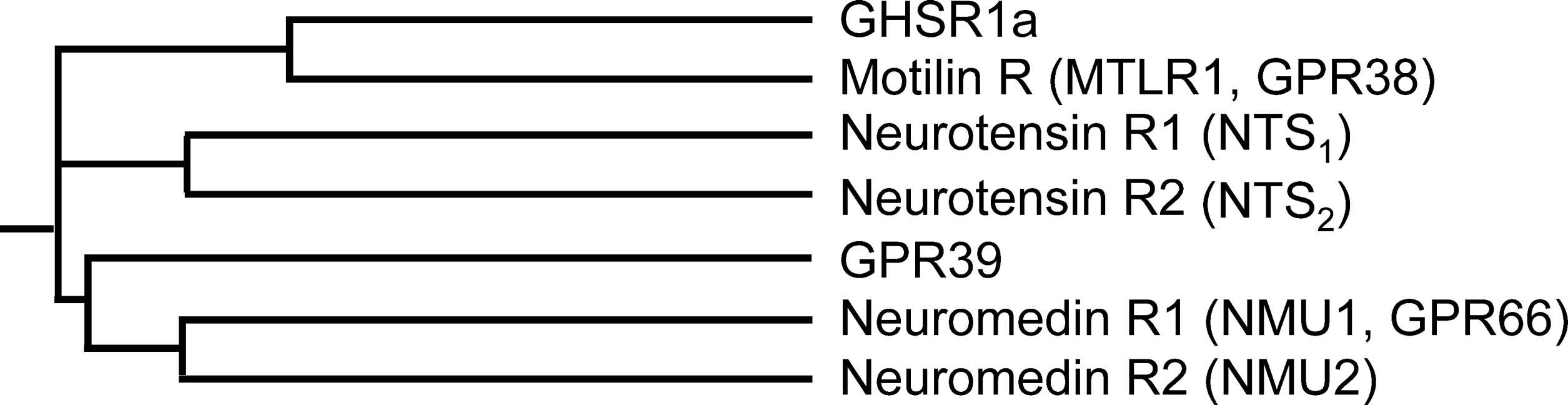
Ipamorelin	Synthetic pentapeptide (Aib-His-D-2-Nal-D-Phe-Lys-NH_2), originally from Novo Nordisk	Being tested for post-operative ileus – phase 2b (Helsinn Pharma) Demonstrated to significantly increase the cumulative fecal pellet output, food intake, and body weight gain in rodent model of post-operative ileus.	(Raun et al., 1998), (Venkova et al., 2009)
L163255	Spiropiperidine. Orally active.	Oral dose of 1 µg/kg increased GH release in pig. IGF-I also elevated. Shown to increase retroperitoneal and perirenal white adipose tissue mass without having any significant influence on inguinal or epididymal fat.	(Chang et al., 1996), (Davies et al., 2009)
Macimorelin acetate (AEZS130) (ARD07) (EP01572)	Synthetic small molecule. Orally active as acetate.	Phase 1 studies completed and phase 2 studies underway to use macimorelin as a treatment of cachexia, Phase 3 studies of a diagnostic test for growth hormone deficiencies in adults have been completed. (Aeterna Zentaris)	(Guerlavais et al., 2003), (MacLean et al., 2009)
RM131 (BIM28131)	Pentapeptide, formerly called BIM-28131	Tested for diabetic gastroparesis in women Currently in Phase 2 clinical trials for diabetic gastroparesis and GI functional disorders. (Rhythm Pharmaceticals)	(DeBoer et al., 2008), (Shin et al., 2013)
SM130686	Oxindole derivative. Orally active.	Partial agonist. EC_{50} = 3.0 nM. From Sumitomo Chemical Co	(Nagamine et al., 2001)
Tabimorelin (NN703)	Modified peptide Orally active	Tested in adults with GH deficiency. Therapeutic effects on growth-hormone deficient adults were limited.	(Hansen et al., 1999), (Svensson et al., 2003)
Ulimorelin (TZP101)	macrocyclic peptidomimetic	Ki=16 nM, EC_{50}=29 nM . Long half life in human, 13 h (Lasseter et al., 2008). Tests in patients with gastroparesis showed minor improvement. Phase 3 trial for post-operative ileus demonstrated compound safety, but ulimorelin showed no advantage compared to placebo. It is reported to have low potency to release growth hormone (Fraser et al., 2008)	(Hoveyda et al., 2011), (Shaw et al., 2013)
Antagonists Many antagonists of GHSR1a have been synthesised, but few have gone forward to clinical trial and most of the compounds are not readily available. Compounds from Abbott, Bayer, Elixir, Merck, Tranzyme and Zentaris have been reviewed (Zhao et al., 2008)			
(D-Lys3)-GHRP-6	Hexapeptide, His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH2	Originally described by Cheng 1989, this is one of the most commonly used antagonists in cell based and animal studies. IC_{50}, 900 nM. However, it also displaces agonists such as hexarelin from CD36 (IC_{50} 6 μM) and it is an antagonist of ghrelin action at GRLRs of osteoblasts (Kim et al., 2005).	(Cheng et al., 1989)

