Ghrelin and Growth

DOI:
10.1159/000475732

Document Version
Accepted author manuscript

Link to publication record in Manchester Research Explorer

Citation for published version (APA):

Published in:
Endocrine Development

Citing this paper
Please note that where the full-text provided on Manchester Research Explorer is the Author Accepted Manuscript or Proof version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version.

General rights
Copyright and moral rights for the publications made accessible in the Research Explorer are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Takedown policy
If you believe that this document breaches copyright please refer to the University of Manchester’s Takedown Procedures [http://man.ac.uk/04Y6Bo] or contact uml.scholarlycommunications@manchester.ac.uk providing relevant details, so we can investigate your claim.
Ghrelin and Growth

Perchard R\textsuperscript{1,2}, Clayton PE\textsuperscript{1,2}

\textsuperscript{1}Division of Developmental Biology & Medicine, School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre

\textsuperscript{2}Royal Manchester Children’s Hospital, Central Manchester University Hospitals Foundation Trust, Manchester M13 9PL

Correspondence address: Prof PE Clayton, 5\textsuperscript{th} Floor (Research – Paediatric Endocrinology), Royal Manchester Children’s Hospital, Oxford Road, Manchester M13 9WL

Abstract
Ghrelin is a pleiotropic hormone, whose effect on growth hormone (GH) secretion, through the growth hormone secretagogue (GHS) receptor, is one of its many actions. Relationships have been identified between GHS receptor gene variants and human height, both in healthy individuals, and in patients with growth disorders. These include constitutional delay in growth and puberty (CDGP), idiopathic short stature (ISS), and isolated growth hormone deficiency (IGHD). In this review, we provide an overview of the role of ghrelin in growth.
Introduction

The key events that have formed the history of our understanding of the growth hormone (GH) axis have spanned more than five decades (Figure 1). Within this sequence of events was the discovery 40 years ago that synthetic small peptides, derived from enkephalins, were capable of inducing GH secretion. It was not until 1996 that a receptor for these GH-releasing peptides, termed GH secretagogues, was cloned – the GH secretagogue receptor (GHS-R). A further three years elapsed before the natural ligand for the GHS-R was discovered. This ligand was named ghrelin, derived from “ghre” the Proto-Indo-European root of the word “grow” [1]. Following extensive research on this ‘new’ hormone, ghrelin has been shown to have roles far beyond its ability to stimulate pituitary GH release via the GHS-R (Table 1).

Ghrelin is predominantly secreted by the P/D₁ cells in the stomach fundus and plays a major role as a centrally acting appetite stimulant [2, 3]. Patients demonstrate reductions in plasma ghrelin following gastrectomy [4] and ghrelin has also been shown to improve weight gain in cachectic patients [5]. Other actions of this pleiotropic hormone on a number of different systems are summarised in Table 1. Overall, ghrelin is considered to play a complex role in the regulation of hunger and metabolism [6]. In this review however we will focus on the relationship between ghrelin and growth.

From gene to peptide

Ghrelin is localized on chromosome 3p25-26 and it comprises five exons (Figure 2). The main in vivo form of ghrelin mRNA, transcript A, is an alternative splicing product
from exons 2 to 4. This mRNA is translated into pre-proghrelin, a ghrelin precursor which consists of 117 amino acids [7] (Figure 3). Pre-proghrelin is then modified by proteolytic cleavage, resulting in either desacyl-ghrelin, or the active species acyl-ghrelin, which has undergone acyl-modification by the enzyme, ghrelin O-acyl transferase (GOAT). Acyl-ghrelin and desacyl-ghrelin consist of 28 amino acids [8]. Acyl-ghrelin binds to the GHS-R and its actions are well understood, in comparison to desacyl-ghrelin whose receptor is unknown and functional role is unclear [9-11].

**Ghrelin and the growth hormone axis**

The discovery of ghrelin followed a long chain of developments in the understanding of the neuroendocrinology of the GH axis (Figure 1). GH release is stimulated by hypothalamic GH-releasing hormone (GHRH) and inhibited by somatostatin (SMS). SMS, discovered in 1973, was the first hypothalamic hormone found to regulate GH secretion [12], inhibiting the response of the pituitary to GHRH, and thereby reducing GH secretion [13]. Although the idea of a hypothalamic hormone stimulating GH secretion had been postulated in the early 1960s, GHRH was not discovered until twenty years later when three peptides were purified and sequenced from pancreatic tumours in acromegalic patients [14, 15], two of which went on to be identified within the human hypothalamus [14]. The physiological control of the GH axis was further characterised by studies involving frequent blood sampling, which defined the pulsatile pattern of growth hormone (GH) release [16]; the pulses corresponding to bursts of GHRH secretion and suppression of SMS secretion, while the inter-pulse intervals were the result of high SMS secretion [17].
As introduced above, a group of compounds were developed from the met-enkephalin molecule that had GH releasing properties [18, 19]. Although they were only able to induce weak GH release, their discovery led to the synthesis of more potent compounds including GHRP-2, GHRP-6, hexarelin and MK-0677; the growth hormone secretagogues (GHSs). An important finding demonstrated by several studies [20-23] was that these GHSs enhanced the release of GH through a different receptor and by different mechanisms than GHRH (Figure 4), introducing the notion of a third pathway of GH regulation.

