

Review Article

Ghrelin: a hormone regulating food intake and energy homeostasis

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Regulation of energy homeostasis requires precise coordination between peripheral nutrient-sensing molecules and central regulatory networks. Ghrelin is a twenty-eight-amino acid orexigenic peptide acylated at the serine 3 position mainly with an *n*-octanoic acid, which is produced mainly in the stomach. It is the endogenous ligand of the growth hormone secretagogue (GHS) receptors. Since plasma ghrelin levels are strictly dependent on recent food intake, this hormone plays an essential role in appetite and meal initiation. In addition, ghrelin is involved in the regulation of energy homeostasis. The ghrelin gene is composed of four exons and three introns and renders a diversity of orexigenic peptides as well as des-acyl ghrelin and obestatin, which exhibit anorexigenic properties. Ghrelin stimulates the synthesis of neuropeptide Y (NPY) and agouti-related protein (AgRP) in the arcuate nucleus neurons of the hypothalamus and hindbrain, which in turn enhance food intake. Ghrelin-expressing neurons modulate the action of both orexigenic NPY/AgRP and anorexigenic pro-opiomelanocortin neurons. AMP-activated protein kinase is activated by ghrelin in the hypothalamus, which contributes to lower intracellular long-chain fatty acids, and this appears to be the molecular signal for the expression of NPY and AgRP. Recent data suggest that ghrelin has an important role in the regulation of leptin and insulin secretion and vice versa. The present paper updates the effects of ghrelin on the control of energy homeostasis and reviews the molecular mechanisms of ghrelin synthesis, as well as interaction with GHS receptors and signalling. Relationships with leptin and insulin in the regulation of energy homeostasis are addressed.

Energy balance: Energy expenditure: Food intake: Ghrelin: Obesity

Over the last 20 years, growing attention has been paid to the various elements involved in the regulation of energy balance; research has identified the critical role of hypothalamic peptide systems in the central regulation of appetite and energy metabolism (Ahima & Osei, 2001; Nakazato *et al.* 2001). The discovery of ghrelin and its influence on appetite, utilisation of energy substrates, weight and body composition – together with its growth hormone (GH) releasing activity – adds a further component to the complex interactions involved in regulating energy balance (Kojima *et al.* 1999; Takaya *et al.* 2000; Tschop *et al.* 2000; Nakazato *et al.* 2001; Kojima *et al.* 2004).

Regulation of energy homeostasis requires precise coordination between peripheral nutrient-sensing molecules and central regulatory networks (Hahn *et al.* 1998). Ghrelin, a twenty-eight-amino acid peptide acylated at the serine 3 (Ser3) position with *n*-octanoic or other medium-chain fatty

acids, produced mainly in the stomach, as well as in other gastrointestinal segments and selected areas of the brain, is the first peripheral orexigenic hormone identified (Kojima *et al.* 1999; Date *et al.* 2000; Tomasetto *et al.* 2000; Tschop *et al.* 2000; Nakazato *et al.* 2001; Wren *et al.* 2001). Des-acyl ghrelin (desacylated or unacylated ghrelin) coexists with acylated forms both in the gastrointestinal tract and plasma (Hosoda *et al.* 2003; Fig. 1).

Ghrelin was initially found as an endogenous ligand of the GH secretagogue (GHS) receptor (GHS-R; Takaya *et al.* 2000) and several studies have provided evidence that ghrelin is involved in the regulation of energy homeostasis. Ghrelin levels increase before and decrease after meals, potentially playing a role in meal initiation and satiety in an inverse pattern to that of insulin (Tschop *et al.* 2000; Cummings *et al.* 2001; Wren *et al.* 2001; Bacha & Arslanian, 2005). In addition, ghrelin is involved in the regulation of energy

Abbreviations: ACC, acetyl-CoA carboxylase; AgRP, agouti-related protein; AMPK, AMP-activated protein kinase; ARC, arcuate nucleus; CART, cocaine amphetamine-related transcript; CNS, central nervous system; GH, growth hormone; GHRH, growth hormone-releasing hormone; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; ICV, intracerebroventricular; LCFA, long-chain fatty acid; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; Ser3, serine 3; VTA, ventral tegmental area.

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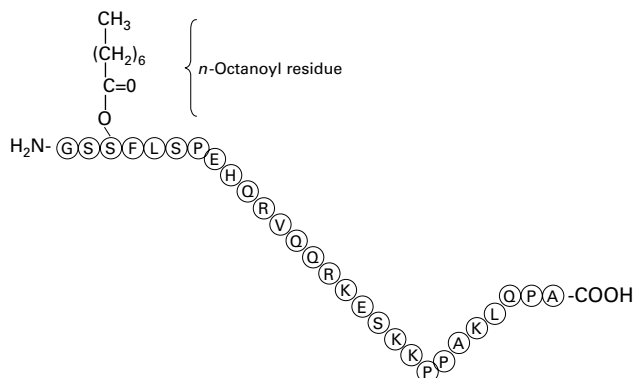


Fig. 1. Structure of human acylated (1–28)-ghrelin. Ghrelin variants with twenty-seven amino acids lack Arg at the C-terminus. The *n*-octanoyl residue at the serine 3 position is essential for neuroendocrine activities of 27- and 28-ghrelin. Other medium-chain fatty acids, namely decanoic (10:0) and decenoic (10:1) acids, can also acylate ghrelin. Des-acyl-ghrelin lacks the *n*-octanoyl residue and seems to have peripheral activities but it does not exhibit any neuroendocrine activity. Amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

balance by increasing food intake and reducing fat utilisation (Tschop *et al.* 2000; Nakazato *et al.* 2001; Wren *et al.* 2001). Moreover, ghrelin regulates glucose metabolism (Patel *et al.* 2006) and possibly is involved in the regulation of insulin activities in man (Murata *et al.* 2002). Likewise, ghrelin appears to be related with the regulation of energy expenditure (St-Pierre *et al.* 2004; Zigman *et al.* 2005; Maffei *et al.* 2006).

Importantly, signalling by circulating ghrelin is mediated downstream by neurons of the arcuate nucleus (ARC) of the hypothalamus; in particular, neurons expressing neuropeptide Y (NPY) and agouti-related protein (AgRP), two potent orexigenic peptides (Guan *et al.* 1997; Kamegai *et al.* 2001; Chen *et al.* 2004; Groppe *et al.* 2005).

Ghrelin has changed our understanding of the central regulation of GH secretion, appetite control and energy balance, and the importance of ghrelin as a unique hormone regulating energy homeostasis is supported by more than 1400 papers published from its discovery until January 2006. In spite of that, it is thought not to be physiologically involved in the regulation of GH secretion, since circulating levels are not correlated with those of GH, either in physiological or in pathological conditions. Nevertheless, co-administration of GH-releasing hormone (GHRH) and ghrelin has synergistic effects on pituitary GH secretion. Ghrelin-induced GH secretion is mostly antagonised by somatostatin via a GHRH-dependent mechanism and also through an independent effect mediated by GHS-R (Casanueva & Diéguez, 2002).

Injected centrally or peripherally ghrelin stimulates the appetite more effectively than does any other molecule except NPY, with which it is fairly equipotent, and it is the only hormone that increases short-term food intake when administered to mice and human subjects (Asakawa *et al.* 2001; Wren *et al.* 2001). In addition, chronic administration of ghrelin increases body weight, due to its effects promoting adipogenesis (Asakawa *et al.* 2001), and decreases energy expenditure (Asakawa *et al.* 2003; Zigman *et al.* 2005; De Smet *et al.* 2006; Maffei *et al.* 2006), fat catabolism and lipolysis (Tschop *et al.* 2000; Muccioli *et al.* 2004). The secretion

pattern of ghrelin is inverse to that of insulin, and its action is opposed to that of leptin, which inhibits the synthesis of NPY and AgRP in the hypothalamus (Rosická *et al.* 2003; Kim *et al.* 2004).

Although the acylated forms of ghrelin have been recognised as the major active orexigenic molecules regulating energy balance (Date *et al.* 2000; Hosoda *et al.* 2000a), recent data provide evidence that when studying the effects of ghrelin on energy balance, differential influences of the acylated and non-acylated forms of the peptide must be considered. Although the correlation between the two forms is good, they may be different hormones with opposite actions, with non-acylated ghrelin being able to antagonise some of the effects of the acylated form (Asakawa *et al.* 2005; Chen *et al.* 2005a,b; Ukkola, 2005).

On the other hand, ghrelin stimulates lactotroph and corticotroph function, influences the pituitary–gonadal axis, inhibits pro-inflammatory cytokine expression, controls gastric motility and acid secretion and influences pancreatic exocrine and endocrine function, as well as impacting on glucose metabolism (van der Lely *et al.* 2004; Otto *et al.* 2005). Moreover, ghrelin also inhibits apoptosis and enhances osteoblast differentiation (Kim *et al.* 2005). Ghrelin also possesses regulatory properties in the cardiovascular system, both in the heart and in the vasculature (Nagaya *et al.* 2001a,b; Wiley & Davenport, 2002; Kleinz *et al.* 2006), it mediates antiproliferative effects in neoplastic cell lines, and influences sleep, memory and anxiety-like behavioural responses (Eisenstein & Greenberg, 2003; van der Lely *et al.* 2004; Ghigo *et al.* 2005). That is possible since GHS-R is expressed not only in the brain but also in peripheral tissues, especially the stomach, intestine, pancreas, thymus, gonads, thyroid and heart (Howard *et al.* 1996; Sun *et al.* 2004). Fig. 2 summarises the biological effects of ghrelin.

The present paper examines only the effects of ghrelin on the control of appetite and energy balance; GHS activity and other peripheral effects are not addressed. The structure and role of ghrelin and its derived molecules, as well as their receptors, are reviewed. Likewise, we have placed emphasis on the central neuronal pathways which mediate the ghrelin effects on energy homeostasis and its suggested molecular mechanisms of action.

Historical background

Since the 1970s it has been known that a number of small synthetic molecules termed GHS act on the pituitary gland and the hypothalamus to stimulate and amplify pulsatile GH release (Bowers *et al.* 1977) and this phenomenon was later shown independent of GHRH (Giustina *et al.* 1997). This was followed by the development of a number of both peptide and non-peptide GHS (Bercu & Walker, 1997).

In 1996, Howard *et al.* (1996) cloned a heterotrimeric GTP-binding protein-coupled receptor of the pituitary and arcuate ventromedial and infundibular hypothalamus of swine and man and it was shown to be the target of the GHS. In addition, on the basis of its pharmacological and molecular characterisation, they suggested that the GTP-binding protein-coupled receptor defined a new neuroendocrine pathway for the control of pulsatile GH release and supported the notion that the GHS mimicked an undiscovered hormone.

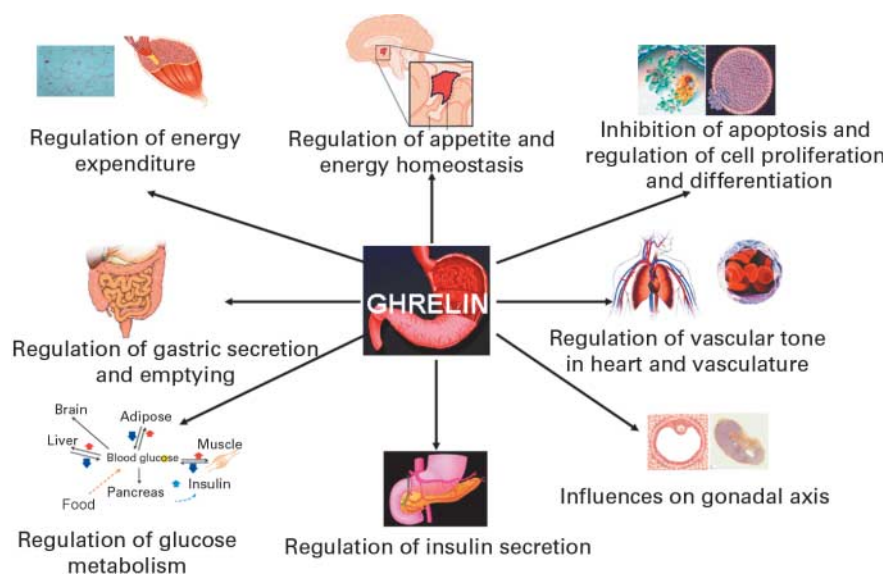


Fig. 2. Main biological functions of ghrelin. Ghrelin is mainly synthesised at the stomach but it can be produced in low amounts in other tissues such as the gastrointestinal tract, the pancreas and the hypothalamus, having endocrine, paracrine or autocrine character. Many of the biological functions of ghrelin are indirectly mediated via the hypothalamus, brainstem and vagal nerve afferents; others, such as the effects on apoptosis and cell proliferation, and regulation of vascular tone in the heart and vasculature, are direct.

In December 1999, Kojima *et al.* (1999) reported for the first time the purification and identification in rat stomach of an endogenous ligand specific for the GHS-R. The purified ligand was a twenty-eight-amino acid peptide named as ghrelin, in which the Ser3 residue was acylated with a molecule of *n*-octanoic acid. The acylated peptide specifically released GH both *in vivo* and *in vitro* and *O*-*n*-octanoylation was essential for the activity. They designated the GH-releasing peptide as 'ghrelin', a term that contains 'ghre' as the etymological root for 'growth' in many languages. 'GH' and 'relin', a suffix for releasing substances in generic names according to United States Pharmacopoeia 'USP Dictionary of USAN and International Drug Names' (www.uspusan.com), also represents an abbreviation for 'growth-hormone release', a characteristic effect of ghrelin (Kojima *et al.* 1999; Hosoda *et al.* 2000b).

In August 2000, Tomasetto *et al.* (2000) also reported the isolation and sequencing of a complementary DNA obtained from mouse gastric epithelium and its expression was examined at both mRNA and protein levels. The novel gene identified and characterised encoded the termed 'pre-pro-motilin-related peptide', had similarity sequence with pre-pro-motilin and coincided with that of ghrelin (Del Rincón *et al.* 2001).

Takaya *et al.* (2000) studied GH-releasing activity and other effects generated by ghrelin in four normal men aged 28–37 years. They demonstrated that ghrelin strongly stimulates GH release in human subjects in a dose-dependent manner. Per mol, ghrelin is more potent for GH release than GHRH. The lowest dose of ghrelin used led to massive GH release, with minimum effects on corticotropin or prolactin. Ghrelin administration did not change serum luteinising hormone, follicle-stimulating hormone or thyroid-stimulating hormone levels.