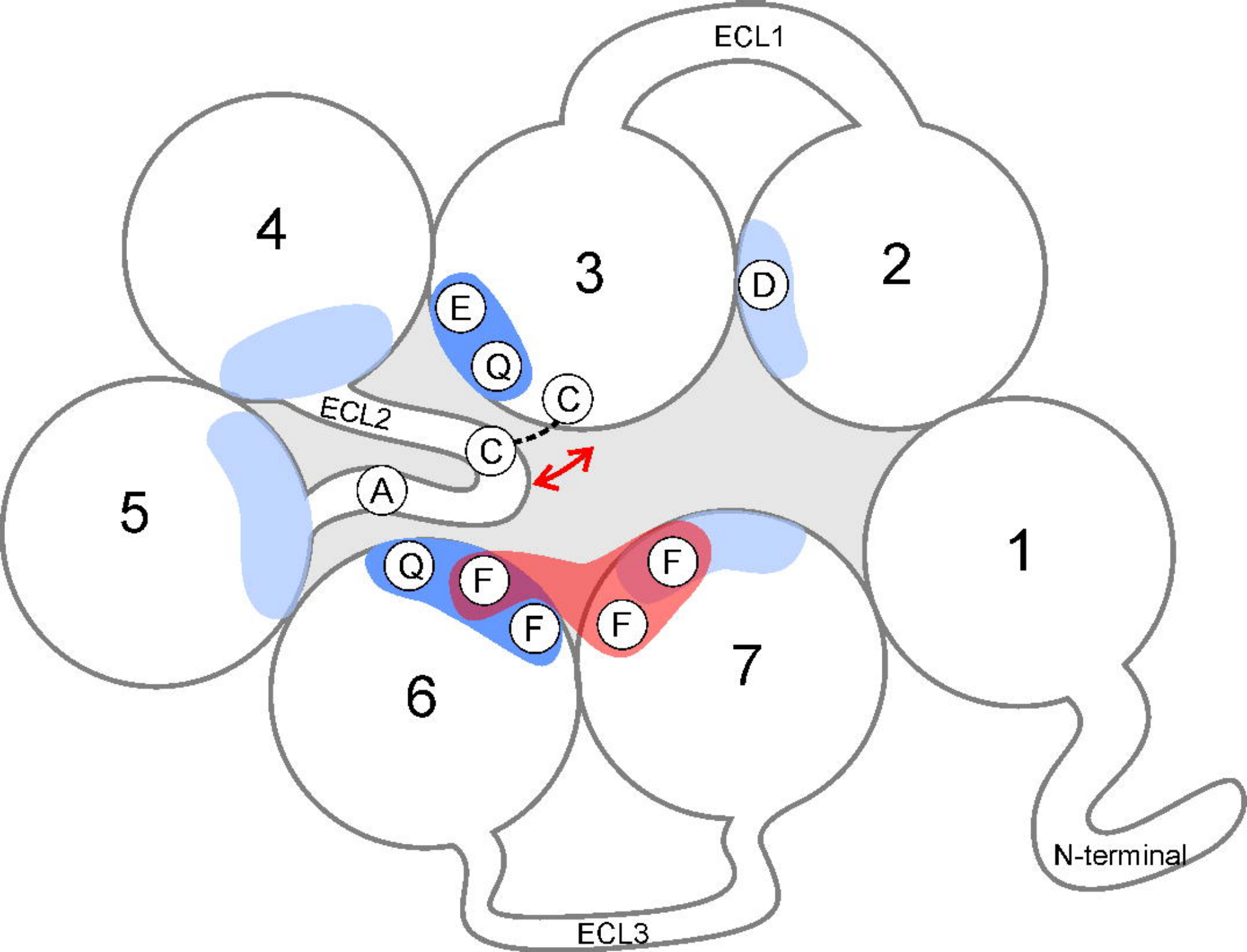
YIL781	Piperidine-substituted quinazolinone derivative. Orally active.	Competitive antagonist with pKB of 7.54 in IP accumulation assay. Centrally penetrant and centrally effective antagonist in rat in vivo. Available commercially for experimental use.	(Esler et al., 2007), (Perdona et al., 2011), (Callaghan et al.)
AEZS123 (JMV2959)	Non-peptide with trisubstituted 1,2,4-triazole structure	IC_{50}, 32 nM and a Kb of 19 nM. No agonist action. Related antagonists are described by Moulin (2008).	(Salomé et al., 2009)
GSK1614343 (Compound 18ad2 in Sabbatini 2010).	Carbohydrazide dipeptide trifluoromethyl derivative	Competitive antagonist with pKB of 8.03 in IP accumulation assay. Not able to antagonize ghrelin-induced food consumption in rat, but unexpectedly stimulated food intake and body weight gain in both rats and dogs, a profile associated with decreased ghrelin plasma levels.	(Sabbatini et al., 2010), (Costantini et al., 2011), (Perdona et al., 2011),
Inverse Agonists acting at GHSR1a			
Modified Substance P (MSP)	Peptide EC_{50} = 5.2 nM	Modified substance P, [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]substance P, is a low-potency antagonist but a high-potency full inverse agonist. A range of related substance P derivatives also had inverse agonist properties.	(Holst et al., 2006)
Substance P derivative, KwFwLL	Hexapeptide EC_{50} = 36 ± 8 nM	A series of derivatives of MSP have been synthesised, leading to Lys-D-Trp-Phe-D-Trp-Leu-Leu-NH2, which has little remaining resemblance to substance P and is an inverse agonist with no residual antagonist (or agonist) activity.	(Holst et al., 2007)