In 1996, the GHS receptor (GHS-R) was cloned [22], and its presence in the hypothalamus and pituitary was recognised (Figure 1). The GHS-R is a G-protein coupled receptor that has seven transmembrane spanning segments as well as three intracellular and extracellular loops. GHS-R1a is the active form of the receptor, whereas GHS-R1b is biologically inactive [22]. Following the cloning of this receptor, its endogenous ligand, ghrelin, was discovered in 1999 [8] with its expression highest in the surprising location of the stomach. In the somatotroph cells of the pituitary gland, GHRH stimulates GH release through binding to the GHRH receptor and increasing cyclic AMP (cAMP) levels (Figure 4), while ghrelin binds to the GHS-R and causes an increase in intracellular Ca^{2+} levels. Ghrelin is now accepted as a key central component of the GH axis, acting through its own distinct pathway. This work clearly identified a role for ghrelin in control of the GH axis.

The clinical relevance of this pathway to GH secretion has been demonstrated in a number of human studies, with ghrelin given as an infusion or bolus demonstrating an excellent short term safety profile [24]. Extensive studies have also been
undertaken with GH secretagogues. In 1996, healthy elderly adults were treated with the oral GHS, MK-0677 (ibutamoren mesylate) once daily, which induced pulsatile GH release and significantly increased IGF-I levels [25, 26]. It was therefore suggested that treatment with MK-0677 may have some benefit in ageing [27]. In 1997, a study of adult men with childhood-onset isolated growth hormone deficiency (IGHD), demonstrated significantly increased GH, IGF-I and IGFBP3 concentrations [28]. Subsequently, in 2001, the same biochemical effects were also observed in children with GHD, following short-term oral administration of MK-0677 [29]. This confirmed MK-0677 as a GH secretagogue even in children with GH deficiency, implying residual function in the hypothalamic-pituitary axis. The authors concluded that further studies testing its effect on promoting growth in such children should be considered.

**Manipulating gene expression in the ghrelin system**

Murine knockout models have contributed further to knowledge on the role of ghrelin [30]. These models have mainly identified and defined ghrelin’s metabolic roles, and have shown that in the mouse, the impact on growth is minimal. The first report of a genetic mouse model [31, 32] involved GHS-R1a null mice. In these mice, injection of ghrelin failed to induce food intake or increase GH release compared to wild-type (WT) mice, providing supporting evidence that the GHS-R1a is the receptor for ghrelin. Consequently, these mice displayed an insulin sensitive and lean phenotype [33] with the leanness thought to be related to elevated energy expenditure, resting metabolic rate and thermogenic capacity [34]. In contrast, ghrelin gene knockout in adult mice conferred no metabolic improvements; there was no alteration in energy or glucose metabolism nor any difference in cholesterol, triglycerides, glucose, leptin
or insulin levels when compared with WT [31]. However, it was found that when mice lacked both ghrelin and GHS-R1 [35], this led to increased energy expenditure, body core temperature and locomotor activity. The resultant phenotype was a mouse with lower body weight (in particular, lower body fat) and lower plasma cholesterol [35]. An alteration in body length, however, was not noted in these knock-outs.

Further studies in murine models have focussed on identifying potential pathways for the treatment of obesity. When GOAT null mice were fed a diet rich in medium chain fatty acids (MCFAs), they demonstrated a low body weight. GOAT null mice have also been studied following calorie restriction, when they demonstrated decreased GH secretion and lower blood glucose levels compared to WT. Following this hypoglycaemia, they eventually became moribund [36] [37]. In summary, the GOAT knockout mouse demonstrated less food intake and weight gain plus increased insulin sensitivity compared to the WT [38].

Further knowledge has been gained from the study of transgenic mice. In contrast to GOAT null mice, GOAT transgenic (Tg) mice, over-expressing GOAT, displayed a higher body weight and adiposity when fed a MCFA rich diet. In line with these findings, Tg mice with increased acyl-ghrelin levels showed late onset impaired glucose homeostasis and reduced insulin sensitivity.