Tschop *et al.* (2000) showed that peripheral daily administration of ghrelin caused adiposity by reducing fat utilisation in mice and rats.

Intracerebroventricular (ICV) administration of ghrelin generated a dose-dependent increase in food intake and body

weight. Rat serum ghrelin concentrations were increased by fasting and were reduced by refeeding or oral glucose administration, but not by water ingestion. Tschop *et al.* (2000) proposed that ghrelin, in addition to its role in regulating GH secretion, signals the hypothalamus when an efficient metabolic state is necessary.

Nakazato *et al.* (2001) demonstrated that ghrelin is involved in the hypothalamic regulation of energy homeostasis. ICV injections of ghrelin strongly stimulated feeding in rats and increased body-weight gain. Ghrelin also increased feeding in rats that were genetically deficient in GH. Anti-ghrelin IgG robustly suppressed feeding. After ICV ghrelin administration, Fos protein, a marker of neuronal activation, was found in regions of primary importance in the regulation of feeding, including NPY/AgRP neurons. Antibodies and antagonists of NPY and AgRP abolished ghrelin-induced feeding. Ghrelin augmented NPY gene expression and blocked leptin-induced feeding reduction, implying that there is a competitive interaction between ghrelin and leptin in feeding regulation. These authors concluded that ghrelin is a physiological mediator of feeding and probably has a function in growth regulation by stimulating feeding and release of GH.

Date *et al.* (2001) demonstrated a role for ghrelin in the central regulation of gastric function. Specifically, ICV administration of ghrelin stimulated gastric acid secretion in a dose-dependent and atropine-sensitive manner. Vagotomy abolished gastric acid secretion. Immunohistochemistry demonstrated the induction of Fos expression in the nucleus of the solitary tract and dorsomotor nucleus of the rat vagus nerve.

Kojima *et al.* (2001) reviewed the role of ghrelin. The peptide is found in the secretory granules of X/A-like cells, a distinct endocrine cell type found in the submucosal layer of the stomach (Date *et al.* 2000). These cells contain round, compact, electron-dense granules and are filled with ghrelin. Ghrelin immunoreactive cells are also found in the small and large intestines.

Cummings *et al.* (2002) investigated plasma ghrelin levels after weight loss induced by diet or by gastric bypass surgery. They found an increase in the plasma ghrelin level with diet-induced weight loss. Gastric bypass was associated with markedly suppressed ghrelin levels, possibly contributing to the weight-reducing effect of the procedure.

Cowley *et al.* (2003) discovered expression of ghrelin in a previously uncharacterised group of neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular and arcuate hypothalamic nuclei. These neurons send efferents onto key hypothalamic circuits, including those producing NPY, AgRP, pro-opiomelanocortin (POMC) products and corticotropin-releasing hormone. Within the hypothalamus, ghrelin is bound mostly on presynaptic terminals of NPY neurons. Using electrophysiological recordings, these authors found that ghrelin stimulated the activity of arcuate NPY neurons and mimicked the effect of NPY in the paraventricular nucleus (PVN) of the hypothalamus, and they proposed that at these sites, release of ghrelin may stimulate the release of orexigenic peptides and neurotransmitters, thus representing a novel regulatory circuit controlling energy homeostasis.

Chen *et al.* (2004) in a series of elegant experiments using knockout mice demonstrated that NPY and AgRP are required for the orexigenic effects of ghrelin, as well as the involvement of the melanocortin pathway in ghrelin signalling.

Two major molecular forms exist in rats and man: ghrelin, which is acylated at Ser3 with a medium-chain fatty acid – usually *n*-octanoic acid – and des-acyl ghrelin (Date *et al.* 2000; Hosoda *et al.* 2000a, 2003). In man, des-octanoyl ghrelin is the predominant circulating form of the peptide (Hosoda *et al.* 2003), which, until recently, was considered biologically inactive. While ghrelin activates GHS-R-expressing cells, the non-modified des-*n*-octanoyl form of ghrelin (des-acyl ghrelin) does not (Hosoda *et al.* 2000a). In addition to those forms, multiple ghrelin-derived molecules produced by post-translational processing of the ghrelin gene (*GHRL*) have been identified. As indicated earlier in the present review, both ghrelin and the ghrelin-derived molecules were found to be present in plasma as well stomach tissue (Date *et al.* 2000; Hosoda *et al.* 2000a, 2003) and, to a lesser extent, in the arcuate, ventromedial and infundibular nuclei of the hypothalamus (Howard *et al.* 1996; Papotti *et al.* 2000; Muccioli *et al.* 2001).

Furthermore, the pre-pro-ghrelin peptide also produces obestatin. Contrary to the appetite-stimulating effects of ghrelin, treatment of rats with obestatin suppressed food intake, inhibited jejunal contraction, and decreased body-weight gain (Zhang *et al.* 2005).

Non-acylated ghrelin, which is present in human serum in far greater quantities than acylated ghrelin, was initially considered to be devoid of any endocrine action. However, it is able to exert some non-endocrine actions including cardiovascular and antiproliferative effects, probably by binding different GHS-R subtypes or receptor families (Date *et al.* 2000; Baldzani *et al.* 2002; Bedendi *et al.* 2003; Cassoni *et al.* 2004; Ariyasu *et al.* 2005; Kleinz *et al.* 2006).

In addition to roles in meal initiation, weight regulation and gastrointestinal activity, ghrelin also regulates the pituitary hormone axis, carbohydrate metabolism, and various functions of the heart, kidney, pancreas, adipose tissues, gonads and cell proliferation (Gnanapavan *et al.* 2002; Corbetta *et al.* 2003; Dixit *et al.* 2004; Tena-Sempere, 2005). Cardiovascular

actions and modulation of the proliferation of neoplastic cells, as well as of the immune system, are also actions of ghrelin and/or other GHS (van der Lely *et al.* 2004). Moreover, ghrelin also inhibits TNF- α -induced apoptosis and suppresses caspase-3 activation as well as enhancing osteoblast differentiation (Kim *et al.* 2005).

Role of ghrelin and its receptors

Structure of the ghrelin gene and diversity of ghrelin molecules

The ghrelin human gene (*GHRL*) is located on 3q25–26 and is predicted to be composed of four exons and three introns (Wajnrajch *et al.* 2000) that encode a molecule designated pre-pro-ghrelin (Kojima *et al.* 1999). The presence of a short non-coding first exon has also been suggested to occur in rat and mouse ghrelin genes (Tanaka *et al.* 2001). Fig. 3 shows the human *GHRL* as well as the main transcriptional and translational products.

Using rat ghrelin cDNA, a human stomach cDNA library was screened and analysis of several clones yielded a deduced amino acid sequence for the designated human pre-pro-ghrelin (a 117-amino acid precursor; Kojima *et al.* 1999). The putative initiation codon ATG is located at nucleotides 34–36, preceded by the consensus initiation sequence, whereas a terminal codon TAG is found 117 codons downstream at position 385–387. A typical polyadenylation signal, AATAAA, is found at position 494–499 (Hosoda *et al.* 2003) similar to that occurring in the mouse and rat *GHRL* (Tanaka *et al.* 2001). The open reading frame starting at the ATG codon encodes pre-pro-ghrelin and the N-terminal twenty-three-residue sequence of pre-pro-ghrelin exhibits features characteristic of a secretory signal peptide; the ghrelin sequence starts from Gly24, which directly follows the signal peptide, and the last two residues of ghrelin Pro-Arg correspond to a processing signal (Kojima *et al.* 1999).

Functional analysis using promoter–reporter constructs containing the 5'-flanking region of the human *GHRL* indicate that the sequence residing within the –349 to –193 region is necessary for human ghrelin promoter function in a human medullary thyroid carcinoma cell line. Within this region existed several consensus sequences for a number of transactivating regulatory proteins, including an E-box site. Destruction of this site decreased the promoter activity to 40%. The upstream region of the promoter has two additional putative E-box sites, and site-directed mutagenesis suggested that these are also involved in promoter activation (Kanamoto *et al.* 2004).

Amino acid identities between rat and human pre-pro-ghrelins are 82.9% and only two amino acids (Arg11–Val12) are replaced in the twenty-eight-amino acid residue segment, indicating that ghrelins are highly conserved between species (Kojima *et al.* 1999; Hosoda *et al.* 2003), particularly in mammals (van der Lely *et al.* 2004). Partial conservation of the ghrelin sequence has been confirmed in other species such as chicken, bullfrog and tilapia (Kaiya *et al.* 2001, 2003; Saito *et al.* 2002). Nevertheless, results indicate that although the regulatory mechanism of ghrelin to induce GH secretion is evolutionarily conserved, the structural changes in the different ghrelins result in species-specific receptor binding

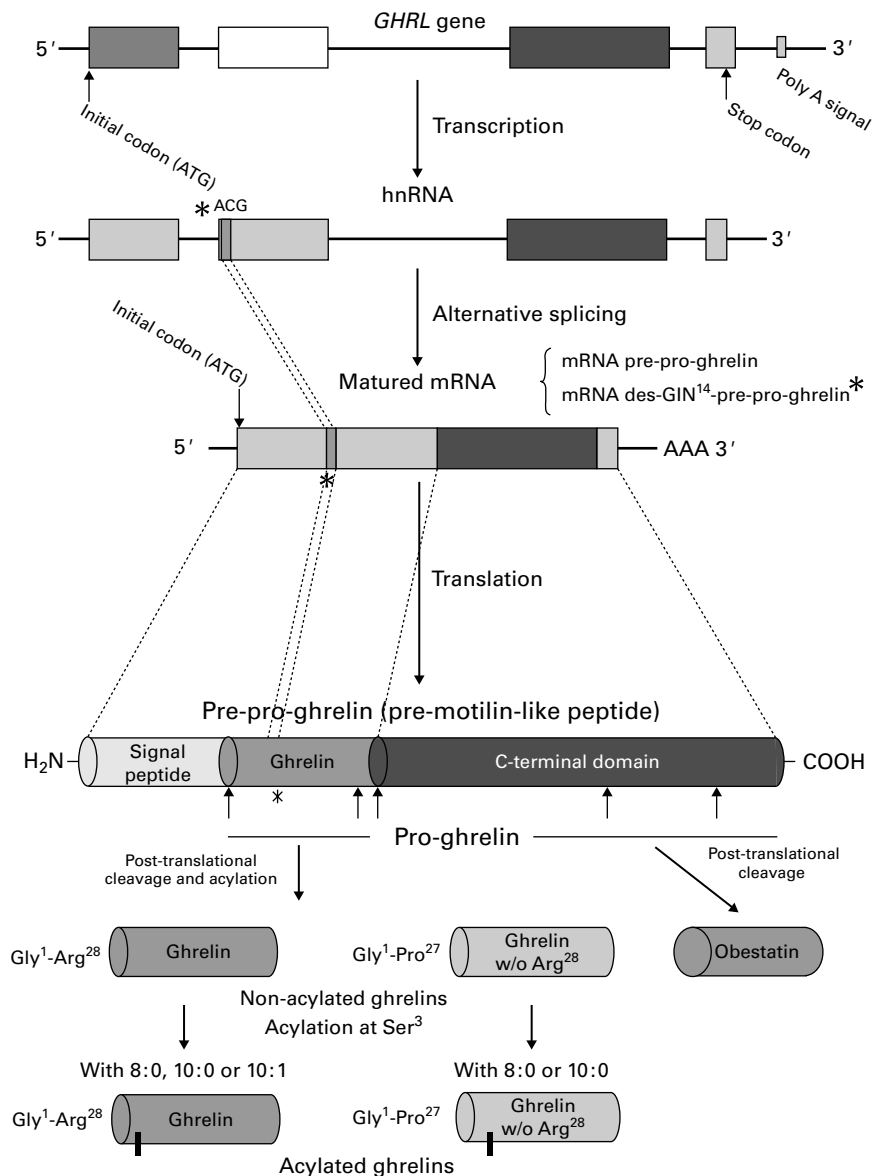


Fig. 3. Structure of the human ghrelin gene, transcription, alternative splicing of high nuclear RNA (hnRNA), translation of matured RNA and post-translational processes leading to the synthesis of acylated and non-acylated ghrelins, as well as the new discovered molecule obestatin. Alternative splicing of the intron 1 at the ACG codon results into two different mRNA, which in turn originate two different pre-pro-ghrelin molecules; one of them is without (w/o) the Arg in position 14 (des-Gln14-pre-pro-ghrelin). This molecule is practically absent in the stomach of man but present in that of rats.

(Kaiya *et al.* 2001; Saito *et al.* 2002). The sequence of human ghrelin and acylation of the Ser³ residue are shown in Fig. 1.

Sequence comparison between ghrelin and motilin shows that both molecules have a number of identical amino acids, which suggest that ghrelin and motilin might have evolved from a common ancestral peptide. However, motilin is not modified by *n*-octanoic acid or by other acyl acids (Kojima *et al.* 2001).

The purification of a second endogenous ligand for GHS-R from rat stomach has also been reported (Hosoda *et al.* 2000b). This ligand, named des-Gln14-ghrelin, is a twenty-seven-amino acid peptide whose sequence is identical to ghrelin except that one glutamine (14th Gln of ghrelin) is missing in des-Gln14-ghrelin. This new GHS-R ligand also has an *n*-octanoyl modification at Ser³ like ghrelin, which is also

essential for its activity. Furthermore, genomic sequencing and cDNA analysis indicated that des-Gln14-ghrelin is not encoded by a gene distinct from ghrelin but is encoded by an mRNA created by alternative splicing of the ghrelin gene. The analysis of the genomic structure of rat ghrelin revealed that an intron exists between Gln13 and Gln14 for the ghrelin sequence (Hosoda *et al.* 2000b; Wajnrajch *et al.* 2000). The 3'-end of the intron has two tandem CAG sequences. The AG parts of these sequences may serve as splicing signals (McKeown, 1992). When the first AG is used for the splicing signal, pre-pro-ghrelin mRNA is produced and the second CAG is translated into Gln14. On the other hand, when the second AG is used, pre-pro-des-Gln14-ghrelin mRNA is generated to produce pre-pro-des-Gln14-ghrelin, which contains 116 amino acid residues, and then by post-translational

cleavage, des-Gln14-ghrelin missing Gln14 (Hosoda *et al.* 2000b). Although nearly all of the cDNA clones isolated from human stomach encoded the pre-pro-ghrelin precursor, a few cDNA clones encoded the pre-pro-des-Gln14-ghrelin precursor (Hosoda *et al.* 2003). The ratios observed between the two precursor populations, ghrelin and des-Gln14-ghrelin, was 5:1 in rat stomach and 6:5 in mouse stomach (Tanaka *et al.* 2001). However, the amount of des-Gln14-ghrelin from the human stomach extracts is negligible.