10n (Pfizer)	Spiro-azetidino-piperidine	One of the first in a series of Spiro-azetidino-piperidine derivatives developed at Pfizer. pKi versus ghrelin 8.13 ± 0.21.	(Kung et al., 2012)
12a (Pfizer)	Spiro-azetidino-piperidine-triazole	pKi versus ghrelin 7 nM.	(McClure et al., 2013)
PF5190457	Spiro-azetidino-piperidine	Orally active. pKi versus ghrelin 8.36. Increases glucose-stimulated insulin secretion in human isolated pancreatic islets. The compound has a predicted human absorption of 86% and half-life of 6.3 h.	(Bhattacharya et al., 2014)

Figure Legends

Fig 1. **The ghrelin receptor family.** Schematic phylogenetic tree of the ghrelin receptor family indicating the relationships of the receptors. For alternative naming of the receptors, see Alexander (2011).

Fig 2. Key sites for agonist activation and constitutive activity of GHSR1a. The diagram represents the receptor seen from its external surface, with transmembrane domains indicated by 1-7. In dark blue are binding surfaces for ghrelin and in light blue other sites involved in agonist interactions, especially interactions of non-peptide agonists. The red area defines a pocket bordered by aromatic amino acids that determines constitutive activity. The extracellular loop 2 (ECL2) is linked through a disulphide to TM3. Mutation of the Ala (A) in this loop to Glu changes ECL2 conformation and reduces constitutive activity.


GHSR1a
Motilin R (MTLR1, GPR38)
Neurotensin R1 (NTS1)
Neurotensin R2 (NTS2)
GPR39
Neuromedin R1 (NMU1, GPR66)
Neuromedin R2 (NMU2)


ECL1
4
3
2
E
Q
D
C
ECL2
C
A
5
1
Q
F
F
F
F
6
7
N-terminal
ECL3

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