The ob/ob mouse has uncontrolled food intake due to inability to produce leptin. Studies in these mice have produced conflicting results. In ob/ob mice with knockout of ghrelin, an improvement in β-cell function and increased peripheral insulin sensitivity was observed [39], whereas in ob/ob mice with GHS-R1a knockout [40],
hyperglycaemia and glucose tolerance worsened. These mice with \textit{GOAT} knockout also failed to demonstrate an improvement in glucose homeostasis \cite{41}.

Overall these murine models with manipulations of the ghrelin system primarily have provided evidence that acyl-ghrelin is involved in metabolism without a major impact on growth.

\textbf{Ghrelin concentrations in infants, children and adolescents}

In view of ghrelin's role in the GH axis, studies were undertaken to characterise physiological levels of ghrelin during childhood and adolescence. In 121 healthy children between the ages of 5 and 18 years, a significant negative correlation was described \cite{42} between ghrelin concentration and age, and this was more marked in boys (Figure 5). This inverse association has also been reported in a cohort of 111 \cite{43} healthy children. The fall in ghrelin concentration over childhood and puberty may suggest that ghrelin is not a direct growth-promoting hormone. When the relationship between ghrelin and the IGF-I axis was explored, ghrelin correlated negatively with IGF-I but positively with IGFBP-1 \cite{42} implying that the fall in ghrelin could facilitate growth during puberty (when IGF-I levels show a dramatic increase and IGFBP-1 levels decrease \cite{44}). In line with these findings, a further study \cite{46} in children with short stature of different aetiologies (GHD, idiopathic short stature (ISS) and neurosecretory dysfunction (NSD) found fasting ghrelin concentrations to be higher in children with lower IGF-I/IGFBP-3 ratios. The authors suggested that lower bioactivity of IGF-I may be a stimulating factor for ghrelin synthesis \cite{45}. Additionally, healthy 0-3 month old infants born small for gestational age (SGA) display higher concentrations of ghrelin \cite{46} compared with appropriate for gestational age (AGA)/
large for gestational age (LGA) infants, possibly suggesting that ghrelin plays a role in the process of catch-up growth in SGA.

Assessment of serum ghrelin, IGF-I and IGFBP-3 levels in constitutional delay/normal variant short stature [47] revealed higher levels compared with controls, and multiple regression analysis identified weight (p<0.0001) and height (p=0.01) as independent negative determinants of ghrelin concentration. A possible explanation is that low weight and/or low height represent a suboptimal nutritional state, leading to a compensatory elevation of ghrelin and hence an orexigenic effect. Overall, these studies highlight the difficulty of attributing a physiological/pathophysiological role for ghrelin in children based on circulating blood levels.

**Variants in the growth hormone secretagogue receptor gene and the relationship with human height**

An alternative approach to examining the role of the ghrelin axis in growth is to assess the impact of variants in *GHS-R* on adult stature. Whilst some genome wide association studies (GWAS) [48, 49] have failed to demonstrate a link between common *GHS-R1A* variants and height, results from a meta-analysis of adult height in 253,288 individuals [50] highlighted many genes relevant to human skeletal growth, one of which was *GHS-R*.

More focused studies that have sequenced *GHS-R* have failed to demonstrate an association with short stature. Wang *et al.* [51] systematically screened the coding region of the *GHS-R* gene, and identified seven sequence variants (five silent SNPs and two novel missense variants) in 43 children with short normal stature. However
these variants were also present in children of normal height, with no difference in frequency observed. Additionally, a genetic study in the French population failed to show any major contribution of common variants of the ghrelin and GHS-R genes to height variation [52].

**The growth hormone secretagouge receptor in short stature of undefined aetiology**

Despite the negative studies outlined above, further investigations in children with undefined growth disorders have implicated abnormalities in the GHS-R as the potential cause for their short stature. In 2006, by sequencing the PCR products spanning the 2 GHS-R coding exons and flanking intronic regions in two families of Moroccan origin with familial short stature, Pantel et al. [53] discovered the first GHS-R functional mutation, a C to A substitution (c.611C→A) in exon 1 (A204E). Data from these families provided evidence for an incomplete dominant mode of inheritance. All individuals with a reduced height for age carried at least one A204E allele, and one patient (with consanguineous parents) carrying a homozygous mutation displayed the most severe height reduction.