Recently, a novel, exon 3-deleted pre-pro-ghrelin isoform has been detected in breast and prostate cancer, although its potential function is unknown (Yeh *et al.* 2005). Exon 4-deleted pro-ghrelin, a novel mouse pro-ghrelin isoform with a unique C-terminal peptide sequence, is also widely expressed in the mouse and thus may possess biological activity (Jeffery *et al.* 2005).

The purification and characterisation of human ghrelin and other minor ghrelin-derived molecules from the stomach has been reported, and the collected ghrnelins have been classified into two groups on the basis of amino acid length and into four groups by type of acylation at Ser3: non-acylated, octanoylated, decanoylated, and possibly decenoylated. Although the major active form of human ghrelin is a twenty-eight-amino acid peptide with an *n*-octanoyl modification at Ser3, the ghrelin-derived molecules observed include octanoyl ghrelin-(1–27), decanoyl ghrelin-(1–28), decanoyl ghrelin-(1–27), and decenoyl ghrelin-(1–28). Moreover, the non-active forms des-acyl ghrelin and des-acyl ghrelin-(1–27) are also present in the human stomach (Hosoda *et al.* 2003). Furthermore, all of these molecular forms of ghrelin were found in human plasma as well as in the stomach. In human stomach, the twenty-seven-amino acid ghrelin:twenty-eight-amino acid ghrelin processing product ratio was observed to be 1:3. It is likely that the twenty-seven- and twenty-eight-amino acid ghrelin molecules isolated are produced through alternative C-terminal processing of the same ghrelin precursor (Weber *et al.* 1983).

Ghrelin has been the first example discovered of a bioactive peptide modified by an *n*-octanoic acid moiety (Kojima *et al.* 1999) and all of the acyl-modified ghrnelins and ghrelin-derived molecules studied have the same potency to induce an increase of Ca²⁺ concentration in the GHS-R-expressing cells and stimulate GH release in anaesthetised rats (Hosoda *et al.* 2003). In addition, the *de novo* synthesis of molecules has demonstrated that octanoic acid is not the only Ser3 modification that will confer full activity to ghrelin (Bednarek *et al.* 2000; Matsumoto *et al.* 2001). In fact, short peptides encompassing the first four or five residues of ghrelin were found to functionally activate GHS-R about as efficiently as the full-length ghrelin and only the segment Gly-Ser-Ser-(*n*-octanoyl)-Phe appeared to constitute the 'active core' required for agonist potency at human GHS-R (Bednarek *et al.* 2000). In addition, more stable ether or thioether bonds of the Ser3 are capable of replacing the octanoyl ester bond in ghrelin, advantageous for the generation of pharmaceuticals with longer stability (Matsumoto *et al.* 2001).

In the human stomach, the octanoylated:decanoylated ghrelin ratio was found to be roughly 3:1 (Hosoda *et al.* 2003). Because acylation of ghrelin is essential for its activity, the enzyme that catalyses this modification step should be an important regulator of ghrelin biosynthesis. However, the

mechanism by which ghrelin is acylated during post-translational processing remains to be clarified.

Very recently, Zhang *et al.* (2005), searching GenBank for orthologues of the human ghrelin gene and comparing pre-pro-ghrelin sequences from eleven mammalian species, have found in addition to the known ghrelin mature peptide, which immediately follows the signal peptide, another conserved region that was flanked by potential convertase cleavage sites. This region encodes a putative twenty-three-amino acid peptide, with a flanking conserved glycine residue at the C terminus, suggesting that it might be amidated. This new ghrelin-associated peptide has been named 'obestatin', a contraction of obese, from the Latin 'obedere' meaning 'to devour', and 'statin' denoting 'suppression'. Contrary to the appetite-stimulating effects of ghrelin, treatment of rats with obestatin suppressed food intake, inhibited jejunal contraction and decreased body-weight gain. Obestatin is bound to the orphan GTP-binding protein-coupled receptor GPR39 that shares homology with GHS-R. Both receptors could have evolved from a common ancestor but diverged in their functions, thus maintaining a delicate balance of body-weight regulation. Thus, two peptide hormones with opposing action in weight regulation are derived from the same ghrelin gene and activate distinct receptors (Zhang *et al.* 2005).

Genetic variability

Korbonits *et al.* (2002) studied the ghrelin gene in a group of seventy tall and obese children. They found ten single nucleotide polymorphisms. One common polymorphism of the ghrelin gene, Leu72 to Met (L72M), corresponding to an amino acid change in the tail of the pre-pro-ghrelin molecule, was significantly associated with children with a higher BMI, and with lower insulin secretion during the first part of an oral glucose tolerance test, although no difference in glucose levels was noted. The authors concluded that variations in the ghrelin gene contribute to obesity in children and may modulate glucose-induced insulin secretion. An Arg51-to-Gln polymorphism (R51Q) in the ghrelin gene associated with obesity has also been reported (Ukkola *et al.* 2001; Vivenza *et al.* 2004).

Hinney *et al.* (2002) screened the ghrelin coding region in 215 extremely obese German children and adolescents (study group 1) and ninety-three normal-weight students (study group 2) by single-strand conformation polymorphism analysis. They found two previously described single nucleotide polymorphisms, R51Q and L72M, in similar frequencies in study groups 1 and 2. Hence, they could not confirm the previous finding. Additionally, two novel variants were identified within the coding region. They detected a non-conservative amino acid change from Gln to Leu at codon 90. They also detected a frameshift mutation in one healthy normal-weight individual. The authors concluded that none of the variants seem to influence weight regulation.

Recently, two novel variants in the pre-pro-ghrelin gene, 36C > T and IVS3 + 715delC, and four known variants, Arg51Gln, Leu72Met, Gln90Leu and IVS1 + 169G >, have been identified in Danish Caucasian subjects. None of the variants showed any significant association with obesity or type 2 diabetes or estimates of glucose and lipid metabolism in glucose-tolerant subjects. Indeed, variation in the pre-pro-ghrelin

gene is not associated with juvenile-onset obesity, type 2 diabetes or related phenotypes among the examined Danish Caucasian subjects (Bing *et al.* 2005; Larsen *et al.* 2005). However, Steinle *et al.* (2005) have also analysed three missense polymorphisms in *GHRL* (Arg51Gln, Leu72Met and Gln90Leu) in subjects of the Amish Family Diabetes Study, and concluded that mutations in *GHRL* may confer risk for the metabolic syndrome.

Tissue distribution and secretion of ghrelin and ghrelin-derived molecules

Both ghrelin and des-acyl ghrelin are expressed in gastrointestinal tissues, with the stomach having the highest concentrations and significant amounts in the duodenum, jejunum, ileum and caecum (Hosoda *et al.* 2000a). Rat ghrelin is present in round, compact, electron-dense granules compatible with those of X/A-like cells whose hormonal product and physiological functions had not previously been clarified. These ghrelin-immunoreactive cells, which are not enterochromaffin-like cells, D cells or enterochromaffin cells, account for about 20% of the endocrine cell population in rat and human oxyntic glands and are associated with the capillary network running through the lamina propria (Date *et al.* 2000).

In contrast to GHS-R1a receptor that is predominantly expressed in the pituitary and at much lower levels in the thyroid gland, pancreas, spleen, myocardium and adrenal gland (Howard *et al.* 1996), ghrelin has been found not only in the stomach and other parts of the gut but also in all the tissues studied (adrenal gland, atrium, breast, buccal mucosa, oesophagus, fallopian tube, fat tissue, gall bladder, human lymphocytes, ileum, kidney, left colon, liver, lung, lymph node, muscle, muscle, myocardium, ovary, pancreas, pituitary, placenta, prostate, right colon, skin, spleen, testis, thyroid and vein; Gnanapavan *et al.* 2002). The biological significance of this widespread distribution of ghrelin is unknown, although ghrelin may have a range of still-unidentified physiological autocrine, paracrine or endocrine effects in both neural and peripheral tissues, using different types of receptors (van der Lely *et al.* 2004).

The ghrelin content of the central nervous system (CNS) is low but ghrelin-responsive neuronal cells can be demonstrated in a very limited region in the hypothalamic ARC (Kojima *et al.* 1999; Hosoda *et al.* 2000b). Likewise, ghrelin mRNA and peptide have been detected in rat and in normal and abnormal human pituitaries (Korbonits *et al.* 2001).

The plasma ghrelin level can be measured by RIA using two polyclonal rabbit antibodies raised against the N-terminal (1–11; Gly1–Lys11) and C-terminal (13–28; Gln13–Arg28) fragments of rat ghrelin (Kojima *et al.* 1999; Hosoda *et al.* 2000a; Ariyasu *et al.* 2002). Ghrelin values obtained by the RIA system using anti-rat ghrelin (1–11) antiserum specifically represent the active, *n*-octanoylated ghrelin. The values acquired by RIA with anti-rat ghrelin (13–28) represent the total ghrelin concentration, including inactive, des-acyl ghrelin.

Adult human plasma has been reported to contain 100–120 fmol total immunoreactive ghrelin/ml (Kojima *et al.* 2001) and, more recently, 61–293 fmol/ml (Murashita *et al.* 2005). Rat plasma levels of total immunoreactive ghrelin range from 220 to 560 fmol/ml (Date *et al.* 2000; Hosoda

et al. 2000a). However, the concentration of active acyl ghrelin is about five to twenty times less, both in rat and human plasma (Date *et al.* 2000; Hosoda *et al.* 2000a; Gauna *et al.* 2004; Murashita *et al.* 2005). In fact, acylated ghrelin ranges from 3.6 to 23.9 fmol/ml in adult human subjects, with an average of 9.1 fmol/ml (Murashita *et al.* 2005). In human cord blood acylated ghrelin ranged from 7.7 to 38.4 fmol/ml and the median for the non-acylated:acylated ghrelin ratio was 13.8, and in neonatal blood acylated ghrelin concentrations ranged from 4.6 to 22.6 fmol/ml, with a non-acylated:acylated ghrelin ratio of 12:50. In both cases, plasma levels of active ghrelin correlated excellently with those of total ghrelin (Yokota *et al.* 2005).

Acylated ghrelin is very rapidly cleared from the circulation because hardly any of it can be found in serum in the following hour after injection (Gauna *et al.* 2004). The half-life of human ghrelin has been reported to be about 10 min (Hosoda *et al.* 2003). Carboxylesterase appears to be the enzyme responsible in rat and human serum for des-octanoylation. In addition, several esterases, including butyrylcholinesterase, contribute to ghrelin des-octanoylation in human serum (De Vriese *et al.* 2004).

Among determinants of ghrelin secretion, the most important appear to be insulin (Mohlig *et al.* 2002; Saad *et al.* 2002; Flanagan *et al.* 2003), glucose (Tschop *et al.* 2000; Nakagawa *et al.* 2002; Nakai *et al.* 2003), and somatostatin (Broglio *et al.* 2002; Barkai *et al.* 2003). Possibly, GH (Muller *et al.* 2002; Tschop *et al.* 2002), leptin (Rosická *et al.* 2003; Tolle *et al.* 2003), melatonin (Mustonen *et al.* 2001), thyroid hormones (Riis *et al.* 2003), glucagon (Kishimoto *et al.* 2003), and the parasympathetic nervous system (Masuda *et al.* 2000; van der Lely *et al.* 2004) also play a role in ghrelin metabolism. In mice, rats, cows and human subjects, ghrelin mRNA expression levels or circulating ghrelin levels are increased by food deprivation and appear to be decreased postprandially (Tschop *et al.* 2000, 2001; Asakawa *et al.* 2001; Cummings *et al.* 2001, 2002; Toshinai *et al.* 2001; Ariyasu *et al.* 2002). This phenomenon, which has been confirmed by several study groups in the recent past, further supports the emerging concept of ghrelin as an endogenous regulator of energy homeostasis.

In addition to fasting, ghrelin expression can be stimulated in rats by insulin-induced hypoglycaemia, leptin administration, and central leptin gene therapy (Toshinai *et al.* 2001). Ingestion of sugar suppresses ghrelin secretion in rats (Tschop *et al.* 2000). These observations indicate a direct inhibitory effect of glucose or energy intake on ghrelin-containing X/A-like cells in the oxyntic mucosa of the rat stomach rather than an exclusively insulin-mediated effect. That insulin is an independent determinant of the circulating ghrelin concentration has recently been shown by several study groups using hyperinsulinaemic–euglycaemic clamps in human subjects (Mohlig *et al.* 2002; Saad *et al.* 2002). These findings add further evidence connecting ghrelin to mechanisms governing energy balance and the regulation of glucose homeostasis. Recent studies have shown that there is a novel islet cell type, the ϵ -cell, that it is specialised in ghrelin synthesis (Prado *et al.* 2004; Wierup *et al.* 2004; Heller *et al.* 2005). Ghrelin is also synthesised by pancreas α -cells, the glucagon-producing cell population (Heller *et al.* 2005).

Because ghrelin is highly expressed in the fetal pancreas (Chanoine & Wong, 2004), it may contribute to islet cell

development, and some evidence suggests that ghrelin may modulate insulin secretion in human adults (Cummings *et al.* 2005; Soule *et al.* 2005) and children (Gil *et al.* 2004; Gil-Campos, 2004).

Interestingly, it has been reported that ghrelin binds a species of HDL associated with the plasma esterase, paraoxonase. Both free ghrelin and paraoxon, a substrate for paraoxonase, can inhibit this interaction. Some endogenous ghrelin is found to co-purify with HDL during density-gradient centrifugation. This interaction links the orexigenic peptide hormone ghrelin to lipid transport and a plasma enzyme that breaks down oxidised lipids in LDL. Furthermore, the interaction of the esterified ghrelin with a species containing an esterase suggests a possible mechanism for the conversion of ghrelin to des-acyl ghrelin (Beaumont *et al.* 2003).

