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Changes in the ghrelin hormone pathway maybe part of an unusual gastric system in monotremes

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ABSTRACT

Ghrelin is a growth hormone (GH)-releasing and appetite-regulating peptide predominately released from the stomach. Ghrelin is evolutionarily highly conserved and known to have a wide range of functions including the regulation of metabolism by maintaining an insulin-glucose balance. The peptide is produced as a single proprotein, which is later proteolytically cleaved. Ghrelin exerts its biological function after *O*-n-octanoylation at residue serine 3, which is catalyzed by ghrelin *O*-acyl transferase (GOAT) and allows binding to the growth hormone secretagogue receptor (GHS-R 1a). Genes involved in the ghrelin pathway have been identified in a broad range of vertebrate species, however, little is known about this pathway in the basal mammalian lineage of monotremes (platypus and echidna). Monotremes are particularly interesting in this context, as they have undergone massive changes in stomach anatomy and physiology, accompanied by a striking loss of genes involved in gastric function. In this study, we investigated genes in the ghrelin pathway in monotremes. Using degenerate PCR, database searches and synteny analysis we found that genes encoding ghrelin and GOAT are missing in the platypus genome, whilst, as has been reported in other species, the *GHSR* is present and expressed in brain, pancreas, kidney, intestine, heart and stomach. This is the first report suggesting the loss of ghrelin in a mammal. The loss of this gene may be related to changes to the platypus digestive system and raises questions about the control of blood glucose levels and insulin response in monotreme mammals. In addition, the conservation of the ghrelin receptor gene in platypus indicates that another ligand(s) maybe acting via this receptor in monotremes.

Introduction

Ghrelin pathway

Ghrelin is a 28 amino acid peptide and endogenous ligand for the growth hormone secretagogue receptor (GHS-R 1a) (Kojima et al., 1999). It was first purified from the rat stomach by Kojima et al. based on its GHS-R 1a stimulating and growth hormone (GH) releasing activities (Kojima et al., 1999), but has since been shown to be expressed in all tissues investigated so far, including pancreatic epsilon cells (ε cells) (Wierup et al., 2002) and gastric P/D_1 cells (known as X/A-like cells in rodents) (Date et al., 2000). In a variety of vertebrate species, ghrelin is known to be involved in a range of activities, not only the stimulation of GH secretion but also food intake, regulation of glucose and lipid metabolism, increasing gastric acid release and motility, decreasing blood pressure and various neuronal functions (Kojima and Kangawa, 2008; Kaiya et al., 2013).

The human ghrelin gene (*GHRL*) encodes a preproghrelin protein, which gives rise to ghrelin and obestatin via posttranslational proteolytic cleavage (Zhang et al., 2005). Acylation at its third serine residue (Ser^3) enables the bidirectional transport of ghrelin across the blood–brain barrier (Banks et al., 2002) and is essential for GHS-R 1a binding and consequent biological activities (Kojima et al., 1999). However, acyl ghrelin only makes up ~25% of the total circulating ghrelin and the majority is nonacylated (des-acyl ghrelin). Although the specific receptor for des-acyl ghrelin is not yet known, some studies have reported effects on food intake, energy expenditure and glucose homeostasis (reviewed by Kirchner et al., 2012). Ghrelin *O*-acyl transferase (GOAT), which belongs to the membrane-bound *O*-acyl transferase (MBOAT) family and is encoded by *MBOAT4*, is the only enzyme known to catalyse the acylation of ghrelin. Moreover, the recognition sequence of GOAT (GXSFX, where X is any residue) is specific for ghrelin, indicating ghrelin is the only

Abbreviations: GH, growth hormone; GHRL, ghrelin; GHS-R, growth hormone secretagogue receptor; GOAT, ghrelin *O*-acyl transferase; GPCR, G-protein coupled receptor; MBOAT, membrane-bound *O*-acyl transferase; Cds, coding sequences.

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substrate of GOAT (Yang et al., 2008b; Ohgusu et al., 2009). Murine GOAT is mainly expressed in the stomach, but is also found in other tissues including pancreas, small intestine and colon (Kirchner et al., 2009; Gutierrez et al., 2008; Yang et al., 2008a). *MBOAT4* mRNA is expressed mostly by ghrelin-producing X/A-like cells of the gastric oxyntic mucosa in mice (Sakata et al., 2009b).

The ghrelin receptor, GHS-R 1a, is a 7 trans-membrane G-protein coupled receptor (GPCR), encoded by the *GHSR*. Alternative splicing of *GHSR* creates two isoforms in humans: the biologically functional GHS-R 1a and the truncated GHS-R 1b (Howard et al., 1996). GHS-R 1b does not bind ghrelin, but forms dimers with GHS-R 1a to inhibit its function (Leung et al., 2007) and expression (Chow et al., 2012). *GHSR* is predominately expressed in the hypothalamus and pituitary gland of the central nervous system (CNS) (Guan et al., 1997) but is also found in peripheral tissues such as the pancreas and spleen (Gnanapavan et al., 2002). High basal signaling activities have led to the idea that GHS-R 1a can also act independently of ghrelin (Holst et al., 2003).

The ghrelin pathway is vital to maintaining growth hormone release and energy homeostasis. Orthologs of genes in this pathway, including *GHRL*, *GHSR* and *MBOAT4*, are highly conserved and have been identified in a range of vertebrate species (Kojima et al., 1999; Tanaka et al., 2001; Kaiya et al., 2008, 2009; Gutierrez et al., 2008; Kojima and Kangawa, 2005).