Functional studies [53, 54] on this mutation have demonstrated that, despite a clear response to ghrelin and efficient translation into a protein, there is reduced expression at the cell surface and loss of constitutive activity (CA). It is recognised that there may be difficulties in ascertaining the extent to which such CA is relevant to the receptor itself, rather than a result of the cellular environment or the assay. One criticism of this work was the paucity of clinical and biological data on patients with the GHS-R1a A204E mutation. Nevertheless, the description of this novel, functionally significant GHS-R mutation provided a basis for studies to look for GHS-R1A mutations in short stature conditions.
Growth hormone secretagogue receptor mutations in constitutional delay in growth & puberty

A further five variations in the GHS-R have been identified (c.-6 G>C, c.251G>T (p.Ser84Ile), c.505G>A (p.Ala169Thr), c.545 T>C (p.Val182Ala), and c.1072G>A (p.Ala358Thr) [56] in subjects with constitutional delay in growth and puberty (CDGP), defined as lack of breast development by the age of 13 years in girls and testicular volume <4.0ml by the age of 14 years in boys, absence of other identifiable causes of delayed puberty, delayed bone age, and spontaneous, complete achievement of pubertal development during follow-up [56]. Of these five, p.Ser84Ile and p.Val182Ala were predicted to be ‘probably’ and ‘possibly damaging’ respectively [57]. In support of this prediction, functional work revealed a decrease in basal activity in both of these mutated receptors. This was thought to be partly explained by a reduction in cell surface expression, but also due to a defect in ghrelin potency in the case of the p.Ser84Ile mutation.

Growth hormone secretagogue receptor 1A mutations in Japanese subjects with short stature

Four rare novel deleterious GHS-R1A mutations (∆Q36, P108L, C173R, and D246A) of varying functional consequence have been identified in Japanese individuals with short stature [58]. Their functional properties have been elucidated by transfection studies, involving HEK293A cells, the WT or mutant GHS-R1A expression construct, and the reporter genes, pSRE-luc and pRL-TK; cells were stimulated with increasing concentrations of ghrelin or substance P (SPA, a neuropeptide that functions as an inverse agonist of the GHS-R [59]). A loss in constitutive signalling activity of the
GHS-R1A receptor was established in all mutated receptors. In addition, C173R caused intracellular retention of the mutated protein which led to a total loss of receptor function, while P108L resulted in a reduced binding affinity to ghrelin and D246A reduced receptor signalling.

In this cohort, a further four previously identified mutations were also found, and considerable variability in CA was noted between the mutant receptors. As previously reported [53, 54], A204E and F279L, showed markedly low CA (4% and 18%, respectively, of the CA of WT, whilst retaining their ability to respond to ghrelin). The ΔQ36 mutant displayed only a subtle reduction. Although it was proposed that this variability may lead to variable phenotypes, clinical and laboratory data on these patients were not published. Nevertheless, the presence of these functionally significant mutations has implicated GHS-R1A mutations as a genetic cause of short stature.

**Growth hormone secretagogue receptor mutations as a cause of growth hormone deficiency**

GHS-R mutations have been identified in a patient with recessive partial IGHD [60], adding further support for the role of ghrelin in growth disorders [41, 42, 45, 53, 56, 58, 61]. Clinical signs in this young boy (first seen at the age of five years) were growth delay (-3.0 SDS) and low body mass index, associated with symptoms of abdominal pain, vomiting, ketosis and hypoglycaemia. Gene sequencing revealed two new molecular defects in exon 1 of the GHS-R gene, with the subject being a compound heterozygote for these defects. The c.6G>A (p.W2X) mutation inherited from his father predicted a premature stop codon, while the c.709A>T (p.R237W)
mutation inherited from his mother was a missense mutation. Of note, transmission of the IGHD phenotype suggested a recessive mode of inheritance, which differs from the incomplete dominant mode of inheritance of the first GHS-R mutation described by this group [53].

To identify further mutations, molecular screening was undertaken in a cohort of 80 index cases with IGHD/combined pituitary hormone deficiency and 20 affected siblings, from 80 unrelated Moroccan families [61]. Of these, GHS-R was sequenced in 46 IGHD index cases, and heterozygous variations were identified in four families. Three families displayed the previously described [53, 58] deleterious substitution mutation in exon 1 (c.611C>A, p.A204E). This mutation was found in 3 index cases and 7 related individuals, of which only the father of one of the probands had a slight height reduction, and only one of the three index cases had a hypoplastic anterior pituitary. Previous studies have demonstrated incomplete penetrance of the short stature phenotype in families carrying this mutation, with the highest reported figure at 66% [53]. The penetrance for heterozygous carriers in this Moroccan cohort was even lower at 40%.