Growth hormone secretagogue receptor and other receptors

Two types of GHS-R cDNA, as a result of alternate processing of pre-mRNA, have been identified, and designated type 1a and type 1b receptors (Fig. 4). GHS-R1a consists of 366 amino acids with seven transmembrane regions and a molecular mass of approximately 41 kDa, whereas GHS-R1b consists of 289 amino acids with only five transmembrane regions. Unlike GHS-R1a, GHS-R1b is not activated by ghrelin or synthetic GHS and it is unclear whether it is a functional receptor. However, a recent study has reported that when GHS-R1b is co-expressed with GHS-R1a in HEK293 cells, the signal transduction capacity of GHS-R1a is attenuated, providing a possible explanation for the existence and the biological significance of the GHS-R1b transcript (Chan & Cheng, 2004).

As commented before, GHS-R1a is highly expressed in the hypothalamus and pituitary gland, consistent with the actions of ghrelin on the control of appetite, food intake and energy balance (Gnanapavan *et al.* 2002). Interestingly, however, GHS-R1a expression has also been reported in other areas of the CNS that affect biological rhythms, mood, cognition, memory and learning, such as the hippocampus, *pars compacta* of the *substantia nigra*, ventral

tegmental area (VTA), dorsal and medial raphe nuclei, Edinger-Westphal nucleus and pyriform cortex (van der Lely *et al.* 2004). In addition, multiple peripheral organs, such as the stomach, intestine, pancreas, thyroid, gonads, adrenal, kidney, heart and vasculature as well as several endocrine and endocrine tumours and cell lines, have been found to express GHS-R1a (Gnanapavan *et al.* 2002; Gaytan *et al.* 2004). In contrast, negligible binding was found in the parathyroid, pancreas, placenta, mammary gland, prostate, salivary gland, stomach, colon and spleen (Papotti *et al.* 2000). All these data are in agreement with previous reports indicating that ghrelin and synthetic GHS possess broader functions beyond the control of GH release and food intake (Tschop *et al.* 2000; Broglio *et al.* 2001) and suggest that a still-unknown receptor subtype may exist in the heart and in other tissues (Smith *et al.* 2005).

Concerning cellular signalling, GHS-R are G-protein coupled and show strong homology across species. GHS-R ghrelin binding activates the phospholipase C signalling pathway through Gq, leading to protein kinase C activation, followed by release of Ca from intercellular stores and diacylglycerol production (Kojima *et al.* 2001; Kohno *et al.* 2003). Similarly, a Gs/protein kinase A pathway appears to be involved in ghrelin action (Casanueva & Diéguez, 2002).

The ghrelin receptor also co-couples to a Gs protein, activating the cAMP/cAMP responsive element binding protein pathway (Holst *et al.* 2003). This secondary pathway could augment signalling by dopaminergic activation of Gs-coupled D1 receptors in the *nucleus accumbens* and the VTA, both located in the mesolimbic area of the brain (Kelley & Berridge, 2002). In the VTA, since activation of Gq or Gs proteins activates neurons (Malagon *et al.* 2003), the ghrelin receptors would probably be found on dopaminergic neurons, rather than on inhibitory neurons expressing γ -aminobutyric acid. Stimulation of γ -aminobutyric acid neurons in the VTA leads to inhibition of dopamine release in target sites (Koob, 1992), decreasing motivated behaviour; however, stimulation of the dopaminergic neurons would enhance dopamine release in these sites, increasing food intake.

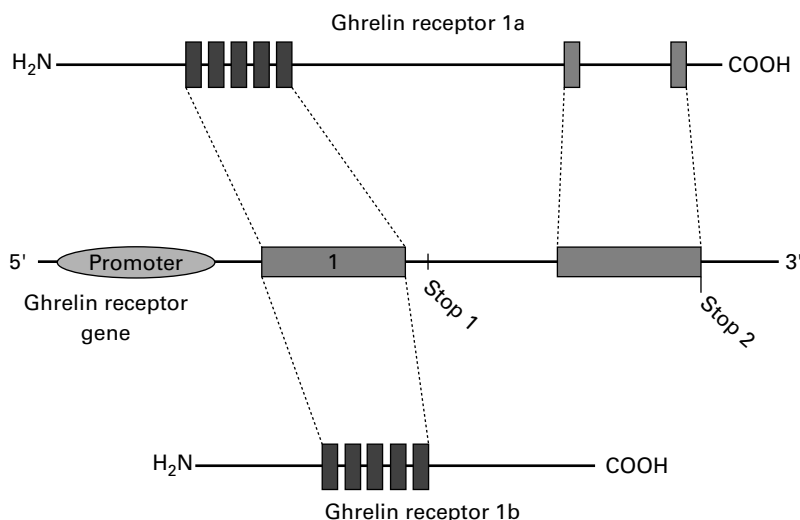


Fig. 4. Structure and translation of the growth hormone secretagogue receptors (ghrelin receptors) 1a and 1b. The former is the known active form, which binds to ghrelin in the hypothalamus and in other peripheral tissues.

Although GHRH and GHS use different receptors and second messengers, co-activation of both receptors induces a cAMP response which is dose-dependent, and is twice that observed after activation of the GHRH receptor alone (Ghigo *et al.* 2001). As we indicated earlier, the acyl group at Ser3 of the ghrelin molecule is essential for binding and activating GHS-R1a. Some carboxy-terminally truncated ghrelin analogues have been found to be able to bind and activate GHS-R1a. These findings indicate that not only the acyl group but also the first seven amino acids of the ghrelin sequence are also essential for GHS-R1a activation (Silva-Elipse *et al.* 2001). Short peptides encompassing the first four or five amino acids and the octanoyl residue are as functional as full-length ghrelin; thus, the Gly-Ser-Ser-(*n*-octanoyl)-Phe segment is considered to be the active core of the molecule (Bednarek *et al.* 2000). However, although these peptides show high receptor affinity, they are unable to stimulate GH secretion from somatotroph cells (Ghigo *et al.* 2001). This sequence of seven amino acids has been universally conserved among different species from fish and reptiles to birds and mammals (Palyha *et al.* 2000), which suggests that ghrelin has an important physiological significance.

The ghrelin receptor is highly constitutively active and this activity could be of physiological importance in its role as a regulator of both GH secretion and appetite control (Howard *et al.* 1996). By measuring inositol phosphate turnover or by using a reporter assay for transcriptional activity controlled by cAMP-responsive elements, the ghrelin receptor showed strong, ligand-independent signalling in transfected COS-7 or human embryonic kidney 293 cells. Ghrelin and a number of the known non-peptide GHS act as agonists stimulating inositol phosphate turnover further. In contrast, the low-potency ghrelin antagonist (D-Arg1,D-Phe5,D-Trp7,9,Leu11)-substance P was surprisingly found to be a high-potency full inverse agonist, as it decreased the constitutive signalling of the ghrelin receptor down to that observed in untransfected cells (Holst *et al.* 2003). These results suggest that inverse agonists for the ghrelin receptor might be particularly interesting for the treatment of obesity.

The GHS-R1a is unlikely to be the only GHS-R (van der Lely *et al.* 2004). It has already been demonstrated that a GHS-R subtype able to bind non-acylated as well as acylated ghrelin exists and probably mediates biological activities (Cassoni *et al.* 2004). Another GHS-R subtype probably mediates the influence of ghrelin on insulin secretion and glucose metabolism, because this effect is not shared by synthetic peptidyl GHS that generally mimic ghrelin actions (Broglio *et al.* 2001).

Recently, the presence of an unknown ghrelin receptor has been suggested that modulates ghrelin actions on weight gain. This assumption is based on the discovery of an analogue of full-length human ghrelin, BIM-28163, which fully antagonises GHS-1a by binding to but not activating the receptor. BIM-28163 blocks ghrelin activation of the GHS-1a receptor, and inhibits ghrelin-induced GH secretion *in vivo* (Halem *et al.* 2004). However, unexpectedly, BIM-28163 acts as an agonist with regard to stimulating weight gain (Halem *et al.* 2005). Additionally, Gauna *et al.* (2005) have reported that glucose output by primary hepatocytes is time- and dose-dependently stimulated by acylated ghrelin and inhibited by des-acyl ghrelin. Moreover, the latter counteracts the stimulatory effect of acylated ghrelin on glucose release. These actions might be

mediated by a different receptor than GHS-R1a and, apparently, we should consider acylated ghrelin and desacyl-ghrelin as separate hormones that can modify each other's actions on glucose handling, at least in the liver.

Ghrelin and central pathways involved in the regulation of energy homeostasis

There are two systems that operate in the regulation of the quantity of food intake: short-term regulation, that is concerned primarily with preventing overeating at each meal, and long-term regulation, which is primarily related with the maintenance of normal quantities of energy stores in the form of fat in the body (Konturek *et al.* 2005; Fig. 5).

The hypothalamus is considered the key region in the CNS involved in feedback control of appetite and food intake, though other brain regions have also been implicated. *Nucleus tractus solitarius* in the brain stem serves as a gateway for neural signals from the gastrointestinal tract to the hypothalamic centres. Also the amygdala, the cortex prefrontalis and the area postrema have been held responsible for feeding disorders and inadequate conservation or storage of energy (Cone, 2005; Horvath, 2005). Additionally, the mesolimbic region, particularly the VTA, which is involved in the reward of feeding, the hippocampus, *substantia nigra*, and dorsal and medial raphe nuclei have also been implicated (Guan *et al.* 1997; Gnanapavan *et al.* 2002).

Early animal experiments with hypothalamic lesions and post mortem examinations with morbid obesity led to a proposal for the 'dual centre' hypothesis, postulating that ventromedial nuclei serve as the 'satiety centre' and the lateral hypothalamic area as the 'feeding or hunger centre' (Druce *et al.* 2004). The ARC and the PVN of the hypothalamus are also considered to be a part of the hunger centre. It appears that the hunger centre is chronically active and that its activity may be transiently inhibited by activity of the satiety centre occurring just after the ingestion of food (Cone, 2005; Konturek *et al.* 2005).

In the short-term regulation of energy intake, the structures in the brain control the intake of a single meal regarding its volume, energy content and duration. Following food ingestion the signals from the oro-pharyngeal and gastric area are conveyed to the *nucleus tractus solitarius* in the brain stem through afferent vagal nerves and other sympathetic nervous system afferents. In addition to mechanical distension, the chemical stimulation of receptors in the gastrointestinal mucosa and various hormones released from the gastrointestinal tract by nutrients contribute to the peripheral signalling with orexigenic and anorexigenic properties (Dockray, 2003; Konturek *et al.* 2004). In the long-term coordination of dietary intake and energy expenditure the CNS receives numerous impulses and peripheral signals, especially from the gastrointestinal tract and fat tissue in response to constantly altered (Halem *et al.* 2004) balance (Druce *et al.* 2004; Horvath, 2005).

In the brain, the mammalian central melanocortin system is defined as a collection of CNS circuits that include: (i) neurons that express hypothalamic NPY and agouti gene-related protein (NPY/AgRP) or POMC and that originate in the ARC; (ii) brainstem POMC neurons originating in the commissural *nucleus tractus solitarius*; (iii) downstream targets of these POMC and AgRP neurons expressing the

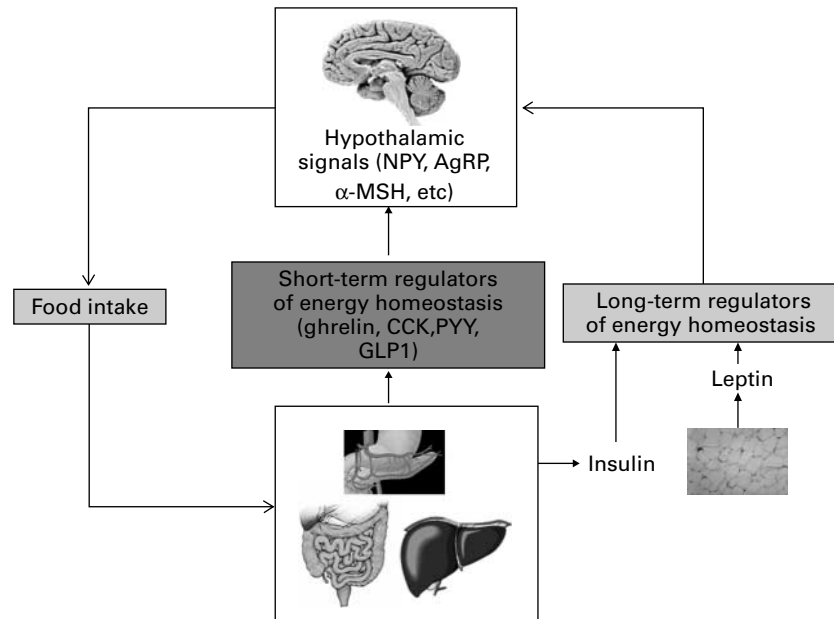


Fig. 5. Schematic view of the short- and long-term regulation of food intake mediated by ghrelin, insulin and leptin. NPY, neuropeptide Y; AgRP, agouti-related protein; α -MSH, melanocyte-stimulating hormone; CCK, cholecystokinin; PYY, peptide YY; GLP, glucagon-like peptide.

melanocortin-3 and melanocortin-4 receptors. In the CNS, melanocortin peptides, namely α -melanocyte-stimulating hormone, are agonists of the melanocortin-3 and melanocortin-4 receptors, whereas AgRP is a high-affinity antagonist of both these receptors. The melanocortin system is unique in that many melanocortin receptor sites in the CNS receive projections from both agonist-expressing POMC fibres and antagonist-expressing AgRP fibres, whereas some seem to receive only agonist innervation (Cone, 2005). Another unique feature of the central melanocortin system is that it acts like a rheostat on energy storage (Huszar *et al.* 1997).