The stomach and pancreas of monotremes

Monotremes (comprising platypus and echidna) represent the most basal lineage amongst living mammals, which diverged approximately 166 million years ago (Bininda-Emonds et al., 2007). Monotremes feature a striking combination of mammalian, reptilian and unique characteristics.

The small and glandless stomach of platypus (*Ornithorhynchus anatinus*) and echidna (*Tachyglossus aculeatus*) is one of the most marked anatomical differences between the monotreme lineage and other mammalian species. The only glands present are the Brunner's glands, which are confined to the submucosa of the distal stomach (Krause, 1971; Griffiths, 1978; Krause and Leeson, 1974).

The platypus genome has been sequenced recently providing important insights into mammalian evolution and into the extraordinary biology of monotremes (Warren et al., 2008). A key feature of the genome analysis of the platypus was the identification and characterization of genes involved in protein degradation (degradome). This revealed the wholesale loss of genes required for gastric function, including genes encoding gastrin (*GAST*) and pepsin (*PGA, PGC*) (Ordonez et al., 2008). The loss of these genes is consistent with the striking physiological and anatomical changes of the platypus digestive tract but raised questions about other genes and pathways related to digestion and metabolic control involving other organs (e.g. the pancreas) in platypus.

Very little is known about the anatomical structure and function of the platypus pancreas although the echidna pancreas has been shown to have distinctive endocrine and exocrine parts (Yamada et al., 1990). Similar to other mammals, the echidna's endocrine islets of Langerhans contain α, β, δ and PP cells (Yamada et al., 1990). However, the existence of ghrelin-producing ε cells in monotremes has not been reported.

Here we investigated genes in the ghrelin pathway in monotremes, which have undergone massive changes in stomach anatomy and physiology accompanied by attrition of the degradome gene repertoire. Our findings suggest that genes encoding ghrelin/obestatin and GOAT are missing in the platypus genome, whilst *GHSR* is present and is expressed in brain, pancreas, intestine, kidney, heart and stomach. These findings raise important questions about the ghrelin pathway and metabolic control in this mammalian lineage.

Materials and methods

Degenerate PCR

Primers were designed using NCBI primer-blast online program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were synthesized at Geneworks (Adelaide, SA, Australia). For *GHRL* degenerate primers (Table 1 No. 1–7) were designed to match the most conserved regions identified by multiple coding sequence (cds) alignments among human (*Homo sapiens*) NM_001134941.1, mouse (*Mus musculus*) NM_021488.4, opossum (*Monodelphis domestica*) XM_001375640.2, chicken (*Gallus gallus*) NM_001001131.1, cow (*Bos taurus*) NM_174067.2 and rabbit (*Oryctolagus cuniculus*) XM_002722463.1 (Supplementary Fig. 1A).

For *MBOAT4* degenerate primers (Table 1 No. 8–10) were designed by multiple cds alignments between human (*H. sapiens*) NM_001100916.1, mouse (*M. musculus*) NM_001126314.2, opossum (*M. domestica*) XM_001372794.2, chicken (*G. gallus*) NM_001199289.1 cow (*B. taurus*) NM_001192257.1 and rabbit (*O. cuniculus*) XM_002709539.1 (Supplementary Fig. 1B).

Genomic DNA was isolated from platypus, echidna, mouse, opossum and chicken liver tissue using a phenol/chloroform/isoamyl alcohol (Sigma Aldrich, USA) method. All PCRs (for *GHRL*, with primer pairs 1&4, 6&7, 2&5, 2&3, 1&3 and for *MBOAT4* with primer pairs 8&10 and 9&10) were performed (and repeated at least twice) in a total volume of 25 μl containing 100 ng genomic DNA,

Table 1
Primers used in this study.

No.	Name	Sequence (5′–3′)	Experimental uses
1	oGHRL for 43	TGCTCTGGATGGAYDTGGC	*GHRL* degenerate PCR
2	oGHRL for 70	GGCTCCAGYTTCCTAAGCCC	*GHRL* degenerate PCR
3	oGHRL rev 110	TGGCTTTTTGGWTTCCTTYC	*GHRL* degenerate PCR
4	oGHRL rev 171	TMTCTACTCCTTCWGGCTGG	*GHRL* degenerate PCR
5	oGHRL rev 212	CCAAYRTCAAAGGGAGCGT	*GHRL* degenerate PCR
6	cGHRL for 54	AGAMAYTGCTHTGGCTGG	*GHRL* degenerate PCR
7	cGHRL rev 137	GKCTCGGCSATGTARTCCTG	*GHRL* degenerate PCR
8	oGOAT for 505	GGCYCTCTGTGTTCCTTC	*MBOAT4* degenerate PCR
9	oGOAT for 722	AAACTSACCTATTACTCYCA	*MBOAT4* degenerate PCR
10	oGOAT rev 956	CCAGGCAGARAAGGCAAAT	*MBOAT4*degenerate PCR
11	pGHSR for 160	TCCAGTTCGTCAGCGAGAGC	*GHSR* genomic PCR & RT-PCR
12	pGHSR rev 747	TAGGGTTGATGGCAGCACTAAAG	*GHSR* genomic PCR & RT-PCR
13	pGHSR rev intron	ATACAGAGAGACCGAGAGAGAGGC	Verification of GHS-R 1b
14	pGHSR for 5'	ATGTGGAAYGCGACGCSSA	*GHSR* degenerate PCR
15	pGHSR rev 190	GGCGGAAAAGGTCCAGGGGC	*GHSR* degenerate PCR

Followed IUB code for mixed base sites (R = AG, Y = CT, M = AC, K = GT, S = GC, W = AT, H = ACT, B = GCT, V = AGC, D = AGT, N = AGCT).