A second, previously described [56] variant (c.1072G>A p.A358T) in exon 2 was identified in another boy with a hypoplastic anterior pituitary. This is recognized as a benign polymorphism (rs150344113) [55] that occurs with a frequency of up to 1.7% in American multi-ethnic and African American cohorts. His father carried the same heterozygous variation and was short (Height SDS −2.2).

Overall there is some evidence that the mutations in GHS-R may be implicated in GH deficiency.
Conclusions

Ghrelin undoubtedly has a key role to play in the complex interplay between appetite and metabolism. In addition ghrelin does play a role in human growth, supported by two distinct lines of genetic evidence: (1) variants in the GHS-R are related to adult stature, and (2) mutations in GHS-R, with well characterised functional consequences, have been found in a range of growth disorders, including isolated GH deficiency. Furthermore there is a case for exploring the use of oral GH secretagogues for treatment of growth disorders.


**Figure Legends**

**Figure 1.** Timeline of the key events in the understanding of the components that control the hypothalamo-growth hormone axis.


**Table 1.** The physiological effects of ghrelin in the human body

**Figure 2.** From the human ghrelin gene to an active peptide.

Figure adapted from Kojima, M. and K. Kangawa, Ghrelin: structure and function. Physiol Rev, 2005. 85(2): p. 495-522 and Genetics Home Reference
https://ghr.nlm.nih.gov/gene/GHRL#location

**Figure 3.** Pre-proghrelin may be acylated to acyl ghrelin, or directly processed without acylation.

Figure adapted from Labarthe, A., et al., Ghrelin-Derived Peptides: A Link between Appetite/Reward, GH Axis, and Psychiatric Disorders? Front Endocrinol (Lausanne), 2014. 5: p. 163.

**Figure 4.** Regulation of growth hormone release from the pituitary


**Figure 5.** The relationship between ghrelin concentration and age

Figure 1. Timeline of the key events in the understanding of the components that control the hypothalamo-growth hormone axis. Figure adapted from Murray, P.G., C.E. Higham, and P.E. Clayton, 60 YEARS OF NEUROENDOCRINOLOGY: The hypothalamo-GH axis: the past 60 years. J Endocrinol, 2015. 226(2): p. T123-40.
Table 1. The physiological effects of ghrelin in the human body

<table>
<thead>
<tr>
<th>System</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamic</td>
<td>↑ appetite</td>
</tr>
<tr>
<td>Non-hypothalamic CNS/SNS*</td>
<td>Taste sensation, reward behaviour, olfaction, learning/memory, circadian rhythm, neuroprotection. ↓ SNS* activity.</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>↑ gastric motility, emptying and acid secretion</td>
</tr>
<tr>
<td></td>
<td>↑ intestinal motility</td>
</tr>
<tr>
<td>Endocrine pancreas</td>
<td>↓ insulin secretion,</td>
</tr>
<tr>
<td></td>
<td>↑ insulin sensitivity</td>
</tr>
<tr>
<td>Hepatic</td>
<td>↑ IGF-I secretion</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>↓ lipid oxidation</td>
</tr>
<tr>
<td></td>
<td>↑ lipogenesis</td>
</tr>
<tr>
<td></td>
<td>↓ inflammation</td>
</tr>
<tr>
<td>Cardiac</td>
<td>↑ cardiac contractility, cardiac output</td>
</tr>
</tbody>
</table>

*CNS= central nervous system,  SNS= sympathetic nervous system*
Figure 2. From the human ghrelin gene to an active peptide


Human ghrelin gene

1. Signal peptide
2. Ghrelin

Exon

1 2 3 4 5 1.0kb

Transcription

Splicing

Transcript A

Transcript B

Translation

Ghrelin precursor protein

1 17

O=C-(CH₂)₆-CH₃

Cleavage & acyl-modification

GSSFLSPEHQRVQQRKESKKPPAKLQPR

n-Octanoyl ghrelin

Chromosome

3p 25-26
Figure 3. Preproghrelin may be acylated to acyl ghrelin, or directly processed without acylation. Figure adapted from Labarthe, A., et al., Ghrelin-Derived Peptides: A Link between Appetite/Reward, GH Axis, and Psychiatric Disorders? Front Endocrinol (Lausanne), 2014. 5: p. 163.
Figure 4. Regulation of growth hormone release from the pituitary. Figure adapted from Kojima, M. and K. Kangawa, Ghrelin: structure and function. Physiol Rev, 2005. 85(2): p. 495-522.
Figure 5. The relationship between ghrelin concentration and age. Figure adapted from Whatmore, A.J., et al., Ghrelin concentrations in healthy children and adolescents. Clin Endocrinol (Oxf), 2003. 59(5): p. 649-54.