The major site of POMC expression in the CNS originates in neurons of the ARC; most of POMC-positive cells also express the anorectic peptide cocaine amphetamine-related transcript (CART). The POMC- and CART-positive (POMC/CART) cell bodies are found throughout the rostrocaudal extent of the ARC and periaruate area of the hypothalamus (Jacobowitz & O'Donohue, 1978; Watson *et al.* 1978). Arcuate POMC cells send a dense bundle of fibres ventral to other brain regions such as the thalamus and the mesolimbic area. As the arcuate NPY/AgRP-expressing neurons express the potent melanocortin-3 receptor and melanocortin-4 receptor antagonist AgRP, they are also a critical component of the central melanocortin system since they are the target of various peripheral hormonal signals such as ghrelin, leptin and insulin (Chen *et al.* 2004). AgRP-immunoreactive fibres appear primarily in a subset of the same hypothalamic and septal brain regions containing dense POMC innervation, with the densest fibres found innervating the PVN, dorsomedial hypothalamus, posterior hypothalamus and septal regions around the anterior commissure (Broberger *et al.* 1997; Haskell-Luevano *et al.* 1999). The interaction of these two POMC/CART and NPY/AgRP systems seems to be the primary driving force in the regulation of energy homeostasis (Huszar *et al.* 1997; Cone, 1999, 2005).

Le Roux *et al.* (2005a) have shown that an intact vagus nerve may be required for exogenous ghrelin to increase appetite and food intake in man. In six men and one woman who all had a previous complete truncal vagotomy with lower oesophageal or gastric surgery, ghrelin stimulated GH release in a dose-dependent manner, confirming bioactivity. However, no change in energy intake was observed with either dose of ghrelin (1 and 5 pmol/kg \times min).

The melanocortin system is not the sole regulator of energy balance within the hypothalamus. The lateral hypothalamic hypocretin-orexin system is important in establishing orexigenic drive associated with arousal (Yamanaka, 2003). Additionally, although ghrelin's effects are mainly mediated at the hypothalamic ARC, the ghrelin receptor is expressed also in other brain sites. These areas are the caudal brainstem, where some gustatory information is processed (Horvath, 2005), the mesolimbic region, particularly the VTA, *nucleus accumbens*, hippocampus, *substantia nigra*, and dorsal and medial raphe nuclei (Guan *et al.* 1997; Ganapavan *et al.* 2002).

Reward for feeding is governed by the mesolimbic dopamine pathway, which consists of dopaminergic cell bodies that reside in the VTA and project to multiple nodes, including the *nucleus accumbens*, amygdala, prefrontal cortex and hippocampus (Kelley & Berridge, 2002). Expression of the ghrelin receptor in the VTA and hippocampus might indicate that ghrelin, normally considered a homeostatic factor, can also act on reward circuitry, perhaps to modulate feeding behaviour. Recently, it has been found that administration of ghrelin into the VTA or *nucleus accumbens* of rats significantly increased feeding (Naleid *et al.* 2005). Intake stimulated from the VTA was at least twice as great as that stimulated from the *nucleus accumbens*. When ghrelin is injected into the VTA, it does not come into contact with known NPY neurons. Therefore, in this region, ghrelin's orexigenic effects are

probably independent of NPY signalling. The most likely mechanism is that ghrelin activates its own receptors in the VTA (Guan *et al.* 1997), thereby increasing dopamine release in target sites.

Fig. 6 shows the main pathways involved in the anorexigenic properties of ghrelin, as well as the opposite effects of leptin and insulin.

Evidence of ghrelin's effects on appetite and regulation of energy balance

The first evidence of ghrelin's involvement in regulating appetite was obtained by Arvat *et al.* (2000) who, in a study of GH release, found that three out of four healthy volunteers injected with ghrelin reported hunger as a 'collateral effect'. These findings were confirmed in subsequent studies (Horvath *et al.* 2001; Nakazato *et al.* 2001; Wren *et al.* 2001; Eisenstein & Greenberg, 2003). At the same time, numerous animal and human studies have added strength to the argument that ghrelin is involved in the regulation of energy balance (Tschop *et al.* 2000; Asakawa *et al.* 2001; Shintani *et al.* 2001; Eisenstein & Greenberg, 2003).

Roles of ghrelin in appetite and short-term food intake

Plasma ghrelin levels are dependent on recent food intake; they are increased by fasting and decline after eating (Tschop *et al.* 2001). For that reason, it has been suggested that they may play a major role in meal initiation (Cummings *et al.* 2001), although the link between the rise in circulating

ghrelin and meal initiation has not been proven. Daytime secretion of stomach ghrelin appears to be suppressed by food intake, whilst it is augmented by night-time fasting. By contrast, the daytime secretion of hypothalamic ghrelin is increased, and nocturnal secretion decreased, thus regulating food intake (Bowers, 2001). In fact, plasma ghrelin concentration shows a nocturnal rise that exceeds the meal-associated increase in lean subjects, although this rise is blunted in the obese (Yildiz *et al.* 2004). In addition, circulating ghrelin levels decrease in normal-weight subjects after mixed meals. However, obese subjects demonstrate a much reduced ghrelin postprandial suppression both in adults and children (English *et al.* 2002; Gil *et al.* 2004; Gil-Campos, 2004; Le Roux *et al.* 2005b).

The main factors promoting ghrelin production are fasting, hypoglycaemia and leptin, whilst the main inhibiting factors are food intake, hyperglycaemia and obesity (Cummings *et al.* 2001; Toshinai *et al.* 2001; Tschop *et al.* 2001; Shiiya *et al.* 2002). High glucose levels and consequently hyperinsulinaemia reduce ghrelin secretion; however, stomach expansion does not display this effect (Shiiya *et al.* 2002). The depth and duration of prandial ghrelin suppression are dose-dependently related to the energy intake (Callahan *et al.* 2004). That means that large meals suppress both ghrelin and hunger more thoroughly than do small meals.

There are conflicting results regarding the specific effects of nutrients on ghrelin postprandial response. Initially, it was proposed that a fat-rich diet decreases plasma ghrelin levels, whereas a protein-rich diet increases them (Lee *et al.* 2002). However, it is now well established that ingested energy

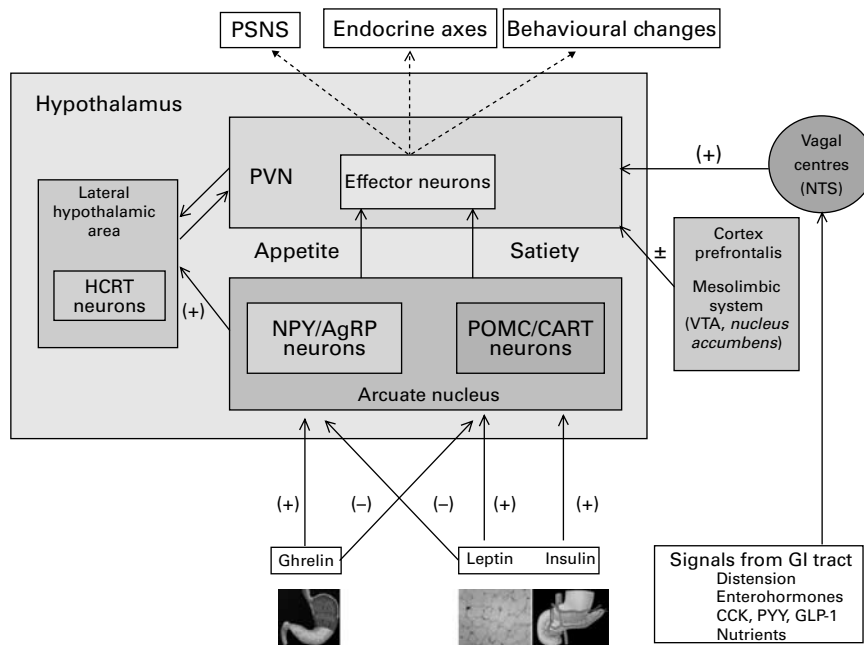


Fig. 6. Regulation of food intake in the hypothalamus by ghrelin and counteracting effects of leptin and insulin. Neuropeptide Y (NPY)/agouti-related protein (AgRP)-expressing neurons, located mainly in the arcuate nucleus of the hypothalamus, are stimulated by ghrelin, which in turn stimulates effector pro-opiomelanocortin (POMC) neurons in the paraventricular nucleus (PVN). These neurons modulate a resulting efferent message. The *nucleus tractus solitarius* (NTS) also receives neuroendocrine signals from vagal afferents, which are activated by a number of factors including mechanical distension of the gastrointestinal (GI) tract, enterohormones and nutrients. The NTS also mediates activation of PVN neurons. Other regions of the brain also have a role in the control of food intake, namely the caudal brainstem and the mesolimbic region, particularly the ventral tegmental area (VTA), *nucleus accumbens*, hippocampus, *substantia nigra*, and dorsal and medial raphe nuclei. PSNS, parasympathetic nervous system; HCRT, hypocretin; CART, cocaine amphetamine-related transcript; CCK, cholecystokinin; PYY, peptide YY; GLP, glucagon-like peptide.

suppresses ghrelin with an efficacy order in rat and human subjects of: carbohydrates > proteins > lipids (Cummings *et al.* 2005). Glucose and amino acids suppress ghrelin more rapidly and strongly (by approximately 70 %) than do lipids (by approximately 50 %); the relatively weak suppression of ghrelin by lipids compared with glucose or amino acids could represent one mechanism promoting high-fat dietary weight gain (Overduin *et al.* 2005). On the other hand, a high-fat diet decreases ghrelin pulse amplitude in obesity-prone rats relative to obesity-resistant rats, which precedes the weight increase (Otukonyong *et al.* 2005). Increasing dietary protein relative to carbohydrate and fat enhances weight loss, at least in part by increasing satiety. Nevertheless, the satiating effect of dietary protein appears to be unrelated to postprandial ghrelin secretion (Moran *et al.* 2005).

Plasma ghrelin was determined together with hunger and satiety ratings and with insulin and glucose concentrations after the ingestion of satiating quantities of carbohydrate-, fat-, protein-, fruit- and vegetable-rich meals (Erdmann *et al.* 2004). Standardised sandwiches were consumed 4 h later. After carbohydrate, ghrelin decreased, whereas fat, protein, fruit, and vegetable ingestion significantly increased ghrelin levels. Considering all test meals, no significant correlation existed between changes of ghrelin levels and satiety ratings, whereas a significant inverse relationship was observed between plasma ghrelin and insulin levels. During the second meal, sandwich consumption was significantly greater after the preceding fruit and vegetable meals, which was significantly correlated with the 4th hour increase of ghrelin. These results suggest that after an overnight fast, ghrelin release appears to depend on the ingested macronutrients more than being a major regulator of acute food intake, although it is of greater importance for the recurrence of hunger and subsequent meal size.

Increasing dietary protein relative to carbohydrate and fat enhances weight loss, at least in part by increasing satiety, although the mechanism for this is unclear. Recently, the effects of isoenergetic test meals with differing protein:fat ratios on fasting and postprandial ghrelin, insulin, glucose, appetite, and energy expenditure before and after weight loss on the respective dietary patterns were compared. Fasting ghrelin increased, and the postprandial ghrelin response improved with weight loss independently of diet composition, suggesting that the reduced appetite observed with increased dietary protein appears not to be mediated by ghrelin homeostasis (Moran *et al.* 2005). On the other hand, before weight loss, there was no significant difference in postprandial ghrelin response between test meals rich in fat or carbohydrates. However, after weight reduction, the ghrelin response was more pronounced after the carbohydrate test meal than after the fat test meal (Romon *et al.* 2006). In obese children, low-fat high-carbohydrate diet-induced weight loss does not change ghrelin secretion, but significantly decreases leptin levels, increases adiponectin levels and improves insulin resistance determined by significantly decreased insulin resistance indices as well as lowered serum insulin levels (Reinehr *et al.* 2005).

Additionally, the postprandial effect of diet composition on circulating acylated ghrelin levels in healthy women has been recently investigated (Al Awar *et al.* 2005). Acylated ghrelin fell significantly after ingestion of both balanced and

high-protein meals and ghrelin persisted at significantly lower levels than baseline for a longer duration following the high-protein meal compared with the balanced meal. Again, in this study, postprandial changes in acylated plasma ghrelin appear to depend on the macronutrient composition of the meal.

On the other hand, a soluble fibre such as arabinoxylan in an enriched meal increases serum ghrelin levels by an unknown mechanism (Mohlig *et al.* 2005). Additionally, high-fructose sweetened beverages suppress ghrelin less well than isoenergetic glucose beverages, probably because of the lower capacity of fructose to increase insulin plasma concentrations compared with glucose. Given that insulin, leptin, and ghrelin function as key signals to the CNS in the long-term regulation of energy balance (see later), decreases of circulating insulin and leptin and increased ghrelin concentrations mediated by chronic consumption of enriched fructose beverages could lead to increased energy intake and ultimately contribute to weight gain and obesity (Teff *et al.* 2004).

Regardless of the effect of ghrelin on appetite in healthy human subjects, this hormone appears to enhance the perceived palatability of the food offered (Druce *et al.* 2006).

Table 1 summarises the observations which support the role of ghrelin in appetite and short-term food intake.

Roles of ghrelin in the regulation of body weight and energy balance

The regulation of body weight is achieved through complex hormonal and neuroendocrine pathways, which result in energy homeostasis whereby energy balance is closely matched over long periods of time (Cummings *et al.* 2005). Critical elements of this control system are hormones secreted in proportion to body adiposity, including leptin, insulin and adiponectin, and the CNS and other peripheral targets upon which they act (Gil-Campos *et al.* 2004a,b). Some of these CNS targets stimulate food intake and anabolic pathways promoting weight gain, whereas others reduce food intake and catabolic pathways promoting weight loss (Batterham *et al.* 2002; Marx, 2003). Moreover, a number of hormones, including leptin, insulin, adiponectin and catecholamines, regulate fuel metabolism, i.e. lipid metabolism, in a number of peripheral tissues, independently

Table 1. Features which support the role of ghrelin in appetite and short-term food intake

1	Most ghrelin is synthesised in the stomach, a well-positioned organ to detect recent intake of food
2	The main effects of intraventricular and blood system ghrelin injection at times of minimal spontaneous intake is to trigger eating and to decrease the latency of feeding
3	Human ghrelin secretion is suppressed immediately after a meal, the depth and duration of the suppression being proportional to the energy intake
4	Ghrelin stimulates gastrointestinal motility, and gastric and exocrine pancreatic secretions
5	Ghrelin stimulates the secretion of neuropeptide Y and agouti-related protein, two well-known orexigens, in the arcuate nucleus of the hypothalamus
6	Some ghrelin gene polymorphisms are associated with alterations in eating patterns

of the regulation of food intake (Baile *et al.* 2000; Yamauchi *et al.* 2002; Gil-Campos *et al.* 2004a,b).

Ghrelin also appears to be involved in the regulation of feeding behaviour and energy homeostasis (Ariyasu *et al.* 2002; Shiiya *et al.* 2002). Thus, serum ghrelin levels are inversely correlated with BMI, age and insulin concentrations in normal children and are markedly increased in the Prader–Willi syndrome (Haqq *et al.* 2003; Park *et al.* 2005), whereas they correlate positively with leptin (Park *et al.* 2005).

Nutritional status also influences ghrelin levels in man. In fact, ghrelin levels increase in response to weight loss resulting from low-energy diets, mixed life-style modifications, cancer anorexia and cachexia, Huntington's disease, anorexia and bulimia nervosa, and chronic failure of the heart, lungs, liver, or kidneys (Horvath *et al.* 2001; Tanaka *et al.* 2002; Tolle *et al.* 2003; Soriano-Guillen *et al.* 2004; Cummings *et al.* 2005). This has led to the suggestion that ghrelin signals the need to conserve energy, and that its secretion is the 'trigger' that counteracts a subsequent energy deficit, preventing cachexia (Horvath *et al.* 2001; Cummings *et al.* 2005).

In contrast to healthy individuals, obese subjects display reduced plasma ghrelin levels, together with low plasma GH and high plasma leptin levels (Tschop *et al.* 2001; Shiiya *et al.* 2002; Rosická *et al.* 2003). Plasma ghrelin concentrations rise rapidly after fasting in normal-weight animals, but this increase is delayed in obese *ob/ob* and *db/db* mice and in fatty Zucker rats (*fa/fa*), suggesting that short-term regulation is modified by excess energy deposit (Ariyasu *et al.* 2002). After eating a test meal, obese human adults do not exhibit the decline in ghrelin levels observed in lean subjects. Fasting ghrelin levels fall by 39.5% in lean subjects 30 min after eating, before returning rapidly towards baseline levels. There was no change in circulating ghrelin in the obese group (English *et al.* 2002).

It is also reported that ghrelin levels are lower in prepubertal obese children than in healthy controls (Gil *et al.* 2004; Gil-Campos, 2004; Bacha & Arslanian, 2005), and that a reduction of up to 50% in the standard deviation of the BMI prompts a significant increase (Soriano-Guillen *et al.* 2004). Moreover, in obese subjects there appears to be a short-term delay in the regulation of ghrelin secretion both in animals and in human subjects (Ariyasu *et al.* 2002). The decline in plasma ghrelin levels recorded in lean subjects after eating a test meal is not observed in adult obese subjects (English *et al.* 2002). Likewise, after the intake of a standardised breakfast, obese children recover plasma fasting ghrelin levels more rapidly than healthy children, which suggests that this biased pattern may have an impact in the increased consumption of foods (Gil *et al.* 2004). Moreover, in a recent study oral glucose tolerance test-induced absolute suppression in ghrelin was about 50% less in overweight *v.* normal-weight children (Bacha & Arslanian, 2005). Also, bariatric surgery, the most effective method to sustain weight in morbid obesity, is associated with reduced ghrelin plasma levels (Cummings & Shannon, 2003; Holdstock *et al.* 2003).

Regardless, ghrelin circulates in proportion to body-energy stores and exhibits compensatory changes in response to fluctuation in fat mass; an adiposity hormone should influence neuronal activity in brain centres known to regulate body weight, and ghrelin's exogenous administration or blockade should alter food intake and energy expenditure (Cummings *et al.* 2005).

Ghrelin enhances food intake by stimulation of NPY and AgRP synthesis in the ARC of the hypothalamus and hindbrain (Bagnasco *et al.* 2003). Likewise, peripheral or central chronic administration of ghrelin increases body weight (Tschop *et al.* 2000). Moreover, the chronic central administration of ghrelin reverses the effects of leptin, primarily by altering food intake, but it also shows regulatory effects on adiposity and plasma insulin levels independent of feeding effect (Kim *et al.* 2004). It can also decrease energy expenditure (Asakawa *et al.* 2001; Kamegai *et al.* 2001), fat catabolism and lipolysis (Tschop *et al.* 2000; Muccioli *et al.* 2004; Barazzoni *et al.* 2005) and adipocyte apoptosis (Kim *et al.* 2004). Des-acyl ghrelin, as well as ghrelin, and short ghrelin fragments act directly as antilipolytic factors on the adipose tissue through binding to a specific receptor, which is apparently distinct from GHS-R1a (Muccioli *et al.* 2004).

Acute ghrelin blockade in animals using anti-ghrelin antibodies, ghrelin receptor antagonists or antisense oligonucleotides has demonstrated decreased food intake and weight loss (Nakazato *et al.* 2001; Asakawa *et al.* 2003; Bagnasco *et al.* 2003). However, knockout ghrelin gene animal models exhibit a subtle increased fat catabolism and leanness (Sun *et al.* 2004; Wortley *et al.* 2004). However, analyses of ghrelin^(-/-) mice demonstrate that endogenous ghrelin plays a prominent role in determining the type of metabolic substrate (i.e. fat *v.* carbohydrate) that is used for maintenance of energy balance, particularly under conditions of high fat intake (Wortley *et al.* 2004).

Table 2 summarises the observations which support the role of ghrelin in the control of body weight and energy expenditure.

Recently, studies in ghrelin knockout mice have suggested that this hormone is not an essential regulator of food intake and gastric emptying since ghrelin^(-/-) mice showed similar body-weight gain and 24 h food intake to that of wild-type animals (Sun *et al.* 2003; Wortley *et al.* 2004). In addition, exogenous ghrelin increased food intake in both wild and ghrelin^(-/-) mice (De Smet *et al.* 2006). Furthermore, adult mice with deletion of the ghrelin gene display a normal sensitivity to high-fat-diet-induced obesity (Grove & Cowley, 2005). However, the disruption of the ghrelin gene may affect not only the synthesis of ghrelin but also obestatin, which is involved in the suppression of food intake (Zhang *et al.* 2005). On the other hand, recent studies by Wortley

Table 2. Features which support the role of ghrelin in the regulation of energy balance

1	Serum ghrelin levels are inversely correlated with BMI, age and insulin concentrations
2	Ghrelin levels are influenced by nutritional status, increasing in response to weight loss resulting from low-energy diets, life-style modifications and diseases leading to malnutrition
3	Obese children and adults have lower ghrelin plasma levels than lean subjects, and they exhibit a lower postprandial decline and a more rapid returning towards baseline levels
4	Bariatric surgery is associated with reduced ghrelin plasma levels
5	Peripheral or central chronic administration of ghrelin increases body weight and reverses the effects of leptin
6	Ghrelin decreases energy expenditure limiting fat catabolism, lipolysis and adipocyte apoptosis
7	Acute ghrelin blockade in animals using anti-ghrelin antibodies, ghrelin receptor antagonists or antisense oligonucleotides decreases food intake and weight loss

et al. (2005) and Zigman *et al.* (2005) demonstrate that both ghrelin^(-/-) and GHS-R^(-/-) mice are resistant to diet-induced obesity when fed a high-fat diet during the early post-weaning period, whereas those mice in the adult period become obese on a high-fat diet. These results suggest a role for ghrelin in the development of metabolic pathways, both central and peripheral, as well as the development of compensatory signals that allow for the maintenance of normal body weight and sensitivity to high-fat diets in the absence of potent orexigenic systems (Grove & Cowley, 2005).

Des-acyl ghrelin in the regulation of food appetite and food intake

Although des-acyl ghrelin was initially considered as a non-functional peptide in the regulation of appetite and food intake, recent studies have shown that it decreases food intake and gastric emptying in mice and rats. In fact, administration of des-acyl ghrelin to mice decreased food intake and gastric emptying rate through an action on the PVN and the ARC in the hypothalamus (Asakawa *et al.* 2005). Moreover, intracisternal administration of des-acyl ghrelin decreases food intake in food-deprived rats and inhibits gastric emptying without altering small-intestinal transit (Chen *et al.* 2005a). In addition, intraperitoneal injection of des-acyl ghrelin has recently been reported to decrease food intake in conscious rats (Chen *et al.* 2005b). Intraperitoneal injection of des-acyl ghrelin enhanced c-Fos expression in the ARC and PVN but not in the nucleus of the solitary tract. Both ICV and intravenous injection of des-acyl ghrelin disrupted fasted motor activity in the antrum but not in the duodenum. Changes in gastric motility induced by intravenous injection of des-acyl ghrelin were completely antagonised by ICV injection of a selective corticotropin-releasing factor 2 receptor antagonist; however, the corticotropin-releasing factor 1 receptor antagonist had no effects. Thus, these results suggest that peripheral des-acyl ghrelin may disrupt fasted motor activity in the stomach by direct activation of corticotropin-releasing factor 2 receptor in the brain and that corticotropin-releasing factor 1 receptor is not involved in this action.

These findings indicate that, in contrast to acylated ghrelin, des-acyl ghrelin induces a negative energy balance by decreasing food intake and delaying gastric emptying; the effect is mediated via the hypothalamus. Although derived from the same precursor, the inverse effects of these two peptides suggest that the stomach might be involved as an endocrine organ in the regulation of the energy balance (Broglia *et al.* 2004; Asakawa *et al.* 2005). This idea is also supported by the new discovery of obestatin (Zhang *et al.* 2005).

Ghrelin and energy expenditure

Although speculative, serum ghrelin may play a role in the regulation of energy expenditure through the induction of metabolic changes that would lead to an efficient metabolic state resulting in increased body weight and fat mass. Thus, despite the known stimulatory effects on appetite and eating behaviour of ghrelin, little information is available regarding its relationship with energy expenditure in both animals and man.

Tschop *et al.* (2000) showed that single subcutaneous ghrelin injections induced an increase of the RQ in mice and that such increased utilisation of carbohydrate and reduced utilisation of fat was congruent with the observed increase in body fat. A selective RQ increase without concomitant increases in carbohydrate intake is unusual and may reflect reduced sympathetic nervous system activity (Snitker *et al.* 1998). In addition, direct stimulation of hypothalamic areas can induce a selective change in RQ (Atrens *et al.* 1985).

The chronic ICV injection of ghrelin increased cumulative food intake and decreased energy expenditure, resulting in body-weight gain (Kamegai *et al.* 2001). On the other hand, in human adults ghrelin enhances the sensation of hunger and reduces fat depot utilisation, increasing carbohydrate consumption through the mediation of γ -aminobutyric acid and the inhibition of anorexigenic substances such as α -melanocyte-stimulating hormone (Casanueva & Diéguez, 2002). Additionally, St-Pierre *et al.* (2004) examined the relationship between serum ghrelin and RMR, the thermic effect of food, fasting and postprandial RQ, physical activity level, peak aerobic capacity (VO_{2peak}), energy intake, and psychological measures of feeding behaviour in young healthy women. They noted significant inverse correlations between ghrelin and RMR and the thermic effect of food and these inverse correlations persisted after statistical control for both fat-free mass and fat mass, which suggest that higher levels of ghrelin are associated with low levels of resting and postprandial thermogenesis.

To elucidate the role of endogenous ghrelin in the regulation of energy homeostasis and gastric emptying, ghrelin knockout mice (ghrelin^(-/-)) were generated (De Smet *et al.* 2006). Although body-weight gain and 24 h food intake were not affected, during the dark period young ghrelin^(-/-) mice had a lower RQ, whereas their heat production was higher than that of the wild-type littermates, inferring a role of ghrelin in the regulation of energy expenditure.

Very recently, changes of ghrelin circulating levels induced by a mixed meal and their relationship with postprandial substrate oxidation rates in overweight and obese children with different levels of insulin sensitivity have been reported (Maffei *et al.* 2006). Insulin sensitivity and postprandial maximal decrease of ghrelin concentration showed a significant correlation. Moreover, the postprandial carbohydrate oxidation rate correlated with the area under the curve for both insulin and ghrelin. These results suggest that the oxidation rate of glucose is affected not only by insulin but also by ghrelin, which in turn will influence the energy expenditure.

On the other hand, Zigman *et al.* (2005) have recently shown that when fed a high-fat diet, both female and male GHS-R-null mice eat less food, store less of their consumed energy, preferentially utilise fat as an energy substrate, and accumulate less body weight and adiposity than control mice. This suggests that ghrelin signalling is required for the control of energy expenditure.

Mechanisms of action of ghrelin as an orexigenic peptide

One of the modes by which ghrelin can influence dietary intake is based on the feature that it acts as a hormone, secreted primarily by the stomach and small intestine into

the bloodstream, from which it gains access to NPY/AgRP neurons in the medial ARC across an incomplete blood–brain barrier at that site. Additional effects may result from circulating ghrelin accessing its receptor at circumventricular sites in the hindbrain, which may subsequently affect ARC neuronal activity via ascending projections.

Evidence for the effects of ghrelin on food intake being mediated by NPY and AgRP has been supported by a number of experimental approaches (Hewson & Dickson, 2000; Nakazato *et al.* 2001; Kamegai *et al.* 2001; Shintani *et al.* 2001; Wang *et al.* 2002), including blockade of ghrelin-induced food intake by either ICV injection of antibodies against NPY and AgRP (Nakazato *et al.* 2001), NPY Y1 receptor antagonists (Nakazato *et al.* 2001; Shintani *et al.* 2001), or peripheral administration of the melanocortin agonist melanotan-II (Tschop *et al.* 2002). Additionally, peripheral administration of ghrelin activates c-Fos expression only in arcuate NPY/AgRP neurons, but not in other hypothalamic or brainstem sites (Wang *et al.* 2002), and ablation of the ARC blocks the actions of ghrelin administration on feeding but not elevation of GH (Tamura *et al.* 2002). Finally, electrophysiological approaches have demonstrated that ghrelin can activate NPY/AgRP neurons and simultaneously reduce the activity of POMC neurons (Cowley *et al.* 2003). Orexigenic activity results from activation of the hypothalamic NPY/Y1 receptor, which antagonises the effects of leptin, an appetite-suppressing hormone from adipose tissues (Shintani *et al.* 2001; Bagnasco *et al.* 2002; Fig. 6).

Surprisingly, disruption of the AgRP and NPY genes either alone or in combination does not alter energy homeostasis to the extent that is predicted from the proposed role of NPY/AgRP neurons in the ARC (Hewson & Dickson, 2000). However, a recent study indicates that induced selective ablation of AgRP-expressing neurons in adult mice by cell type-specific expression of a diphtheria toxin receptor and the administration of diphtheria toxin results in acute reduction of feeding, demonstrating direct evidence for a critical role of these neurons in the regulation of energy homeostasis (Gropp *et al.* 2005). In addition, co-administration of ghrelin with AgRP into the hypothalamic PVN during the light and dark phases of feeding in rats did not produce a synergistic effect on food intake, suggesting that ghrelin induction of feeding occurs by recruiting AgRP as one of the obligatory mediators of its orexigenic effect (Shrestha *et al.* 2006).

It has been suggested that two ghrelin-mediated networks exist for energy balance: the NPY network exerting an acute effect and the AgRP network a chronic effect (Muccioli *et al.* 2004). A number of studies have shown that ghrelin acts, at least partially, by activating genes coding for these powerful promoters of food intake, NPY and AgRP, and its action is mediated by receptors different from those causing the GHS effect (Seoane *et al.* 2003; Thompson *et al.* 2004; Cone, 2005; Horvath, 2005).

Another model to explain activation of NPY/AgRP neurons by ghrelin is the vagal pathway. It is based on the assumption that ghrelin, produced mostly in the stomach, acts on the hunger centre and does not cross the blood–brain barrier. Thus, there should be an indirect pathway through which peripherally administered ghrelin can activate the hypothalamic appetite-regulatory neurons. This pathway may be via the vagus nerve system, since the appetite-stimulating effect of

ghrelin is suppressed by vagotomy (Date *et al.* 2002). Moreover, ghrelin administration at low doses in mice improves the efferent activity of this nerve, which stimulates stomach contraction, secretion and filling (Asakawa *et al.* 2001). Despite activating c-Fos only in ARC NPY neurons, peripheral ghrelin may access the ARC through vagal afferents (Horvath, 2005).

Although models by which ghrelin stimulates NPY/AgRP neurons are universally accepted, one additional model has been proposed in the scientific literature. Ghrelin could act as a neuropeptide, released from hypothalamic ghrelinergic neurons that synapse with NPY/AgRP cells and other neurons involved in energy homeostasis (Cowley *et al.* 2003; Toshinai *et al.* 2003; Cone, 2005; Horvath, 2005). However, determining what pathways are the most critical mediators of ghrelin's anabolic actions will require tissue and/or cell-specific elimination of ghrelin signalling.

Fig. 7 exhibits the neurons and pathways involved in ghrelin hypothalamic action.

Molecular mechanisms of action

To control energy homeostasis, hypothalamic energy centres must gather nutritional information from multiple signals that were initially included within the glucostatic and adipostat–lipostatic hypotheses (Mayer, 1955; Lam *et al.* 2005). However, how the hypothalamus converts these diverse signals into a cogent and coordinated response to changes in nutrient availability is mostly unknown. Recently, it has been proposed that the hypothalamic sensing of fatty acids provides a viable biochemical explanatory framework (Lam *et al.* 2005) and that 5'-AMP-activated protein kinase (AMPK) is an important signalling molecule that integrates nutritional and hormonal signals and modulates feeding behaviour and energy metabolism (Andersson *et al.* 2004; Minokoshi *et al.* 2004; Kim & Lee, 2005; Lam *et al.* 2005).

The hypothesis advanced is that circulating lipids such as long-chain fatty acids (LCFA) regulate feeding behaviour and glucose production by generating an increase in the cellular LCFA-CoA pool in the hypothalamus. In turn, LCFA-CoA signal an energy 'surfeit' within the hypothalamus, which activates neural pathways designed to curtail both food intake and liver glucose production (Lam *et al.* 2005). Consistent with this hypothesis, intravenous infusion of a lipid emulsion is sufficient to suppress food intake in baboons (Woods *et al.* 1984). Thus, circulating lipids (triacylglycerol, glycerol and LCFA) seem to generate a signal of nutrient 'surfeit' (Lam *et al.* 2005). This signal is independent of measurable changes in plasma insulin and does not require gastrointestinal nutrient absorption (Woods *et al.* 1984; Matzinger *et al.* 2000). Likewise, sustained ICV infusion of brain fuels, such as glucose, glycerol and β -hydroxybutyrate, causes a decrease in body weight and food intake (Davis *et al.* 1981). The well-established biochemical link between cellular carbohydrate and lipid metabolism must be important in modulating the hypothalamic sensing of fatty acids. Thus, it is likely that this central nutrient-sensing mechanism is able to respond to increased availability of lipids, carbohydrates or both (Lam *et al.* 2005).

On the other hand, *in vivo* administration of leptin, which leads to a reduction in food intake, decreases hypothalamic AMPK activity (Andersson *et al.* 2004; Minokoshi *et al.*

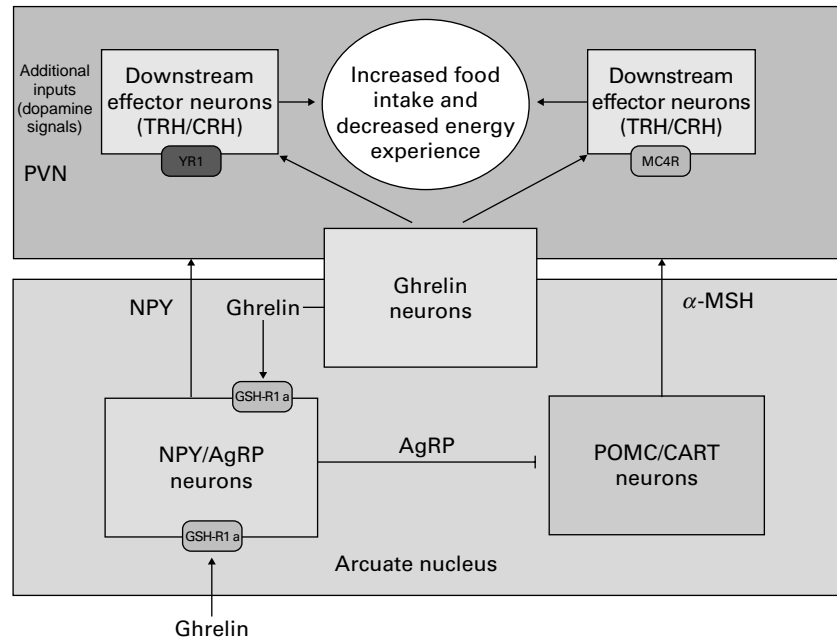


Fig. 7. Integrative regulatory circuits in the regulation of appetite and food intake mediated by ghrelin in the central nervous system. Neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons are activated by the binding of ghrelin to its receptors. These neurons co-express NPY, which activates effector neurons in the paraventricular nucleus (PVN) of the hypothalamus, and AgRP, which inhibits the binding of melanocyte-stimulating hormone (α -MSH) to melanocortin receptor 4 (MC4R). All these signals are integrated in the PVN and modulate a resulting efferent message, which is believed to be mediated in part by thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH). Ghrelin-expressing neurons are adjacent to the third ventricle between the ventromedial hypothalamus, the dorsal hypothalamus, the PVN and the arcuate nucleus. Efferents of ghrelin-expressing neurons project to key circuits of central energy balance regulation and may contribute to balance the activity of orexigenic NPY/AgRP with anorexigenic pro-opiomelanocortin (POMC) neurons. YR1, GHS-R1a, ghrelin receptor; CART, cocaine amphetamine-related transcript.

2004). By contrast, injection of ghrelin *in vivo*, which increases food intake, stimulates AMPK activity in the hypothalamus. Consistent with the effect of ghrelin, injection of 5-amino-4-imidazole carboxamide riboside, a pharmacological activator of AMPK, into either the third cerebral ventricle or directly into the PVN of the hypothalamus significantly increased food intake (Andersson *et al.* 2004).

5'-AMPK is known as a cellular 'energy sensor', as its activity is sensitively changed by the cellular energy state. AMPK controls a number of metabolic processes to help the restoration of energy depletion in the peripheral tissues (Hardie *et al.* 1999; Kemp *et al.* 2003) and is also expressed in the neurons of the CNS (Turnley *et al.* 1999; Culmsee *et al.* 2001). AMPK was first described as an enzyme capable of phosphorylating and inactivating hydroxymethylglutaryl-CoA reductase and acetyl-CoA carboxylase (ACC), key enzymes in the synthesis of cholesterol and fatty acids (Beg *et al.* 1973; Carlson & Kim, 1973). It was later named AMPK, because its activity is highly dependent on the presence of 5'-AMP (Yeh *et al.* 1980). In fact, AMPK activity is regulated by the AMP:ATP ratio (Hardie *et al.* 1999). Thus, an increase in the AMP:ATP ratio activates AMPK and inactivates ACC (Carlson & Kim, 1973), causing a decrease in intracellular levels of malonyl-CoA, an inhibitor of carnitine palmitoyl transferase-1 (Ruderman *et al.* 2003). As a result, AMPK activation causes the stimulation of mitochondrial fatty acid oxidation (Ruderman *et al.* 2003).

Recent studies have demonstrated that adipocyte-derived hormones, leptin and adiponectin, increase fatty acid oxidation through AMPK activation in skeletal muscle (Tomas *et al.*

2002; Yamauchi *et al.* 2002; Minokoshi *et al.* 2004). AMPK is also required for mitochondrial biogenesis in response to chronic energy deprivation in skeletal muscle (Zong *et al.* 2002). AMPK activation in hepatocytes inhibits fatty acid and cholesterol synthesis by inactivating ACC and hydroxymethylglutaryl-CoA reductase (Beg *et al.* 1973; Carlson & Kim, 1973). In contrast to its action in muscle cells, AMPK activation in 3T3-L1 adipocytes has little effect on glucose transport at the basal state, but inhibits insulin-stimulated glucose transport. However, the role of AMPK in the regulation of lipolysis is not presently settled (Kim & Lee, 2005).

The mechanisms by which AMPK activity in hypothalamic neurons affects feeding behaviour are still not fully understood. However, hypothalamic AMPK controls feeding behaviour, at least in part, through the regulation of NPY and AgRP expression. Over-expression of AMPK in the medial hypothalamus decreases NPY and AgRP mRNA expression in *ad libitum*-fed rats, whereas over-expression of constitutively active AMPK augments the fast-induced increase in NPY and AgRP expression (Minokoshi *et al.* 2004). It is likely that increased AMPK activity associated with high ghrelin levels at fasting would decrease hypothalamic malonyl-CoA levels by lowering the activity of ACC. Consistent with this idea, the decreased cellular levels of malonyl-CoA would in turn activate carnitine palmitoyl transferase-1 activity and increase food intake through a cellular decrease of LCFA-CoA (Yeh *et al.* 1980). On the other hand, AMPK could also modulate the transcription of hypothalamic neuropeptides independently of its effects on ACC or malonyl-CoA (Minokoshi *et al.* 2004).

Fig. 8 shows a potential mechanism by which ghrelin may signal on hypothalamic LCFA-CoA and indeed on energy homeostasis.

Interactions between ghrelin, leptin and insulin

The regulation of ghrelin secretion and its biological effects appear to be opposed to those of leptin (Shintani *et al.* 2001; Williams & Mobarhan, 2003). However, they are complementary molecules within the same regulating system for informing the CNS about the current acute and chronic energy balance (Tschop *et al.* 2000, 2001). It has been suggested that circadian rhythms in the afferent signal of leptin from adipocytes and from ghrelin in the stomach code for a corresponding discharge pattern in the NPY-dependent hypothalamic system (Kalra & Kalra, 2003; Kalra *et al.* 2003).

Leptin does not appear to be a major regulatory factor of ghrelin levels, at least in animals (Ariyasu *et al.* 2002). In fact, no correlation has been found between ghrelin and leptin levels in obese children and adolescents (Ikezaki *et al.* 2002). However, other studies suggest that there is a complex interaction between insulin, leptin and ghrelin, and that leptin may regulate ghrelin levels and affect changes in body weight (Williams & Mobarhan, 2003). In a recent report, it has been shown that leptin inhibits both the secretion of gastric ghrelin and the stimulation of feeding by ghrelin (Kalra *et al.* 2005).

A reciprocal rhythmic pattern of the two afferent hormonal signals, anorexigenic leptin and orexigenic ghrelin, imparts rhythmicity to the NPY system, the final common pathway

for appetite expression in the hypothalamus. It has been shown that leptin inhibits both the secretion of gastric ghrelin and the stimulation of feeding by ghrelin. It has been proposed that this dual leptin restraint is the major regulatory arm of the feedback communication between the periphery and the hypothalamus for weight homeostasis (Barazzoni *et al.* 2003; Cummings & Foster, 2003; Konturek *et al.* 2004), and disruption in the rhythmic communication at any locus in the leptin–ghrelin–NPY feedback loop impels loss of hypothalamic control, leading to abnormal weight gain and obesity (Kalra *et al.* 2005).

Barazzoni *et al.* (2003) tested the hypothesis that increased plasma leptin prevents the potential increase in plasma ghrelin concentration during moderate energy restriction in lean rats. These authors found that moderate hyperleptinaemia prevents an increase of plasma ghrelin during moderate short-term energy restriction and satiety-inducing effects of leptin include suppression of gastric orexigenic signals and disruption of a potential feedback mechanism between body-weight changes and plasma ghrelin in lean adult rats. This cross-talk between leptin and ghrelin has been termed as the ‘ghrelin–leptin tango’ in body-weight regulation (Cummings & Foster, 2003; Konturek *et al.* 2005). Moreover, increased plasma ghrelin concentration was reported during diet-induced weight loss in obese human subjects, suggesting that ghrelin contributes to adaptive increment in appetite associated with energy restriction and lower leptin levels (Moran *et al.* 2005).

The ‘ghrelin–leptin tango’ hypothesis implies that weight-reducing effects of leptin are mediated not only by its direct

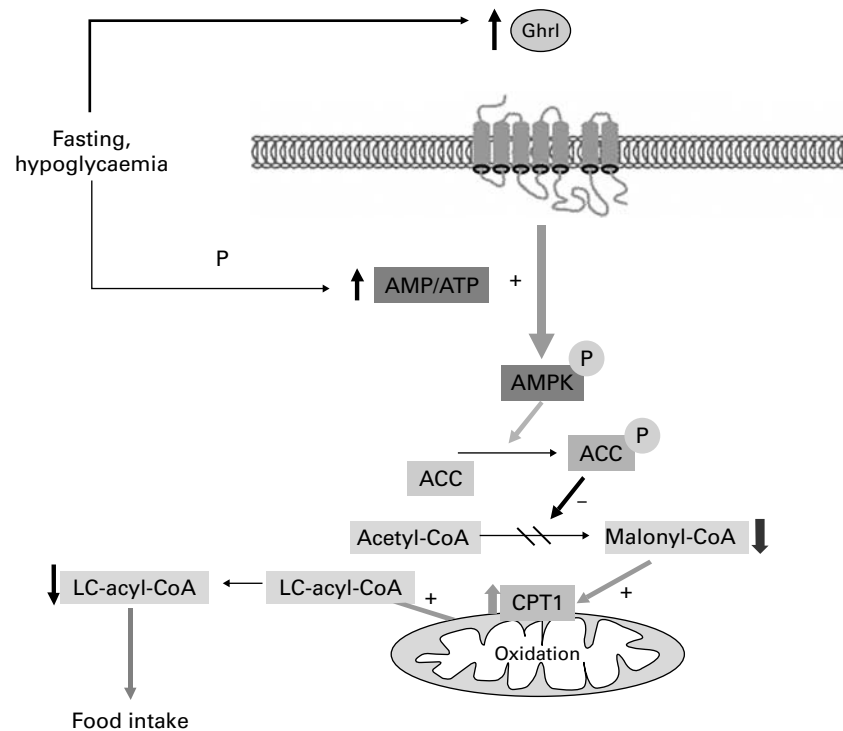


Fig. 8. Role of the 5'-AMP-activated protein kinase (AMPK) in the maintenance of energy homeostasis mediated by ghrelin (Ghrl) in the hypothalamus. It is likely that increased AMPK activity, associated with high Ghrl levels at fasting, would decrease hypothalamic malonyl-CoA levels by lowering the activity of acetyl-CoA carboxylase (ACC). Consistent with this idea, the decreased cellular levels of malonyl-CoA would in turn activate carnitine palmitoyl transferase-1 (CPT1) activity, increasing the transport of long-chain fatty acids (LC-acyl-CoA) to the mitochondria as well as their oxidation. Decreased intracellular levels of LC-acyl-CoA would result in enhanced food intake mediated by a higher expression of neuropeptide Y.

central action on the hypothalamus but also through its peripheral inhibitory effect on the release and action of ghrelin. According to that, parenteral administration of ghrelin in rats at a dose that raises plasma hormone to the level observed under fasting conditions significantly attenuates plasma levels of leptin, while markedly increasing food intake (Konturek *et al.* 2003). Moreover, immunoneutralisation of circulating plasma ghrelin with specific IgG anti-ghrelin antibodies causes a marked increase in plasma leptin and decrease in food intake. In contrast, exogenous leptin, at the dose that raises plasma leptin to the level occurring postprandially, markedly reduced plasma levels of ghrelin and attenuated food intake; these effects can be reversed by the administration of specific IgG anti-leptin antibodies (Konturek *et al.* 2003).

It has long been thought that glucose homeostasis is finely regulated by the brain (Mayer, 1955; Lam *et al.* 2005). The brain receives nutritional and hormonal signals from peripheral tissues, giving rise to efferent signals that control peripheral glucose metabolism, including insulin secretion, hepatic glucose production, and glucose uptake and glycogen synthesis in skeletal muscle (Minokoshi *et al.* 1999; Obici *et al.* 2001, 2002).

The observations that insulin and ghrelin have reciprocal 24 h profiles and that both are involved in the physiological response to food intake has led to hypothesise that insulin regulates ghrelin secretion and vice versa (Cummings *et al.* 2005). Ghrelin's action on energy metabolism appears to be associated with a reduction in insulin synthesis (Broglio *et al.* 2001) and it has been suggested that postprandial insulin is responsible for reducing plasma ghrelin levels following food intake (Lucidi *et al.* 2002). However, most studies designed to evaluate whether insulin inhibits ghrelin have concluded that insulin can suppress ghrelin only at doses higher than those observed physiologically and that insulin is not absolutely required for postprandial ghrelin suppression, although it allows the duration of the response, at least in animals (Cummings *et al.* 2005).

How ghrelin regulates insulin is also controversial. At least three different models have been proposed to explain the action of ghrelin in decreasing insulin secretion. First, several observations suggest a potential model of ghrelin as a counter-regulatory hormone that blocks insulin secretion and action to maintain blood glucose levels. In fact, a variation in the ghrelin gene increases weight and decreases insulin secretion in obese children (Korbonits *et al.* 2002). Moreover, a recent work has shown that ghrelin is suppressed by glucagon, although it does not mediate glucagon-related GH release (Hirsh *et al.* 2005). Ghrelin can also stimulate GH, cortisol and adrenaline (Nagaya *et al.* 2001 *a,b*). In addition, ghrelin has been demonstrated to down-regulate the expression of adiponectin in differentiating adipocytes (Ott *et al.* 2002), an adipocytokine involved in the sensitisation of tissues to insulin, which enhances fatty acid oxidation and that has been involved in the pathogenesis of obesity and insulin resistance (Gil-Campos *et al.* 2004b). Furthermore, it has been shown that GHS-R is found in hepatoma cells, raising the possibility that ghrelin modulates insulin activities in man. In fact, it has been demonstrated that exposure of those cells to ghrelin caused up-regulation of several insulin-induced activities including tyrosine phosphorylation of insulin

receptor substrate-1, association of the adapter molecule growth factor receptor-bound protein 2 with insulin receptor substrate-1, mitogen-activated protein kinase activity, and cell proliferation. Unlike insulin, ghrelin inhibited Akt kinase activity as well as up-regulated phosphoenolpyruvate carboxykinase gene expression, a key enzyme in gluconeogenesis (Murata *et al.* 2002).

A recent study in human volunteers has added support to the hypothesis that insulin is a negative regulator of ghrelin secretion in the postprandial state (Soule *et al.* 2005). Thus, basal ghrelin was decreased significantly by 15 min after glucagon administration, then remained suppressed relative to the basal value until 240 min after glucagon, and there was an inverse statistical relationship between the increase in insulin over the first 120 min and the decrease in ghrelin.

Gauna *et al.* (2004) investigated the metabolic actions of ghrelin in human subjects by examining the effects of acute administration of acylated ghrelin, des-acyl ghrelin, and the combination in eight adult-onset GH-deficient patients. They followed glucose, insulin and NEFA concentrations before and after lunch and with or without the presence of GH in the circulation. They found that acylated ghrelin, which is rapidly cleared from the circulation, induced a rapid rise in glucose and insulin levels. Des-acyl ghrelin, however, prevented the acylated ghrelin-induced rise in insulin and glucose when it was co-administered with acylated ghrelin. Finally, acylated ghrelin decreased insulin sensitivity up to the end of a period of 6 h after administration. This decrease in insulin sensitivity was prevented by coinjection of unacylated ghrelin. This combined administration of acylated and unacylated ghrelin even significantly improved insulin sensitivity, compared with placebo, for at least 6 h.

The role of AMPK in the regulation of glucose homeostasis has been suggested by a study that demonstrated glucose intolerance and insulin resistance in α 2-AMPK knockout mice (Viollet *et al.* 2003). Since the pancreas, skeletal muscle and adipose tissue express GHS-R1a (Howard *et al.* 1996) and ghrelin has been shown to activate AMPK in the ARC, it might be speculated that ghrelin would influence glucose homeostasis not only at the hypothalamic level but also through a direct interaction with peripheral tissues.

One of the most important features of the metabolic syndrome is insulin resistance (Reaven, 2005). An inverse association between endogenous ghrelin and the metabolic syndrome has been recently reported in older adults, which is largely explained by the strong ghrelin–BMI association. Nevertheless, a significant association independent of BMI was also observed between insulin and ghrelin (Langenberg *et al.* 2005).

The associations between plasma ghrelin concentration and metabolic parameters in children and adolescents have also been reported (Park *et al.* 2005). Fasting plasma ghrelin concentration was negatively correlated with height, weight, BMI, percentage body fat, waist circumference and hip circumference in both boys and girls. Fasting plasma ghrelin levels were significantly negatively correlated with triacylglycerols and fasting insulin levels and positively correlated with HDL-cholesterol in boys, but not in girls. All these results suggest that higher plasma ghrelin levels have beneficial effects on metabolic parameters in boys and that the relationships between fasting plasma ghrelin levels and metabolic parameters differ according to sex.

The role of ghrelin in childhood obesity, a state associated with hyperinsulinaemia and insulin resistance, is not fully understood. Recently, Bacha & Arslanian (2005) have investigated the dynamics of ghrelin suppression after an oral glucose tolerance test in normal-weight *v.* overweight children and the relationship of ghrelin suppression to insulin sensitivity. Fasting ghrelin levels were significantly lower in overweight *v.* normal-weight youth and were mainly influenced by insulin sensitivity independently of adiposity. Oral-glucose-tolerance-test-induced absolute suppression in ghrelin was approximately 50% less in overweight *v.* normal-weight children and the suppression of ghrelin correlated positively with the whole-body insulin sensitivity index and negatively with the change in insulin at 30 min. Fasting ghrelin, the change in insulin, and the change in glucose during the oral glucose tolerance test were the significant independent variables contributing to the variance in absolute suppression of ghrelin. Thus, alterations in ghrelin suppression in overweight children may be yet another manifestation of the insulin resistance of obesity. However, whether this is responsible for differences in satiety in overweight individuals merits additional investigation.

Rat adipose tissue expresses GHS-R1a mRNA, suggesting ghrelin may directly influence adipocyte function. Indeed, the effects of ghrelin on insulin-stimulated glucose uptake in isolated white adipocytes *in vitro* have been investigated (Patel *et al.* 2006). Ghrelin increased insulin-stimulated deoxyglucose uptake by 55% in isolated white adipocytes extracted from the epididymal fat pads of male Wistar rats. However, ghrelin administration in the absence of insulin had no effect on adipocyte deoxyglucose uptake, suggesting that ghrelin acts synergistically with insulin. Des-acyl ghrelin had no effect on insulin-stimulated glucose uptake. All these results suggest that ghrelin may play a role in adipocyte regulation of glucose homeostasis.

The inhibitory effect of ghrelin on insulin secretion was suggested to be due to a tonic regulation of pancreatic β -cells, prompting inhibition of both insulin and pancreatic somatostatin secretion (Egido *et al.* 2002). However, more recently, it has been shown that ghrelin is produced in a new type of islet cell, the ϵ -cell, and that the production of the hormone in those cells would affect β -cells via a paracrine mechanism that may require higher local levels of ghrelin than those found in plasma (Prado *et al.* 2004; Wierup *et al.* 2004). In fact, it has been proposed that that insulin and ghrelin cells share a common progenitor and that Nkx2-2 and Pax4, two homeodomain proteins, are required to specify or maintain differentiation of the β -cell fate. This finding also suggests that there is a genetic component underlying the balance between insulin and ghrelin in regulating glucose metabolism (Prado *et al.* 2004). Recently, definitive evidence has proven that the ghrelin hormone is also synthesised by pancreas α -cells, the glucagon-producing cell population, and confirmed the presence of single-hormone ghrelin-producing cells devoid of any of the four classical islet hormones, termed ϵ -cells (Heller *et al.* 2005). All these findings taken together raise the possibility that ghrelin modulates insulin activities in man.

In conclusion, some evidence suggests that ghrelin has a key role in the control of appetite and food intake and most probably in energy expenditure. The mechanism of action for ghrelin involves several pathways including hormonal

actions on hypothalamus and hindbrain neurons. The interaction of ghrelin with its receptor GHS-R1a appears to be the most important mechanism by which ghrelin mediates the synthesis of the orexigenic NPY and AgRP in the hypothalamus. In addition, a neuroendocrine vagal pathway and a neuropeptide system, working in the brain have also been reported, although their biological significance is practically unknown. Moreover, new receptors for ghrelin and desacyl-ghrelin seem to contribute to diverse central and peripheral actions of ghrelin. A role for LCFA acyl-CoA and AMPK in the regulation of hypothalamic signalling associated with food intake and energy expenditure has been recently described. Both LCFA acyl-CoA and AMPK would act as 'fuel sensors' within the hypothalamus and will inform about the current energy body status.

Leptin inhibits both the secretion of gastric ghrelin and the stimulation of feeding by ghrelin. In turn, ghrelin suppresses the secretion of leptin in the stomach and it has been proposed that this dual leptin restraint is the major regulatory arm of the feedback communication between the periphery and the hypothalamus for weight homeostasis.

How ghrelin regulates insulin is a matter of controversy and three different models have been proposed to explain the action of ghrelin in decreasing insulin secretion. Furthermore, recent data suggest that ghrelin may play a role in the regulation of glucose homeostasis in adipocytes and new findings claim that ghrelin may act as an anti-hypoglycaemic hormone modulating insulin tissue activities.

Perspectives and future research

Given the wide spectrum of biological activities of ghrelin and related peptides, it is evident that the discovery of ghrelin has opened many new perspectives within neuroendocrine and metabolic research and even has an influence on fields of internal medicine such as gastroenterology, immunology, oncology and cardiology. It is therefore increasingly likely that ghrelin and its GHS analogues, acting as either agonists or antagonists on different physiological and pathophysiological processes, might have clinical impact and therapeutic potential (van der Lely *et al.* 2004). However, a better understanding of the roles of the various different forms of ghrelin and ghrelin-related peptides, *i.e.* des-acyl ghrelin and obestatin, in the intricate balance of energy homeostasis and body-weight control may be essential for the successful treatment of obesity.

In addition, due to the complexity of GHS-R, it is clear that further studies are required to clarify whether ghrelin is the sole ligand or one of a number of ligands activating the GHS-R1a and whether that receptor used for ghrelin isolation is the sole receptor or one of a group of receptors for such ligands. Likewise, much work needs to be done to establish the physiological role of ghrelin in meal initiation and body-weight regulation and to establish its mechanism or mechanisms of action.

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